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A Multifunctional Chemical Probe for Local Micropolarity and Microviscosity in Mitochondria**

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Abstract

The measurement of physicochemical parameters in living cells can provide information specific to individual cell organelles, helping us to understand subcellular function in health and disease. While organelle-specific chemical probes have allowed qualitative measurements of micropolarity or microviscosity in organelles such as the mitochondria, the simultaneous quantification of mitochondrial local microviscosity (η_m) and micropolarity (ϵ_m), along with structural concurrent structural variations, has remained an unmet need. Here, we describe a new multifunctional mitochondrial probe (MMP) for simultaneous monitoring of η_m and ϵ_m . This new probe enabled highly precise, quantitative measurements of local mitochondrial microviscosity and micropolarity in the presence of a variety of agents perturbing cellular function, and the changes observed using the probe could also be correlated with alterations in mitochondrial network morphology and motility. The strategy described represents a promising tool for the analysis of subtle changes in organellar structure.

Graphical Abstract

A new fluorescent probe enables highly precise, quantitative measurements of local mitochondrial microviscosity and micropolarity in the presence of a variety of agents perturbing cellular function, where the observed changes could also be correlated with alterations in mitochondrial network morphology and motility.

[**]Supporting information for this article is given via a link at the end of the document.

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Supporting Information. TCSPC procedure and imaging, confocal imaging collection for all swelling agents. Plasma membrane potential depolarization treatment.

Keywords

Mitochondria; Cellular Microviscosity; Cellular Polarity; Fluorescent Probes

Measuring subcellular activity in living cells with fluorescent sensors is a powerful means to collect information specific to a particular subcellular microenvironment.^{1–9} In particular, mitochondria – the powerhouses of the cell – possess a variety of physicochemical parameters such as membrane potential, micropolarity, local microviscosity, proton gradient, ionic transport, redox environment and temperature that are of interest due to their influence on the efficiency of mitochondrial metabolism.^{3, 10–20} Mitochondrial micropolarity (ϵ_m), mitochondrial membrane potential (ψ_m) and local microviscosity (η_m) are particularly important as they affect cellular bioenergetics, ATP production, reactive oxygen species (ROS) production and regulation, and the initiation of cell death pathways.

The development of imaging tools to monitor fast and, often subtle, changes in mitochondrial microenvironment is a challenging goal given the difficulty of specific intracellular delivery of functional probes that can accurately assess physiochemical phenomena.²¹ Many probes embed features required for mitochondrial localization, including hydrophobicity and delocalized cationic charge, into the structures of fluorescent sensors. Hydrophobic character is important given the lipophilic nature of the mitochondrial membranes, and cationic charge helps molecular cargo traverse the mitochondrial membrane by responding to the negative potential across this barrier.

Intrinsic mitochondrial localizers have enabled ratiometric fluorescence imaging of cellular polarity, as well as self-calibrated viscosity sensing in this organelle.^{10–11, 18, 22} Fluorescent molecular rotors featuring BODIPY and julolidine derivatives have also enabled the measurement of local microviscosity, and probes including rhodamine derivatives and JC-1 helped elucidate charge polarization and ψ_m changes.^{23–25} A limitation of these probes, however, is that the localization is controlled by the same chemical moieties that serve as reporters, which can complicate the interpretation of fluorescence profiles. Delivery vectors that employ separate functional groups for mitochondrial import and chemical sensing have been described,³ but primarily rely on the triphenylphosphonium (TPP) cation, which has limited compatibility with many neutral and anionic sensors of interest. The development of more versatile and multifunctional intramitochondrial sensors would benefit from the use of

an adaptable delivery vector that could import molecular sensors with appropriate chemical functionalities for multiparameter monitoring through different detection channels such as fluorescence lifetime and intensity outputs for local microviscosity and polarity, respectively.

Here, we describe the design and study of a new fluorescent probe (**MMP**, Figure 1) that takes advantage of a peptide-based vector for the delivery of sensors enabling simultaneous mitochondrial micropolarity and local microviscosity monitoring. Measuring these parameters with a single integrated probe is critical as the system fundamentally acts as a double-referenced reporter that can measure these two phenomena simultaneously. The delivery vector is a mitochondria-penetrating peptide,^{21, 26–27} which is a cell permeable, mitochondria-specific vector having no membrane disruptive activity. We describe the characterization of this molecular sensor and the first systematic study using a mitochondria-targeted probe to obtain quantitative ϵ_m and η_m values concurrently.

Mitochondria-targeted probe design and subcellular localization.

Our strategy to develop a multifunctional mitochondrial probe, **MMP**, appropriate for the simultaneous monitoring of microviscosity and micropolarity was to combine a fluorescent phenylquinoxaline-derived molecular rotor that undergoes internal rotation at a rate dependent on local microviscosity²⁸ (but independent of polarity variations), with coumarin 343, a solvatochromic polarity probe²⁹ with blocked internal bond rotations that avoid local microviscosity crosstalk. Attaching these two dyes to the termini of a mitochondria-penetrating peptide separates the functional modules of the probe, and thus, the electric dipolar nature and solvatochromic character of the coumarin moiety is purely directed by the polarity of the cellular microenvironment. On the other hand, the fluorescence decay profile of the neutral phenylquinoxaline rotor is solely governed by changes in its local microviscosity and its large Stokes shift prevents deactivation via energy transfer. The use for these two sensors allows the environmental measurements to be made by monitoring two different phenomena; microviscosity is measured by monitoring the fluorescence lifetime of the phenylquinoxaline sensor, while micropolarity is measured by monitoring the emission profile of the coumarin sensor.

The **MMP** conjugate was synthesized using solid-phase synthesis and characterized as described in the Supporting Information. As shown in Figure 1, the **MMP** exhibits highly specific localization in the mitochondria of a human cell line. The conjugate allows clear visualization of the intracellular mitochondrial network (Figure 1B), and when colocalized with a mitotracker dye (Figure 1C), it is readily apparent that the multifunctional sensor exhibits strong and specific mitochondrial localization.

Characterization of the phenylquinoxaline sensor for viscosity measurements.

The fluorescent molecular rotor selected for the **MMP**, phenylquinoxaline, was characterized to evaluate its behavior as part of the sensor conjugate. Solvent viscosity influences the rate of the nonradiative decay process resulting from intramolecular rotation within the fluorophore,³⁰ and this relationship can be represented in a Förster-Hoffmann

logarithmic plot relating the fluorescence quantum yield (Φ_F) to viscosity³¹ as shown in Figure 2A.

A viscosity calibration study measured the fluorescence lifetimes (τ) of the MMP at different viscosities using methanol:glycerol mixtures with time-correlated single photon counting (TCSPC). Both fluorophores were monitored with the fluorescence green channel (from 400 nm to 570 nm) which encompasses both the phenylquinoxaline (τ_1) and the coumarin 343 (τ_2) emission profiles (Figure 2B). The fluorescence lifetime of τ_1 increased from 1.19 ± 0.17 to 4.89 ± 0.32 ns as viscosity was increased while τ_2 exhibited a much smaller increase. The small change in the coumarin 343 lifetime likely reflects that the polarity of the solvent mixtures slightly increases as viscosity is varied.

Characterization of coumarin 343 as a solvatochromic polarity probe.

As noted above, the coumarin 343 moiety in the **MMP** does not exhibit significant changes in fluorescence lifetime with increasing viscosity. However, the fluorescence spectra shown in Figure 3A illustrate significant sensitivity to solvent polarity, where the coumarin emission maximum exhibits a strong enhancement as the polarity of the solvent is decreased. When the ratio of fluorescence intensity at 420 nm versus 570 nm was quantified, a linear relationship was observed with a regression coefficient of $R^2 = 0.99$.

Local microviscosity variations in mitochondria.

Intracellular mitochondrial microviscosity was monitored using the **MMP** under conditions promoting swelling to assess the ability of the probe to track dynamic changes. Swelling is perhaps one of the most common physical changes observed in mitochondria, where the organelle morphology within the interconnected mitochondrial network can vary from elongated filamentous to swelled spherical forms.³² These morphological changes give rise to pronounced physicochemical variations in the organelle microenvironment.³³

Table 1 shows the fluorescence lifetime values and corresponding microviscosity values when cells are exposed to nystatin and monensin. Each value represents a single FLIM image pixel recording, while cell by cell averages are presented in Figure 4. Both swelling agents caused detectable changes. In the case of monensin a subtle lifetime increase is observed, consistent with the microviscosity varying from 63.5 to 91.2 cP. Nystatin, on the other hand, caused a strong effect on microviscosity, with microviscosity values changing from 63.5 to 132.2 cP. The microviscosity values using the **MMP** are comparable to the values reported in the literature for other microviscosity probes,¹⁸ but this is the first study to resolve the dynamics of microviscosity fluctuations. Further analysis with different swelling agents is presented in the supporting information file, Table S1. Fluorescence intensity controls for swelling and apoptotic agents as well as are shown Figure S4.

To visualize these changes at the single cell level, we acquired FLIM images of HeLa cells incubated with **MMP** and treated with monensin and nystatin (Figure 4). The green channel fluorescence was detected at 510 nm using an appropriate optical filter set. The lifetime changes described above are reflected qualitatively in the FLIM images and the pronounced changes in viscosity observed with nystatin are readily apparent.

Mitochondrial polarity monitoring using the MMP.

To analyze the changes in mitochondrial polarity, HeLa cells treated with swelling agents were analyzed using ratiometric confocal imaging (Figure 5). As mentioned above, the phenylquinoxaline group fluorescence in the **MMP** is unaffected by polarity variations and serves as a ratiometric reference for excitation at 402 nm, thus allowing the monitoring at 420 – 480 nm as compared to 576 – 613 nm emission.

The polarity calibration plot presented in Figure 3B was used to quantitate this parameter. In the case of nystatin, a significant increase in polarity was observed in addition to the swelling described above, with the dielectric constant changing from $\epsilon = 18.9 \pm 0.5$ to 24.6 ± 1.1 (Figure 5A). This is consistent with a mitochondrial accumulation of Na^+ and water molecules as a result of an increase in the Na^+ influx through the nystatin pores with a simultaneous osmotically driven H_2O influx.³⁴ Similar results were obtained with monensin. Small variations were observed with rotenone, artesunate and artemisinin. In the case of cyclosporine A, no significant polarity variations were observed (Figure 5B, 5C and S2). However, this strong nystatin effect could be a consequence of both, microviscosity and polarity parameters.

Previous studies have indicated that cell polarity in dying cells is lower than in normal cells.¹¹ To evaluate if the **MMP** is able to report on losses in mitochondrial polarity we analyzed the response of the **MMP** when cells were incubated with the apoptotic agents etoposide, cisplatin and hydrogen peroxide. Figures 5D, 5E, and 5F show (in addition to changes in cell morphology) that the mitochondrial polarity decreases to 14.76 ± 1.10 , 9.81 ± 1.65 and 7.12 ± 3.92 for etoposide, cisplatin and H_2O_2 , respectively. It is noteworthy that treatment with H_2O_2 was challenging to follow since **MMP** fluorescence was drastically diminished after 15 min.

Mitochondria morphology monitoring with the MMP in HeLa cells.

To analyze changes in mitochondrial morphology in detail we conducted time-dependent experiments using monensin and nystatin. During confocal fluorescence experiments, each image was manually focused and excitation laser light was fully shielded between recordings to prevent artifacts and photobleaching.

A series of time-lapse images is shown in Figure 6 before and after treatment with nystatin. The images of cells treated only with the **MMP** show no significant changes 20 minutes later. However, after the nystatin treatment the degree of co-localization decreased dramatically. Extensive mitochondrial motility is observed, and the filamentous shape of the mitochondria changed to more ovoid structures with concomitant mitochondrial fragmentation, thus causing a decrease in the form factor values. Moreover, overall cell morphology was also affected following nystatin treatment; Figure 6B shows the initial and final morphology after treatment where a subtle decrease in cell size is observed (continuous vs. dashed lines), this is a consequence of a general mitochondrial network fragmentation and motility.

Quantitation of mitochondrial shape was achieved by calculating form factor, integrated area and motility descriptors. The mitochondrial form factor is defined as $F = (\text{perimeter})^2 / (4 \cdot \pi \cdot A)$. Figure 6C displays the average effect of monensin and nystatin on the form factor, and the results are consistent with the images in Figure 6A indicating a strong change in mitochondrial shape with nystatin treatment. Not surprisingly, monensin treatment did not cause significant changes to the form factor. Figure 6A shows the time lapse change in form factor upon nystatin application for one individual mitochondria.

The influence of pH and membrane potential changes on probe response were also explored (Figure S3). Using 100 nM nigericin, the mitochondrial pH gradient (pH_m) was effectively abolished since nigericin promotes a K^+/H^+ equilibration ($[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$, therefore $[\text{H}^+]_{\text{in}} = [\text{H}^+]_{\text{out}}$ and $\text{pH}_m = 0$). The live cell experiments with **MMP** were always corrected by these control parameters.

The effects of mitochondrial membrane polarization were also investigated to determine its impact on **MMP** fluorescence (Figure S5–6). Mitochondrial hyperpolarization with NaCl resulted in a 15% increase in **MMP** uptake, while the effect of nystatin and monensin on the fluorescence intensity resulted in a decrease overtime, however the remaining fluorescence signal was still sufficient to monitor mitochondrial parameters. Addition of the ionophore nigericin was also found to have negligible effects on **MMP** fluorescence, indicating that varying pH has little impact on signal readout of the probe.

The multifunctional probe presented here is a powerful new tool for the simultaneous monitoring of local microviscosity, micropolarity, and morphology variations in mitochondria. The probe was shown to reliably monitor the effects of swelling- and apoptosis-inducing agents on the physicochemical properties of the mitochondria. Particularly, the strong effect of nystatin not only on morphology but also on the micropolarity and microviscosity of mitochondria were quantitatively analyzed for first time, using a self-calibrating targeted probe. The dual probe thus offers a reliable platform for these parameters to be monitored in real time under very subtle input stimuli. Given the importance of microviscosity and micropolarity on mitochondrial function and bioenergetics, this probe is an enabling tool for further investigation of these parameters in human health and disease.³⁵ Moreover, coupling this type of multifunctional probe with super-resolution spectroscopy will provide a powerful tool for cell characterization.³⁶

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(**A**) Structure of the multifunctional mitochondrial probe (**MMP**). The solvatochromic, polarity-dependent coumarin 343 is represented in green, the phenylquinoxaline molecular viscosity rotor is displayed in blue, and the mitochondria-penetrating peptide fragment is displayed in black. (**B**) Intracellular localization of the MMP in live HeLa cells. (**C**) Correlation of the **MMP** localization (green) with the mitotracker dye (red). The high level of overlap for the localization of these two molecules is indicative that the **MMP** is mitochondria-specific. Scale bars represent 20 μm .

Figure 2.

(A) Förster-Hoffmann plot of viscosity (η) vs. fluorescence quantum yield (Φ_F) for the **MMP**, as well as the ratio of fluorescence maxima at 512 and 488 nm. Hollow and filled symbols are $\log \Phi_F$ and ratio values, respectively. (B) Fluorescence lifetimes in nanoseconds of 10 mM **MMP** in solutions with viscosities ranging from 0.6 cP (methanol) to 1420 cP (glycerol). A TCSPC system exciting at 402 nm was used to characterize the biexponential lifetime profile (See Figure S1). The instrument response function (IRF) was measured by using coumarin 6 probe and potassium iodide as a quenching agent.

Figure 3.

(A) Variation of fluorescence profile for 40 μM MMP in water: dioxane mixtures. (B) Calibration of observed solvatochromism as a function of dielectric constant (ϵ). Filled and hollow symbols are *ratio* and *weight %* values, respectively.

Figure 4.

(A) FLIM images of HeLa cells incubated with the **MMP**, and cells also treated with (B) monensin and (C, D) nystatin detected in the green channel. The calibration bar represents the change in fluorescence lifetime values ranging from 0.7 to 3.8 ns with event counts of 2200 ± 100 . Viscosity values corresponding to these images are tabulated in Table 1.

Figure 5.

Effect of swelling agents on the mitochondrial polarity of HeLa cells stained with 10 μ M MMP. Pseudo-color images show increases in polarity 1 hour after treatment with: (A) 10 μ g/mL nystatin; (B) 10 μ M monensin and (C) 10 μ M rotenone. Left images represent non-treated cells and the right images represent treated cells. Apoptotic agents caused a notable polarity decrease when treated with (D) 2 μ M etoposide; (E) 25 μ g/mL Cisplatin and (F) 50 μ M H_2O_2 , the left images are non-treated cells; the right images are 4 and 8 hours post-

treatment. **(G)** Dielectric constant (ϵ) values for swelling and apoptotic agents. Mean r values are interpolated with Figure 3B.

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Figure 6.

Effect of nystatin on the mitochondrial morphology and fluorescence intensity of HeLa cells stained with **MMP**. **(A)** Time course monitoring morphology changes: the cells are stained with 10 μM **MMP** in the initial state (in red) and two images were recorded after 10 min and 20 min for control, then 10 $\mu\text{g/mL}$ nystatin was added and the images were recorded after each 5 min (in green). Nystatin triggers mitochondrial motility and shape variations as evidenced by loss in colocalization (yellow variation). **(B)** Superposed images after 1 min (continuous line) and 20 min (dashed line) post-nystatin addition to show the change in cellular footprint. **(C)** from left to right: (left) quantitative evaluation of mitochondria form factor (F) after 15 min nystatin and 20 min monensin addition; (right) time dependence of form factor changes.

Table 1.

Dynamics of microviscosity changes caused by monensin and nystatin on the fluorescence lifetimes obtained by FLIM measurements in HeLa cells stained with **MMP**. The interpolated microviscosity values are highlighted in bold. Values in parentheses represent standard errors calculated from replicate experiments.

Time	+ Monensin		+ Nystatin	
[min]	time [ns]	viscosity[cP]	time [ns]	viscosity[cP]
0	3.110(3)	63.5	3.110(3)	63.5
5	3.132(2)	75.5	3.124(6)	73.7
10	3.145(3)	80.3	3.263(1)	106.4
15	3.159(2)	86.0	3.319(2)	126.4
20	3.160(8)	88.9	3.375(3)	130.9
25	3.173(6)	91.2	3.384(5)	132.1
30	3.175(2)	91.9	3.389(2)	132.2