Novel nontoxic mitochondrial probe for confocal fluorescence microscopy

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Abstract. We propose a 2,5-Bis[1-(4-N-methylpyridinium)ethen-2-yl)]-N-methylpyrrole ditriflate (PEPEP) as a novel nontoxic, nonpotentiometric mitochondrial probe for confocal fluorescence microscopy. PEPEP is a representative chromophore of a large family of heterocyclic fluorescent dyes that show fluorescence emission in aqueous media and great DNA affinity. We check its cytotoxicity and intracellular localization in mammalian and yeast cell cultures. We demonstrate that PEPEP is a very efficient dye for fluorescence confocal microscopy and a valuable alternative to the most frequently used mitochondrial stains.
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Keywords: confocal fluorescence microscopy; chromophore; donor acceptor system; mitochondrial stain; fluorescence.

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1 Introduction

Detailed monitoring of a physiological process in living cells is one of the main goals in biological and medical sciences. To this aim, laser scanning confocal fluorescence microscopy (LSCFM) offers higher resolution and sensitivity than conventional fluorescence microscopy and allows 3-D analysis. Among commercial dyes, few fluorophores are characterized as mitochondrial stains, most of them being potentiometric. 2,5-Bis[1-(4-*N*-methylpyridinium)ethen-2-yl)]-*N*-methylpyrrole ditriflate (PEPEP) (see Fig. 1) is a representative chromophore of a large family of heterocyclic fluorescent dyes that shows fluorescence emission even in aqueous media, an important prerequisite for cellular fluorescence microscopy.[1,2] *In vitro* interaction studies with biomolecules show that PEPEP has DNA affinity.[3] In view of possible applications of PEPEP as a fluorescent marker in living cells, we checked its cytotoxicity and intracellular localization in different cell cultures such as human umbilical vein endothelial cells (HUVEC), human lymphoblast U937, immortalized monkey kidney COS7, and yeast *Saccharomyces cerevisiae*.

2 Materials and Methods

2.1 *Chemicals*

3,6-diamino-9-(2-(methoxycarbonyl) phenyl-, chloride (R123) and dimethylaminostyryl-methylpyridinium iodide (DASPMI) were purchased from Molecular Probes (Eugene, Oregon). The stock solution concentrations of R123 and

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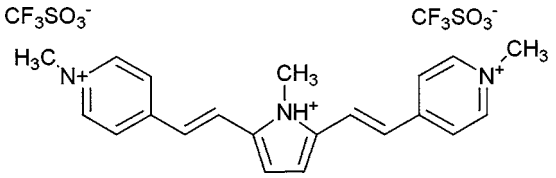

Fig. 1 Molecular structure of PEPEP.

DASPMI were determined by spectrophotometric methods. PEPEP was prepared in our laboratory.[1] In phosphate buffer (pH 7), PEPEP shows a single absorption at 525 nm, with an extinction coefficient of 52,000±3000 M^{-1} cm^{-1}, and a fluorescence emission peak at 605 nm with a ~0.1 fluorescence quantum yield.[3] Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was purchased from Sigma.

2.2 *Cell Cultures*

We used four different cell cultures. 1. Human umbilical vein endothelial cells (HUVEC) from the American Type Culture Collection (ATCC) cultured in M199 medium containing 10% fetal calf serum, ECGF (150 mg/ml), and heparin (5 U/ml) on 2% gelatin-coated six well multidishes. Cells were subcultured using 0.05% trypsin 0.02% EDTA solution. 2. U937 cells cultured in RPMI medium containing 10% fetal calf serum. 3. Immortalized monkey kidney COS7 cells cultured in DMEM medium containing 10% fetal calf serum. 4. Yeast (bakery) *Saccharomyces cerevisiae*, in stationary growth phase. All culture reagents were from Sigma.

2.3 *Citotoxicity*

Cytotoxicity of PEPEP has been assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) kit, designed for the spectrophotometric measurement of cell growth as a function of mitochondrial activity in living cells. The experiment has been performed on U937 and HUVEC cultured in 96-well plates. Cells have been treated with PEPEP solution (5 μM) for 24 e 48 h, and then incubated with MTT for 4 h at 37 °C. MTT reduction by intact cells was performed in plates containing 150-μL medium/150-μL dye solution. Absorbance values were determined at 570 nm.

2.4 *Sample Preparation*

HUVEC were seeded in 4-cm Petri dishes and allowed to grow at 37 °C and 5% CO_2 up to 50 to 70% confluence. U937 and COS7 were cultured in flasks at 37 °C and 5% CO_2. The medium was then removed and the cells were washed twice with phosphate buffered saline (PBS) and incubated at 37 °C and 5% CO_2 in the growth medium containing the appropriate dye for each specific staining. In details, either 5-μM PEPEP solution was added for 30 min or 24 h, or 1-μM R123 solution for 10 min, or else 10-μM DASPMI solution for 15 min. For the double staining experiment, cells were incubated with 5-μM PEPEP solution for 20 min, then 1-μM R123 solution was added for 10 min. For the FCCP experiment, the cells, treated with 5-μM PEPEP solution for 30 min as described earlier, were added up with 2-μM FCCP solution for 5 to 10 min; after incubation, the cells were washed twice with PBS. To prevent cell drying, few microliters of PBS were added before the coverslip was placed on. For the yeast experiments, the cells suspended in PBS were treated with 5-μM PEPEP solution for 30 min and then seeded on the microscope slide.

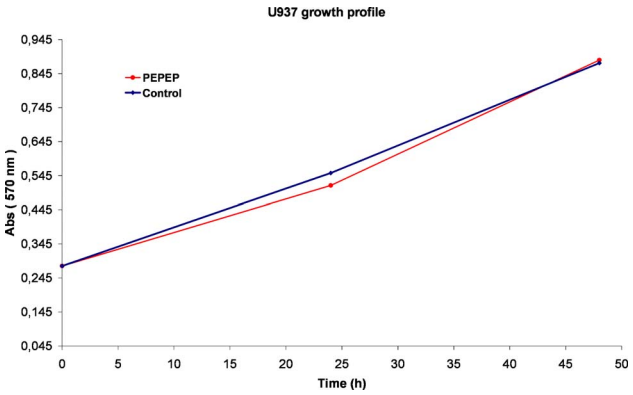

Fig. 2 PEPEP viability test. U937 were treated with dye solution (10^{-5} M) for 24 and 48 h, followed by 4 h of MTT reduction at 37 °C. Absorbance values were determined at 570 nm. Absorbance is directly related to viable cell numbers.

2.5 *Confocal Laser Scanning Fluorescence Microscopy*

The intracellular distribution of the dyes was studied with LSCFM using a laser scanning confocal microscope MRC-600 (Bio-Rad Microscience Division, United Kingdom) coupled to an epifluorescence microscope Nikon Optiphot II. A 60× oil immersion objective with NA=1.4 or a 20× air objective (NA=0.4) were employed. Dye fluorescence was excited with the 488-nm argon laser line and detected through a long-pass filter above 515 nm, by the fast photon counting mode of the MRC-600 microscope. In the double staining experiment dye fluorescence was excited at 488 nm, and the two fluorescence emissions were detected through a band-pass filter at 540 nm for R123 (green channel) and through a long-pass filter above 600 nm for PEPEP (red channel). For

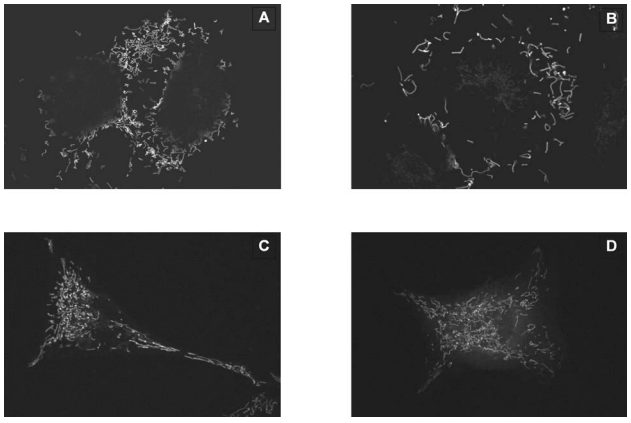

Fig. 3 Fluorescence images of mitochondrial localization of PEPEP in HUVEC. Cells were incubated with: (a) 5-μM PEPEP for 30 min; (b) 5-μM PEPEP for 24 h; (c) 1-μM R123 for 10 min; and (d) 10-μM DASPMI for 15 min.

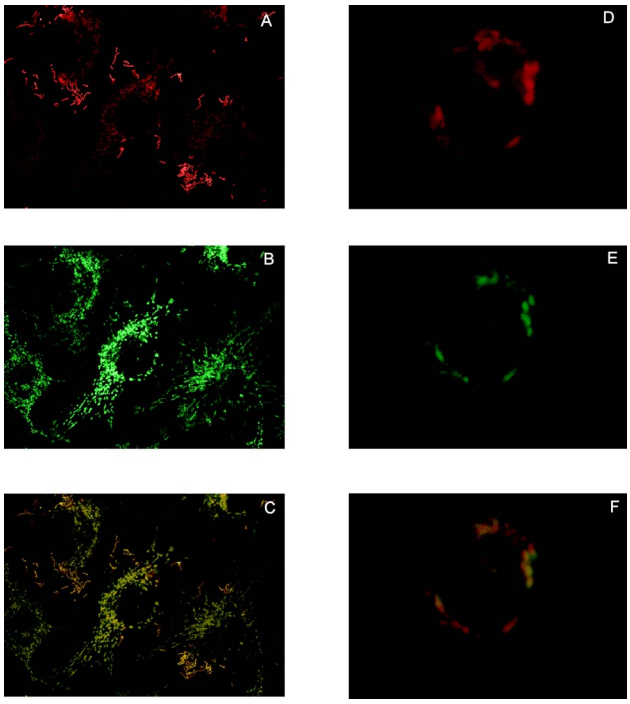

Fig. 4 Fluorescence images of mitochondrial colocalization of PEPEP and R123 in HUVEC and U937. Cells were incubated in the presence of 5-μM PEPEP for 20 min, then 1-μM R123 was added for 10 min. HUVEC cells: (a) red channel, PEPEP fluorescence; (b) green channel, R123 fluorescence; and (c) merged image. U937 cells: (d) red channel, PEPEP fluorescence; (e) green channel, R123 fluorescence; and (f) merged image (color online only).

the experiment with FCCP, to image the same field of view before and after the addition of FCCP, a 20× air objective was employed.

3 Results and Discussion

PEPEP cytotoxicity has been evaluated using the assay based on MTT reduction via mitochondrial dehydrogenase activity of living cells. Up to 48 h of incubation with 5-μM PEPEP, the growth of U937 (Fig. 2) and HUVEC is not affected, showing that the new dye does not compromise cell viability and can be used as nucleic acid probe in living cells.

The intracellular localization of PEPEP in different cell lines was investigated by LSCFM using the MRC-600 confocal microscope with argon laser excitation at 488 nm and photon counting detection. The high sensitivity of this detection allowed us to employ a low power laser excitation (0.1 mW) at the entry of the optical head,[4,5] an essential condition for the study of active mitochondria—known to be damaged by light[6–9]—in living cells.

Cells incubated with PEPEP at a final concentration of 5 μM for 15 to 30 min were found to be permeable to the dye. We report the intracellular distribution of PEPEP in HUVEC for short [Fig. 3(a)] and long [Fig. 3(b)] incubation times. In both cases, the compound was found to localize selectively in mitochondria, with an intense fluorescent signal and a higher accumulation at 24 h.

To estimate the efficiency of PEPEP in staining mitochondria, we present for comparison the HUVEC intracellular localization of two well known potentiometric vital probes, R123 [Fig. 3(c)] and DASPMI [Fig. 3(d)], whose mitochondria affinity is well documented in the literature.[10,11]

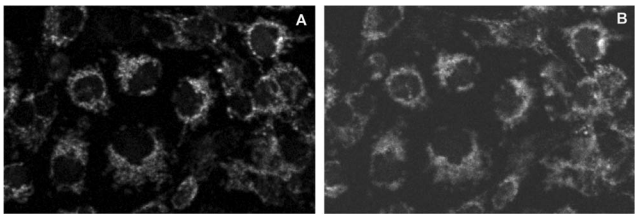

Fig. 5 Images of mitochondrial localization of PEPEP in COS7 cells in (a) the absence and (b) the presence of 2-μM FCCP for 5 to 10 min.

To validate PEPEP as a mitochondrial probe, we studied its intracellular distribution in different cell cultures. In addition to HUVEC, we considered U937 (Fig. 4) and COS7 (Fig. 5) as model systems for physiological and pathological mammalian cultures, and yeast *Saccharomyces cerevisiae* for its relevance in biotechnology and bioengineering (Fig. 6). In particular, we performed colocalization experiments of PEPEP and R123 in HUVEC and U937. Colocalization of the two probes is evident in the merged images reported in Fig. 4, and strongly supports the mitochondrial specificity of PEPEP. In addition, colocalization levels in the two images for HUVEC and U937 cells were evaluated by using the colocalization test plug-in for image J (Wright Cell Imaging Facility, Toronto, Canada). The Pearson correlation coefficient ranges at about 95% for HUVEC and 80% for U937 cells.

Stimulated by these results, we investigated whether the mitochondrial uptake of this new probe was driven by the organelle membrane potential or by its high DNA affinity. We studied the fluorescence distribution of PEPEP in COS7 cells treated with a membrane-potential uncoupler, the protonophor carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). The images of stained cells in the absence and in the presence of 2-μM FCCP are reported in Figs. 5(a) and 5(b), respectively. Unambiguously, the dye fluorescence intensity and localization are not appreciably affected by the uncoupler, suggesting that mtDNA is the target of PEPEP, as expected taking into account its affinity for DNA in solution.[3] We confirmed that no nuclear localization was observed in viable cells, in agreement with what was already reported for other nucleic acid probes in living intact cells.[4,8,9,12] The impact of alterations in mitochondrial membrane potential ($\Delta\Psi m$) and oxidant stress on PEPEP staining in mitochondria with eventual relocalization of the dye will be the object of future investigations.

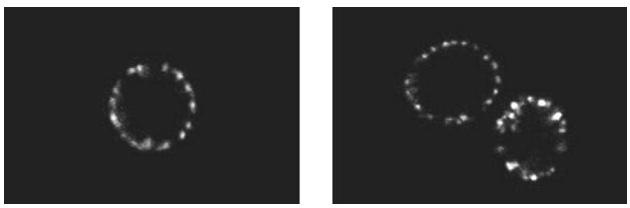

Fig. 6 Fluorescence images of mitochondrial localization of PEPEP in *Saccharomyces cerevisiae* cells. Cells were incubated in the presence of 5-μM PEPEP for 30 min.

In conclusion, our results indicate that PEPEP enters easily into living cells, is not cytotoxic and localizes in mitochondria with a nonpotentiometric mechanism. All of these data unequivocally prove that PEPEP is a very efficient dye for fluorescence confocal microscopy, and a valuable alternative to the most frequently used mitochondrial stains.

Acknowledgments

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