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ARTICLE

A fluorescent molecular rotor showing vapo-chromism, aggregation-induced emission, and environmental sensing in living cells

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Among the plethora of recently proposed molecular sensors, those belonging to the class of fluorescent molecular rotors (FMRs) have attracted much attention owing to their peculiar photophysical properties that enable an unprecedented sensitivity towards environmental microviscosity. The usual FMR synthetic design prescribes chromophores characterized by an intramolecular rotation between two well-defined excited states, a locally excited state and a twisted internal charge transfer (TICT) state, where the sensing capabilities arise from a dual competition of the corresponding radiative/non-radiative decay processes. However, we have recently demonstrated a different *modus operandi* of a new subclass of solvatochromic FMRs, which exploit a solvent-independent, barrier-free intramolecular rotation of the excited dye. The rotational dynamics is modulated by local viscosity and, in turn, manifested through a variable spectral signal. In order to translate the same rotational mechanism in a versatile sensor of polarity and viscosity, we designed and thoroughly characterized a novel FMR, namely 4-(triphenylamino)-phthalonitrile (**TPAP**). Remarkably, in addition to a high sensitivity versus solvent polarity and viscosity, **TPAP** is also able to form stable fluorescent nanoparticles characterized by aggregation-induced emission, via a simple sonochemical treatment. Such peculiar features are tested in different applications aiming at illustrating its capability to report on solvatochromic and vapo-chromic effects, as well as to provide detailed intracellular information through bioimaging studies.

Introduction

Fluorophores whose emission changes in response to variations in their environment have emerged as an interesting family of compounds with widespread applications in analytical, environmental, optical, and biochemical areas. In this context, sensors for polarity and viscosity are the most expedient ones, owing to their widespread applicability in several life-science studies.¹ The polarity sensitivity is generally based on the change in dipole moment of the fluorophore upon photo-excitation and the resulting dipolar relaxation of the surrounding molecules or residues. This effect is called solvatochromism and is accounted for by several theoretical descriptions, among which stands the Lippert-Mataga model² for its simplicity and effective interpretation

of many experimental data.³⁻⁵

Fluorophores sensitive to local viscosity are referred to as *fluorescent molecular rotors* (FMR).⁶ FMRs perceive local viscosity through intramolecular twisting motion(s) that occur at excited state.⁷ In most cases, intramolecular distortion leads to a twisted internal charge transfer (TICT) state, which is almost non-emissive. Thus, slowing the twisting motions as viscosity increases leads to stronger emission and longer lifetime decay.⁸ The Forster-Hoffmann model of molecular rotors⁹ affords a reliable quantitative description of fluorescence dependence on viscosity for most FMRs.¹⁰ Nonetheless, a common drawback of FMRs is the difficulty to predict their sensitivity towards local polarity, owing to the complex dipolar properties of accessible excited states.¹¹ Some FMRs are even insensitive to the dielectric properties of their surrounding environment.¹² In this context, probes reporting predictably on both polarity and viscosity are a desirable tool in the field of fluorescence molecular sensors, since they may allow for a thorough physicochemical characterization of their local environment.

We recently described a prototype for a new class of molecular rotors, namely 4-(diphenylamino)-phthalonitrile (**DPAP**), which combines strong fluorochromism and viscosity response within one molecule.¹³ This unique feature is attributed to an unprecedented and distinctive *modus operandi*, which is based on an unusual barrier-less and solvent-independent flexibility that finely modulates its photophysical properties. Motivated by these

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findings, we here present 4-(triphenylamino)-phthalonitrile (**TPAP**, Fig. 1a), a FMR designed to exploit the same atypical mechanism with an increased charge transfer distance. **TPAP** is easily synthesized in a few steps via a simple and convergent route and makes use of an amino electron donor unit and two nitrile electron acceptor units to facilitate intramolecular charge transfer (ICT) upon photoexcitation. The extended π -conjugated system of **TPAP** is designed to facilitate the formation of a charge transfer state, which, in turn, does affect its electronic and spectroscopic features, as well as its sensitivity towards the environment. In this work, we embarked on a thorough photophysical and computational study to analyze **TPAP** solvatochromism and viscosity response. On this account, **TPAP** has been tested in three different applications requiring environmental sensing. Our goal was to assess the feasibility and *modus operandi* of **TPAP** in different contexts, including its capability to report fluorescence emission and lifetime differences in the cellular environment.

First, **TPAP** was shown to exhibit excellent vapochromic response when exposed to a range of different solvent vapors. Hence, **TPAP** was demonstrated to give rise to the formation of highly-fluorescent organic nanoparticles based on an aggregation-induced emission (AIE) mechanism.¹⁴ Finally, **TPAP** was investigated as a dual polarity and viscosity probe in living cells, on account of the fundamental relevance of electrostatic interactions¹⁵⁻¹⁷ and diffusion-controlled reactions to sustain life.⁶ The sensing capability of **TPAP** is exploited by a new imaging approach, named *phasor analysis*, which relies on a simple graphic analysis of spectral and lifetime fluorescence images following a mathematical transformation.¹⁸ Overall, our results do support the use of **TPAP** as an outstanding probe in the field of environmental sensors.

Materials and methods

Synthesis of TPAP

TPAP was prepared and purified following the reported procedure.¹⁹ **TPAP** was characterized by ¹H and ¹³C NMR, elemental analysis, mass spectrometry and IR, and the data obtained, that can be found in the Supporting Information, matched with the values reported in the literature.

Preparation of TPAP AIE dispersions

For the preparation of the **TPAP** dispersions in a 87/13 water/methanol mixture, a solution of **TPAP** in methanol (286 μ L) was added to deionized water (1914 μ L) under vigorous stirring (final volume of the prepared solvents mixture = 2200 μ L). A similar procedure, but using different ratio of the solution of **TPAP** in methanol and deionized water, has been used for the preparation of the other mixtures which present different water/methanol ratio. The water/methanol mixtures at different **TPAP** concentration (from 1.1×10^{-6} up to 5.0×10^{-4} M) were obtained using methanol solutions at different **TPAP** concentration.

Preparation of TPAP films for vapochromic studies

TPAP films for vapochromic studies were prepared by uniformly spreading a hexane solution of **TPAP** on a glass slide and letting the solvent to evaporate. Residual traces of the

solvent were eliminated by introducing the **TPAP**-coated glass slides in a vacuum chamber. The vapochromic studies were carried out by placing these coated slides into a chamber saturated with the vapours of different solvents while illuminating the slides with a 365 nm lamp.

Cell cultures

CHO cells were grown in Dulbecco's modified Eagle medium: F-12 nutrient mix (D-MEM/F-12) purchased from Invitrogen (Carlsbad, CA) supplemented with 10% fetal bovine serum and 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere. For fluorescence imaging, 120000 cells were plated onto a 35-mm glass-bottom dish (WillCo-dish GWST-3522).

More details on quantum mechanical (QM) calculations, fluorescence microscopy imaging of living cells, photophysical studies of **TPAP** in solution and dispersions, cryogenic transmission electron microscopy (Cryo-TEM) studies and dynamic light scattering (DLS) measurements can be found in the Supporting Information section.

Results and discussion

TPAP structural analysis

The peculiar chemical structure of **TPAP** confers significant rotational freedom to its phenyl rings, while the backbone of the molecule is constrained to a Y-shaped configuration. From the computed optimized structure, it has been observed that the nitrogen atom is coplanar with its covalently linked carbon atoms, according to a sp^2 hybridization. Due to steric hindrance, the amine phenyl groups assume a propeller-like configuration, as shown in Fig. 1b, and their rotational motion is therefore concerted. On the other hand, the 1,2-dicyanobenzene moiety can rotate rather freely with respect to the rest of the molecule (see Fig. S1), showing relatively small energetic barriers (<3 kcal/mol) along the computed torsional potential.

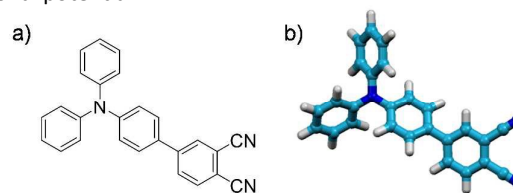


Fig. 1: a) Molecular and b) optimized structure of **TPAP**.

TPAP optical properties and solvatochromism

Optical absorption and emission spectra of **TPAP** were recorded in various solvents and solvent mixtures of different polarity (Table S1). The absorption spectra of **TPAP** in cyclohexane, *o*-xylene, THF and acetonitrile are shown in Fig. 2a. All spectra are characterized by two broad bands with maxima at about 390 nm and 285 nm. QM calculations have assigned these bands to transitions from S₀ to S₁ and S₄, respectively (see Fig. S2 for the computed optical spectra).

Extinction coefficients of about $2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ were observed in all solvents. However, a strong increase in absorption with extinction coefficients rising to $1 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ was noted in pyridine (Fig. S3). This finding may suggest that π - π interactions with the solvent are responsible for the observed absorption enhancement.

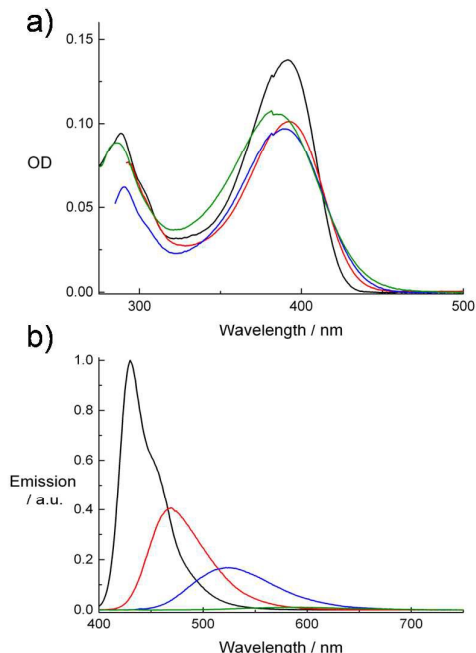


Fig. 2: a) Absorption and b) emission spectra ($\lambda_{\text{exc}} = 392 \text{ nm}$) of **TPAP** ([**TPAP**] = $5 \times 10^{-6} \text{ M}$ and $1 \times 10^{-6} \text{ M}$ in a) and b), respectively) in cyclohexane (black), *o*-xylene (red), THF (blue) and acetonitrile (green).

Emission spectra recorded in cyclohexane, *o*-xylene, THF and acetonitrile are shown in Fig. 2b upon excitation at the absorption maximum (392 nm). In contrast to absorption, a strong solvatochromic effect is observed in the recorded emission spectra, affecting both emission energies and intensities. It is worth noting that a single, broad emission signal evolves in all solvents except for cyclohexane for which an additional small shoulder was detected. In this case, QM calculations have confirmed the nature of the emission as occurring from a relaxed S1 state and have nicely matched the observed solvatochromism in going from apolar to polar solvents (see Fig. S2).

For a detailed description of **TPAP** solvatochromism, the Lippert-Mataga polarity parameter (Δf) was evaluated for an extended set of solvents and solvent mixtures according to eq S1 (Table S1).^{20,21} Fig. 3a shows the correlations between the optical absorption and emission maxima and Δf .

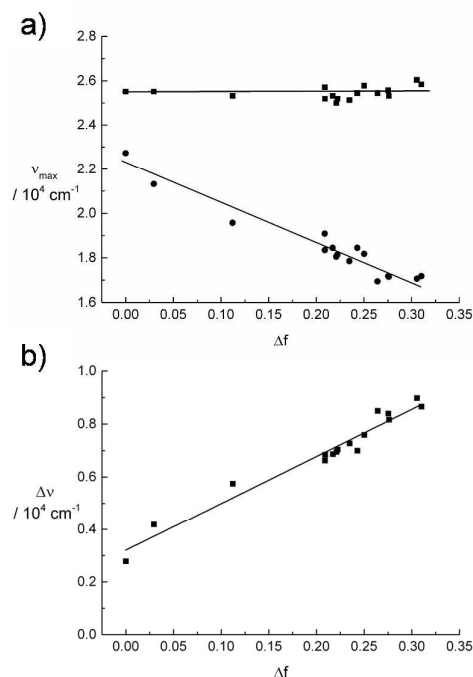


Fig. 3: a) Plot of the absorption (squares) and emission (circles) maxima of **TPAP** as a function of the solvent polarity parameter (Δf). b) Lippert plot of **TPAP** as a function of Δf .

As previously shown for a few solvents, polarity has a little effect on the absorption maximum of **TPAP**, while the emission is associated with a significant solvatochromic effect. Remarkably, in strongly polar solvents, a Stokes shift as large as 200 nm is observed. The good linear correlation with Δf furthermore suggests that the nature of the fluorescent state is virtually equivalent in all applied solvents.

Next, to estimate the polarity sensitivity of **TPAP**, a Lippert plot was created by plotting the Stokes' shift against Δf according to the Lippert-Mataga equation (eq. 1), resulting in a linear correlation (Fig. 3b). Here, $\Delta\mu$ describes the change in dipole moment upon excitation and can be used as a measure for solvent sensitivity of the fluorophore.²¹ From the slope of the Lippert Plot, we could estimate a $\Delta\mu$ value of 17.4 D for **TPAP** (Note that computed $\Delta\mu$ for **TPAP** are 17.7 D and 12.7 D in acetonitrile and hexane, respectively). This significant dipole change is attributed to the strong ICT character of the excited state, owing to the extended π -system that enables a better charge separation between the oxidation and reduction centers of the molecule, and thereby increasing $\Delta\mu$ in a significant way.

$$\Delta\nu = \Delta\nu_0 + \frac{2(\mu_e - \mu_g)^2}{hcr^3} \Delta f \quad (\text{eq. 1})$$

Fluorescence quantum yields (Φ) and emission lifetimes (τ) of **TPAP** were determined in several solvents and are listed in Table S1. Φ values of **TPAP** are generally very high in apolar solvents, while they are low (< 0.2) in polar and protic milieu (Fig. S4). The Φ linearly decreases with increasing solvent

polarity and, in turn, it confirms the trend established in the emission and Lippert plots.

All emission time decays were fitted by a mono-exponential function. Interestingly, τ values were found to increase linearly up to $\Delta f = 0.22 - 0.25$, while in the high polarity region a sudden decrease set in (Fig. S5). This odd behaviour prompted us to separate out the radiative (k_r) from the non-radiative (k_{nr}) decay rate constants by eq. S2 (Table S1). k_r values showed good linearity when plotted versus Δf (Fig. 4a). In contrast, k_{nr} vs. Δf was associated with a different trend (Fig. 4b). For solvents with $\Delta f < 0.22-0.25$, the polarity of the medium had no influence on k_{nr} . However, in the high-polar solvent region ($\Delta f > 0.22-0.25$) a sudden increase in k_{nr} was detected. This effect was extremely pronounced in protic polar solvents such as methanol and ethylene glycol, suggesting a possible hydrogen-bonding stabilization of the ICT state.

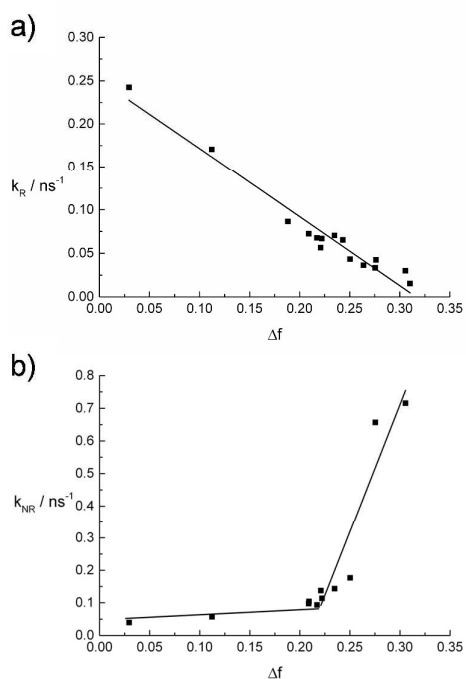


Fig. 4: Plot of the solvent parameter Δf versus a) the fluorescence radiative decay rate constant (k_r) and b) estimated non-radiative decay rate constant (k_{NR}) for TPAP.

Interestingly, beside the above mentioned solvatochromic response, TPAP also exhibit a strong vapochromic response. The emission wavelength of TPAP films ($\lambda_{exc} = 365$ nm) strongly varied when exposed to vapors of solvents with different polarity as observed by naked eye (Fig. 5). In all cases, solvent vapors could be reversibly adsorbed by and removed from the films, with no apparent loss of emission efficiency. These results support the use of TPAP and structurally-related compounds²² as a new chromogenic material suitable for the detection of volatile organic compounds (VOCs). Notably, the recent application of the hue parameter of the HSV color-space for spectral fluorescent sensing²³ could open the way to

a cost-effective way to identify vapours and vapour mixtures by means inexpensive RGB cameras.

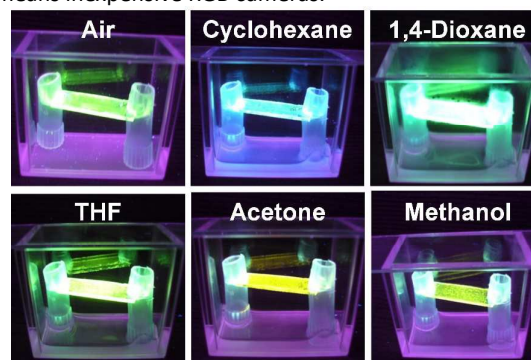


Fig. 5: Vapochromism of TPAP films on glass exposed to air and vapors of different solvents. Excitation wavelength for all samples was 365 nm.

Characterization of TPAP triplet state

Nanosecond transient absorption experiments with excitation at 355 nm were carried out in oxygen-free THF solutions to characterize the triplet state of TPAP. The triplet state of TPAP showed a maximum at 700 nm that decayed over several μ s (Fig. S7). Quenching experiments with molecular oxygen confirmed the triplet nature of the detected absorption, with a quenching constant of $6.5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. Fitting the transient absorption decay traces resulted in a triplet lifetime of 26 μ s for TPAP in THF (Figure S7, inset).

In this context, singlet oxygen quantum yields, gathered at 1275 nm for TPAP in different solvents, helped us to confirm the solvent dependency of the triplet state formation (Fig. S8).^{24,25} The largest production of singlet oxygen was detected in *o*-xylene ($\Phi_{\Delta} = 0.16$), the less polar solvent, followed by THF ($\Phi_{\Delta} = 0.13$). Notably, comparison of these figures with the Φ in the same apolar solvents indicated that intersystem crossing to triplet accounts for a large fraction of the non-radiative depopulation of the singlet excited state. Conversely, in polar solvents like acetonitrile almost no singlet oxygen could be detected ($\Phi_{\Delta} = 0.01$). The low Φ of TPAP in polar solvents suggested that non-radiative decay channels connecting singlet excited and ground states become largely predominant.

Transient absorption measurements on TPAP solutions

To shed light on the excited state processes and the deactivation pathways of TPAP, femtosecond transient absorption spectroscopy was carried out in different solvents (*o*-xylene, THF, acetonitrile and methanol). Photoexcitation (pump) was set at 387 nm, and transient spectra were collected at different times in the 400–1100 nm range (Fig. 6). In all solvents, a large positive absorption appeared at 450–500 nm; smaller positive bands were detected between 550–1100 nm (Fig. 6a). Notably, the band in the 500–600 nm region was found to change in < 10 ps, witnessing the dipolar relaxation of the surrounding solvent molecules (*i.e.*, 1–10 ps is the characteristic time of apolar solvent rearrangement around solvatochromic dyes).²⁶

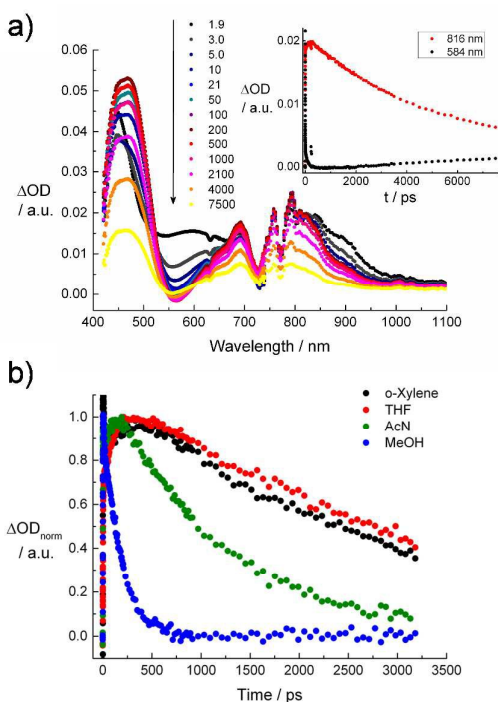


Fig. 6: a) Differential absorption spectra of **TPAP** obtained upon femtosecond flash photolysis in argon-saturated THF with several time delays between 0 and 7500 ps (see legend for details). OD at the 387 nm excitation wavelength was 0.2 – 0.3. Inset: time-absorption profiles in THF monitoring intersystem crossing with singlet decay (red) and triplet formation (black). b) Time-absorption profiles in *o*-xylene (black), THF (red), acetonitrile (green) and methanol (blue) monitored at 480 nm.

Owing to its intensity, the high-energy absorption at 450–500 nm was followed over time from 1 to 3500 ps. Time-plots of absorption at 480 nm showed faster kinetics in polar and protic solvents (acetonitrile and methanol) as compared to medium-polarity or apolar milieu (Fig. 6b). In all cases, the transient profile decayed almost biexponentially (Table S2). The faster component (rate constant k_1) referred to a 10–100 ps kinetic process. The slower component (rate constant k_2) was attributed to the return to the ground state and was characterized by a time constant similar to fluorescence lifetime values. Plot of k_1 and k_2 vs. the solvent polarity parameter Δf resembled the relationship established for the non-radiative decay constants k_{NR} – *vide supra* (Fig. S6). These findings highlight the strong influence of solvent polarity in determining ICT formation and depopulation.

Viscosity dependence of **TPAP** fluorescence

Along with a strong solvatochromism, **TPAP** also presents a good response to the solvent viscosity (η), especially in polar and protic solvents where non-radiative deactivation of excited state is predominant (*vide supra*). Increasing the solvent viscosity should lead to a decrease in the rotor's flexibility with a smaller interval and lower total values of the average dihedral angle being accessible. This influences the

rotor's deactivation pathways by shifting towards a more radiative decay.²⁷

Experimentally, we measured fluorescence emission, in media characterized by high and constant polarity values and variable viscosities. Fig. 7 shows the emission changes for **TPAP** in protic solvents such as methanol ($\eta = 0.52$ at 298 K), ethylene glycol ($\eta = 16.2$ at 298 K), and glycerine ($\eta = 950$ at 298 K). Upon increasing the solvent viscosity, we observed a clear increase of emission intensity accompanied by a significant blue-shift. It is worth noting that shift of the emission wavelength upon changing viscosity is generally not observed for TICT rotors, where emission emerges from an energetically invariable locally-excited state.¹⁰

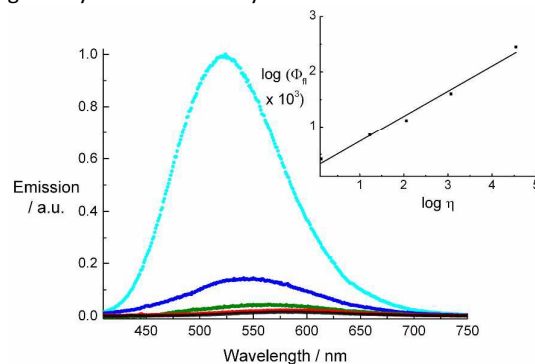


Fig. 7: Emission of **TPAP** in solvents of different viscosity: Methanol (black), ethylene glycol (red), ethylene glycol/glycerine (1:1) (green), glycerine at 295 K (blue), and glycerine at 265 K (cyan). The optical density at the 395 nm excitation wavelength was 0.09 for all samples. Inset: Double-logarithmic plot of the solvent viscosity (η) versus the fluorescent quantum yield (Φ_f) for **TPAP** according to the Förster-Hoffmann equation.

Next, we investigated further the system plotting η vs. Φ_f for five solvents/solvent mixtures. Data complied with the Förster-Hoffmann equation (eq. 2) (Fig. 7, inset),²⁸ which predicts linearity on a double logarithmic scale on account of the response of conformational motions to the solvent viscosity. **TPAP** shows high sensitivity towards viscosity changes as witnessed by the Förster-Hoffmann slope $x = 0.46$. Notably, this value is in keeping with the figures determined for most fluorescent rotors.²⁹

$$\log \Phi_{fl} = x \log \eta + C \quad (\text{eq. 2})$$

As high solvent polarity mainly affects the non-radiative decay pattern, transient absorption spectroscopy experiments were additionally carried out by inspecting the absorption band located at 480 nm. Upon increasing the viscosity from methanol to glycerol, lifetimes increased significantly by a factor of 10 for the short-lived compound k_1 (formation) and by a factor of 4 for the longer-lived compound k_2 (decay) (Table S2). Similarly, a double-logarithmic plot according to the modified Förster-Hoffmann equation was created and revealed linear dependency in the investigated viscosity interval for both k_1 and k_2 (Fig. S9).

Aggregation-induced emission studies on **TPAP**

To evaluate the dependence of **TPAP** emission upon aggregation, we investigated **TPAP** nanoparticles formed in aqueous media by the reprecipitation method.³⁰

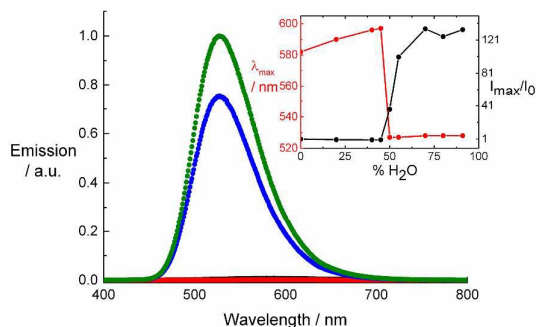


Fig. 8: Fluorescence emission ($\lambda_{\text{exc}} = 390$ nm) of **TPAP** ($[\text{TPAP}] = 4.5 \times 10^{-5}$ M) in methanol (black) and in 45/55 (v/v) (red), 55/45 (v/v) (blue) and 87/13 (v/v) (green) water/methanol mixtures. Inset: Relative emission intensity (right) and emission maximum wavelength (left) of water/methanol mixtures of **TPAP** as a function of the water content.

Notably, the fluorescence of **TPAP** was found to quench upon addition of a small amount of water to a methanol solution. This effect was attributed to the increase in solvent polarity that favoured non-radiative deactivation. However, when the water content exceeded 50% (v/v), the emission gradually intensified reaching a maximum for a water content of around 70% (Fig. 8, inset). The astounding emission increase (120-fold) was attributed to **TPAP** aggregation and it largely surpasses the emission enhancement obtained with **DPAP** (20-fold emission increase).³¹ At the same time, a blue-shift of the emission maximum of ca. 70 nm (from 600 to 528 nm) and a red shift of the absorption maximum from 390 (methanol) to 414 nm (87/13 (v/v) water/methanol mixture) was noticed. Absorption spectra were accompanied by a broad tail reaching to almost 800 nm, which give further evidence to the formation of **TPAP** aggregates (Fig. S10). In addition, upon freezing the **TPAP** water/methanol mixture, a further emission increase (ca. 1.3-fold) was observed (Fig. 9).^{32,33}

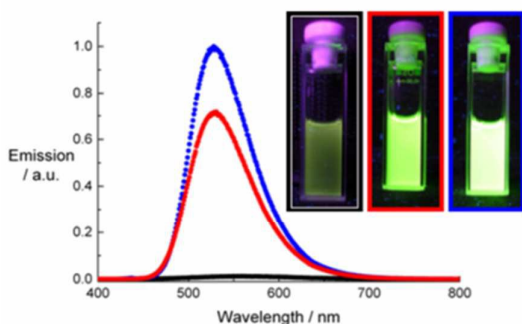


Fig. 9: Fluorescence emission ($\lambda_{\text{exc}} = 390$ nm) of **TPAP** (4.5×10^{-5} M) in methanol (black) and 87/13 (v/v) water/methanol mixtures at room temperature (red) and frozen (green). **TPAP** concentration in both solution and dispersions is 4.5×10^{-5} M. Inset: Pictures of the three cuvettes used for

the fluorescent experiments upon illumination at 365 nm. The coloured boxes around each picture follow the same colour-code used for the fluorescence experiments.

Lifetime studies helped to better rationalize the observed photophysical behaviour. As compared to methanol solution ($\tau = 0.17$ ns), fluorescence lifetime increased up to 12.9 ns for the AIE dispersions and even further (14.3 ns) upon freezing the **TPAP** dispersion (Fig. S13, Table S3). The lifetime increase was consistent with the increase in their emission intensities (*vide supra*). The emission for **TPAP** nanoparticle dispersions decays biexponentially. Time-resolved emission spectroscopy (TRES) highlighted a clear blue-shift of about 20 nm in the 0.5–20 ns time range. This finding suggested the presence of a faster component emitting at shorter wavelengths and a slower component emitting at longer wavelengths (Fig. S14). Indeed, an analysis of the deconvoluted, time-resolved emission spectra corroborated this concept (Fig. S15). From these observations and literature data,^{34,35} we speculated that in the **TPAP** aggregates the local charge-transfer within a monomer is accompanied by intermolecular delocalization of the charge-separated state which deactivates via a long-lived and red-shifted emission.

Dispersions at different **TPAP** concentrations were prepared in an 87/13 (v/v) water/methanol mixture.³⁶ These studies showed that AIE set in above 1 μM **TPAP** (Fig. S11), which is much lower compared to the figure relevant to **DPAP** (20 μM). **TPAP** dispersions were found to be reasonably stable for a few hours; also, ultrasonication did not prompt changes in structure, emission and stability.

The structural properties of **TPAP** aggregates were studied by dynamic light scattering (DLS) and cryogenic-transmission electron microscopy (cryo-TEM) techniques. DLS showed that the **TPAP** aggregates correspond to nanoparticles with radius below 300 nm. The nanoparticle size decreased upon increasing the water content of the dispersing medium (Fig. 10). On the other hand, **TPAP** concentration did not affect the nanoparticle radius (Fig. S12).

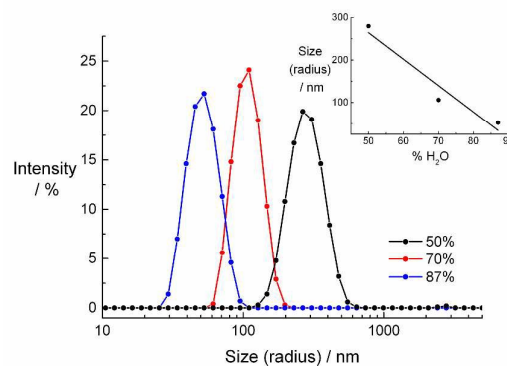


Fig. 10: Dynamic light scattering (DLS) measurements of **TPAP** dispersions ($[\text{TPAP}] = 4.5 \times 10^{-5}$ M) in different water/methanol mixtures (50/50 (v/v) (black), 70/30 (v/v) (red) and 87/13 (v/v) (blue)). Inset: Plot of the average hydrodynamic radius vs. the water content.

Consistently with DLS results, cryo-TEM on a 87/13 (v/v) water/methanol highlighted both spherical and fragmented material with an average size of 100-200 nm (Fig. 11).

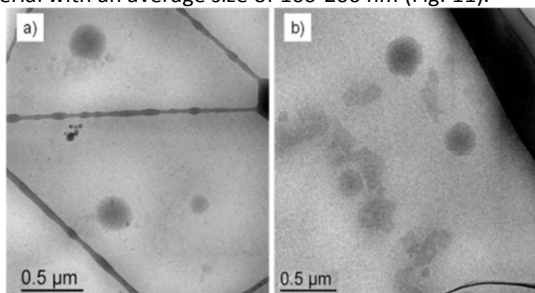


Fig. 11: a,b) Cryo-TEM pictures of 87/13 (v/v) water/methanol dispersions of TPAP at 4.5×10^{-5} M.

TPAP as environmental fluorescent probe in living cells

The last set of experiments established TPAP as environmental probe in living cell, on account of its sensitivity to local viscosity and polarity. At first, TPAP was added to the external medium of Hamster Ovary Cells (CHO) and its intracellular distribution was evaluated by confocal imaging. Within a few minutes from administration, TPAP was found to internalize effectively inside the cells, without affecting their viability. The dye mostly distributed in the endoplasmic reticulum (ER), and in internalization vesicles (Fig. 12). This partition into internal membrane organelles follows the moderate lipophilic nature of TPAP, and it is in agreement with the intracellular distribution of other neutral and lipophilic dyes.³⁷

The ability of TPAP to evaluate local polarity and viscosity was assessed by two complementary techniques, which independently monitor the spectral and lifetime features of the probe, respectively. Both techniques are based on the “phasor analysis” approach developed by the Gratton’s group.^{42,43} In the phasor analysis, the spectral or lifetime profile of each pixel is Fourier-transformed to produce a vector (phasor) represented in a 2D plot named “phasor plot”. Two cartesian (G and S) or polar (phase and modulation) coordinates pinpoint any phasor. In the *spectral* phasor plot, the angular coordinate (phase) is proportional to the spectral center of mass; the radial coordinate (modulation) is inversely related to the spectral width. A red-shift in wavelength leads to an increase of the angular position, whereas a decrease in spectral width increases the radius of the phasor. In the *lifetime* phasor plot, longer lifetimes are associated with larger phase and smaller modulation values. Notably, monoexponential lifetimes map out onto a semicircle of radius $\frac{1}{2}$ and center (1/2,0).

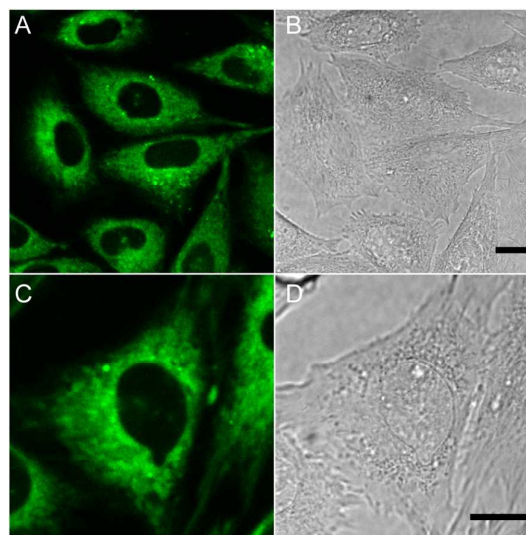


Fig. 12: A,C) Fluorescence confocal images revealing intracellular localization of TPAP in CHO cells and B,D) corresponding transmission images. Scale bar in B,D: 10 μ m.

A cluster of points in any phasor plot corresponds to pixels with similar spectra or lifetimes. Phasor clusters in different positions identify different population/structures in target samples. Thus, the phasor approach provides an ideal tool for hyper-spectral and/or lifetime imaging analysis. Importantly, phasor combinations follow simple vectorial addition rules. For instance, the phasor of a two-component mixture inevitably falls on a line connecting the two phasors of the pure species. The distance from the resultant phasor to the individual “reference” phasors is inversely proportional to the contribution of that component, allowing for the calculation of the intensity fraction associated with each species. Several groups, including us, applied successfully the phasor analysis to follow a broad range of intracellular processes.^{38,39}

The intracellular polarity analysis by TPAP was carried out by sequentially collecting 57 fluorescence confocal images from 430 to 600 nm on a target cell region. Fig. 13A shows the sum of all images and represents the whole emission in the 430–600 nm range. Fig. 13B displays the *spectral* phasor plot relevant to the spectral acquisition. We investigated the phasor clouds relevant to two different cell organelles, namely internalization vesicles (Fig. 13A, cyan arrow) and the ER (Fig. 13A, yellow arrow). The pixels relevant to the two organelles (Fig. 13C) mapped out different phasor regions, which were enclosed by coloured squares in Fig. 13B. Notably, these regions were unlike in term of phase angles (ER: -135° to -180° ; vesicles: -225° to -300°), indicating large spectral differences between these two organelles. Indeed, vesicles were associated with a significant wavelength blue-shift ($\lambda_{\max} = 480$ nm) as compared to ER ($\lambda_{\max} = 500$ nm) (Figure 13D), although the Lippert-Mataga calibration of TPAP highlighted rather hydrophobic environments in both cases. These findings may suggest that TPAP sensed a lower polarity in vesicles than in the ER. Alternatively, the higher TPAP concentration in vesicles

(see intensity differences in Fig. 13A) led to significant dye aggregation and yielded blue-shift likewise what observed for TPAP nanoparticles (*vide supra*).

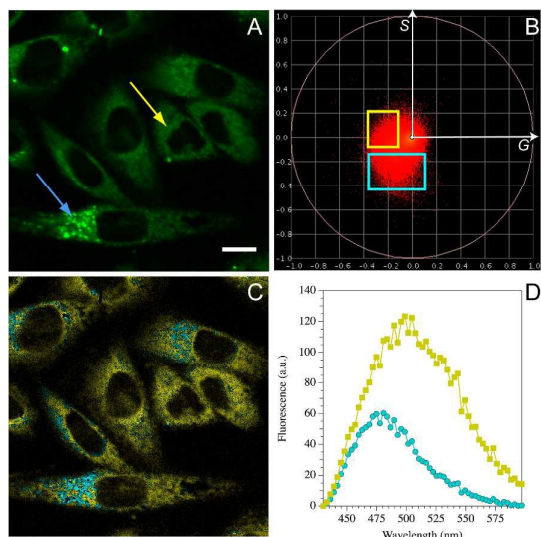


Fig. 13: Polarity analysis of living CHO cells by TPAP emission according to the phasor approach. A) Global fluorescence confocal image of CHO cells obtained scanning the emission interval from 430 to 600 nm in 59 steps; two representative cell vesicle and ER regions are indicated by a cyan and a yellow arrow, respectively; B) Spectral phasor of the 430–600 nm stack of images: two distinguishable phasor regions were enclosed in two colored squares; C) Phasor separation in the confocal image: the pixels corresponding to phasors in the cyan and yellow squares of panel B) are colored accordingly, highlighting cell vesicles and the ER; D) Spectra of phasor regions enclosed in the cyan and yellow squares: each spectrum has the same color code. Scale bar in A): 10 μm .

Next, we carried out intracellular viscosity measurements by inspecting the fluorescence lifetime of TPAP. Fig. 14A reports the collective *lifetime* phasor plot relevant to 19 cell fields. Notably, TPAP showed phasor clouds falling within the universal circle (Fig. 14A). This indicates that TPAP emission is no more monoexponential. Nonetheless, multiexponential decays are often encountered when rotors are embedded in complex cellular domains and reflect the environmental heterogeneity at nanoscale.^{40,41} A second striking feature of the phasor clouds was their almost linear distribution. This arrangement prompted us to consider each TPAP phasor as an intensity-weighted sum of two reference phasors lying on the universal circle, on account of the vector additivity rule of phasors.⁴² The reference phasors *H* and *L* were identified by the intersections between the universal circle and the linear fit of the global phasor cloud associated with all normal cells.

As a matter of fact, this approach correspond to a graphical deconvolution of the lifetime decay into two monoexponential components ($\tau_H = 4.37$ ns, $\tau_L = 1.47$ ns) for each pixel of any image.⁴³

H and *L* identify two limit conditions of high and low local viscosities, respectively.⁴¹ The map of intensity fraction relevant to *H* (X_H) was calculated for each image by simple

vector algebra.⁴⁴ Inspection of the *H* maps (Fig. 14D,G) provided a simple and straightforward way to compare relative viscosities of intracellular domains. TPAP entrapped in internalization vesicles (Fig. 14B white arrow sensed higher viscosity ($\langle X_H \rangle = 0.827 \pm 0.043$) than in the ER (Fig. 14B, purple arrow; $\langle X_H \rangle = 0.707 \pm 0.036$). Consistently, endosome vesicle membranes are about 3 times richer in cholesterol (the most relevant stiffening factor of biomembranes) than the ER.⁴⁵ Higher viscosity could be also brought about by dye aggregation in vesicles, inducing concomitantly a blue-shift of emission as discussed before.

Even more remarkable was the capability of TPAP to report the local viscosity of regions where its fluorescence is rather dim, namely chromatin in the cell nucleus (Fig. 14B,E, red arrow), and plasma membrane (Fig. 14E yellow arrow). In this context, the dividing cell of Figs. 14E-G nicely shows the fluidity of chromatin environment ($\langle X_H \rangle = 0.598 \pm 0.038$) as compared with the rigid environment of plasma membrane ($\langle X_H \rangle = 0.732 \pm 0.068$).

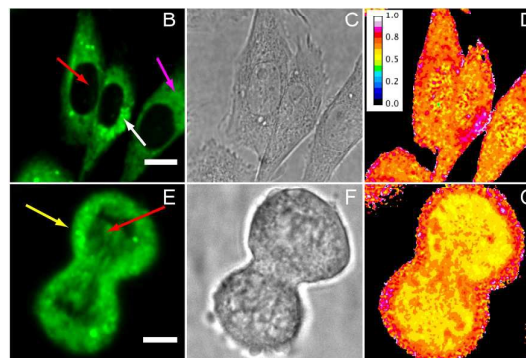
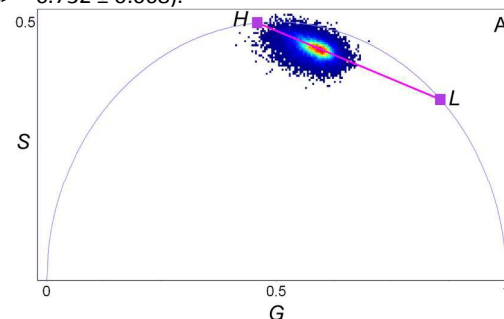


Fig. 14: Viscosity analysis of living CHO cells by TPAP lifetime according to the phasor approach. A) Collective *lifetime* phasor plot relevant to 19 cell images; the calibration line connecting the two reference phasors *H* and *L* is also reported; B,E) Fluorescence confocal images and corresponding C,F) transmission images and D,G) maps of *H* intensity fraction (X_H). Scale bars: B) 10 μm , E) 5 μm .

Conclusions

In summary, in this work we described the complete photophysical and computational characterization of TPAP, a new FMR. TPAP represents a derivative of highly-flexible molecular rotors, which exert barrier-less rotations independent of the solvent. Therefore, it belongs to the same

FMR family of **DPAP**, as its *modus operandi* lacks the presence of a TICT state. Instead, the emissive deactivation of the solvent-stabilized ICT state is either accompanied by triplet formation in non- and medium-polar solvents, or by direct non-radiative conversion into the ground state in polar and protic solvents. As compared to its parent analog (**DPAP**), **TPAP** is 2-3 fold more emissive and it is associated with a solvatochromism that spans an interval of 150 nm, resulting in Stokes' shifts larger than 200 nm in high polar solvents. At the same time, a sensitive viscosity dependence of the **TPAP** emission was established. Hence, with the help of spectroscopic and microscopic techniques, information on the emissive and structural properties of **TPAP** nanoparticles has been also gathered. In particular, **TPAP** nanoparticles show a notable enhancement of emission intensity with lifetimes of up to 14 ns, as obtained from time-resolved studies, which is more than twice as long as lifetime of **TPAP** molecules in solution. On account of **TPAP** peculiar capability to report on local polarity and viscosity, we applied the probe to image confocally these features at intracellular level by a simple and straightforward graphic method named *phasor analysis*. Pleasantly, **TPAP** returned relative polarity and viscosity values of intracellular organelles (*e.g.*, endosomes, endoplasmic reticulum) that are in line with their compositions and structures. Further information on biological systems might be accessible by targeting specific subcellular regions via biofunctionalization of the rotor.

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