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A mitochondria-targeted protonophoric uncoupler derived from fluorescein†

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Linking decyl-triphenyl-phosphonium to fluorescein yields a fluorescent probe that accumulates in energized mitochondria, facilitates proton transfer across membranes and stimulates mitochondrial respiration. This features a mitochondria-targeted uncoupler, being of potential interest for therapeutic use against oxidative stress-related diseases.

An electrochemical proton gradient across energy-coupling (either mitochondrial or photosynthetic) membranes resulting from oxidative or photosynthetic electron transfer is employed to drive ATP synthesis. This fundamental principle of bioenergetics was validated primarily by using uncouplers – agents capable of dissipating the proton gradient and electrical potential difference by facilitating transmembrane proton movement thereby leading to uncoupling of electron transfer and phosphorylation. The protonophoric uncouplers have attracted much attention not only from the mechanistic point of view, but also as promising agents potentially curing obesity. Of note, the anti-obesity use of the uncoupler dinitrophenol (DNP), later dismissed because of deaths caused by occasional overdosing,[1] was demonstrated very early along with its protonophoric activity.[2] Recently, limited uncoupling, *i.e.* a reduction of proton-motive force to a level being still sufficient for maintaining ATP synthesis but diminishing reactive oxygen species (ROS) production,[3] was found to be beneficial, in particular by protecting living organisms from a variety of pathologies associated with oxidative stress.[4] On the way to therapeutic use of mild uncouplers, it is of importance to carefully examine their interaction with different cell compartments and tissues, for which brightly fluorescent compounds as reporters of their intracellular localization could be useful. On the other hand, direct targeting to mitochondria could reduce the therapeutic concentration and side effects of uncouplers.

Earlier attempts were made to render uncouplers specific to mitochondria by their conjugation to the triphenylphosphonium (TPP) cation preferentially accumulating in the negatively charged interior of mitochondria. Unfortunately, the obtained compounds either lacked uncoupling activity,[5] or showed rather weak activity which was mediated by the mitochondrial carrier adenine nucleotide translocase (ANT).[6] To combine three essential requirements such as protonophoric activity, intense fluorescence and mitochondria targeting, we designed here a compound containing a TPP moiety attached to fluorescein *via* an alkyl linker (mitofluorescein, mitoFluo), taking into account that the use of lipophilic cations such as alkylTPP has enabled targeting of a great variety of molecules to mitochondria.[7] The rationale of the design is based on our previous study, where we observed the submicromolar uncoupling activity of dodecyl and octyl fluorescein esters, brightly fluorescing compounds, which did not accumulate in mitochondria in an energy-dependent manner.[8] The uncoupling activity of fluorescein alkyl esters depended non-monotonically on the alkyl chain (C_nH_{2n+1}) length with an optimum at $n = 8$ (n – the number of carbon atoms). Here we chose an alkyl linker with $n = 10$, because conjugation with positively charged TPP was expected to reduce the hydrophobicity of the chimeric compound.

The synthesis of mitoFluo, 10-[2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoyl]oxydecyl-triphenyl-phosphonium bromide, involved quaternization of triphenylphosphine and then synthesis of fluorescein ester by reaction with the resulting 10-bromdecyl-triphenyl-phosphonium bromide (Scheme S1, ESI†).

Most of the conventional uncouplers are weak acids with a pK_a close to physiological pH, which are able to permeate the membrane both in charged and neutral forms.[1,9] These substances usually show maximal activity at pH values higher than pK_a due to a lower membrane permeability of a negatively charged form than that of a neutral form. Being also a weak acid with a pK_a of 6.9, as measured by fluorescence titration (Fig. S1, inset, ESI†), mitoFluo could be expected to possess protonophoric activity under physiological conditions. Actually, the addition of mitoFluo to aqueous bathing solutions at both sides of a planar bilayer lipid membrane

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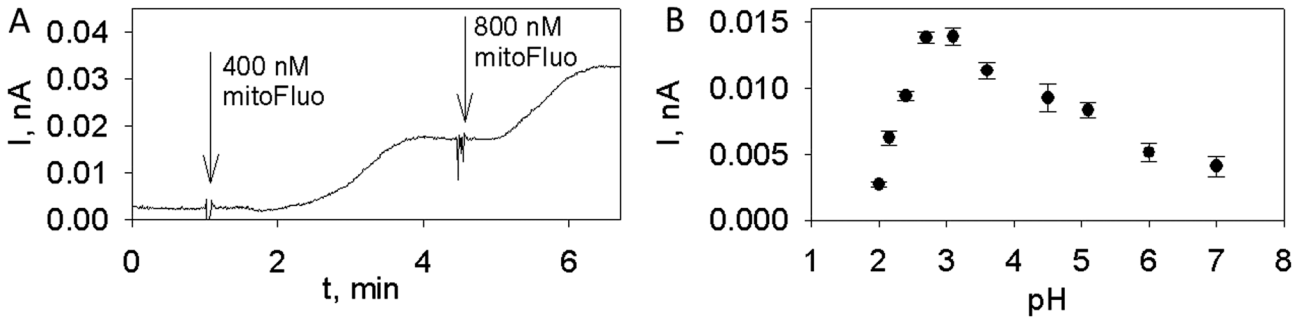
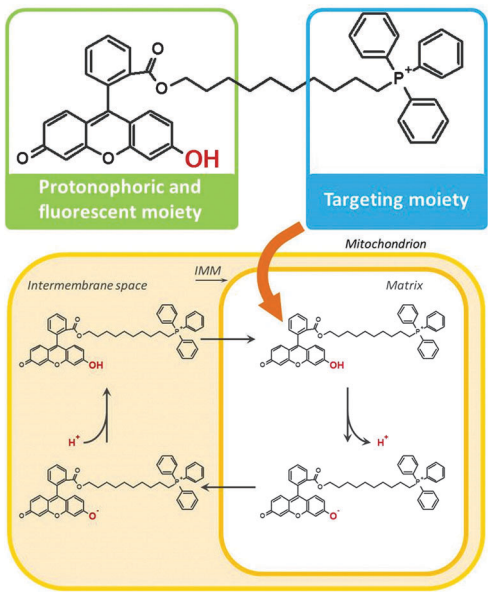


Fig. 1 Response of the transmembrane electrical current to mitoFluo. (A) Changes in the electrical current across planar BLM formed from the *Escherichia coli* polar lipid extract at the applied voltage of 50 mV upon successive additions of mitoFluo. (B) pH dependence of mitoFluo-mediated electrical current across planar BLM formed from DPhyPC at the applied voltage of 50 mV (mitoFluo concentration 400 nM).

(BLM) caused an increase in membrane proton conductance (Fig. 1A) with a maximum at pH 3 (Fig. 1B), as detected by recording the electrical current across BLM under voltage-clamp conditions. In view of the known bell-shaped pH dependence of the activity of conventional protonophores with the optimum pH depending on the limiting step of proton transfer across BLM,[9] the above data allowed us to conclude that mitoFluo-mediated proton conductance is limited by membrane permeability for the protonated cationic form of the compound. Thus, the proton transfer cycle is suggested to involve deprotonation of mitoFluo and movement of the zwitter ion through the membrane, followed by protonation and back transfer of the cation, the latter being the rate limiting step (Scheme 1). In support of this idea, proton selectivity of mitoFluo-mediated permeability of BLM was demonstrated by measuring current–voltage dependencies under symmetrical and asymmetrical conditions (Fig. S3, ESI†).

The finding of mitoFluo protonophoric activity on BLM encouraged us to examine its effect on mitochondria respiration.



Scheme 1 Schematic representation of mitoFluo accumulation and uncoupling activity.

As seen in Fig. 2A, the addition of 200 nM mitoFluo to the suspension of isolated rat liver mitochondria (RLM) energized by succinate resulted in significant acceleration of mitochondria respiration. The mitoFluo-induced increase in the respiration rate was ANT-independent, as shown by its insensitivity to the ANT inhibitor atractyloside (Fig. 2A, ii). Concentration dependence of the mitoFluo effect on the respiration rate (Fig. 2B) appeared to be non-monotonic: it reached maximum at a mitoFluo concentration of 1 μM, which was followed by a modest gradual decline of the effect upon further increase in concentration, in contrast to an abrupt drop in the uncoupling activity of fluorescein octyl ester (C_8-FL). Such a gap between the uncoupling and inhibiting concentrations of mitoFluo, not characteristic of known protonophores,[10] could be associated with its self-limiting potential-dependent behavior.[5,6,11] Simultaneous recording of RLM respiration using a Clark oxygen electrode and that of membrane potential using a tetraphenylphosphonium (TPP^+)-selective electrode (Fig. S4, ESI†) demonstrated the applicability of the latter for monitoring the concentration of mitoFluo in the medium (showing mitoFluo uptake by mitochondria upon energization with its subsequent partial release upon the addition of the potent conventional uncoupler carbonyl cyanide *m*-chloro phenyl hydrazone (CCCP) and oxygen exhaustion) and also correspondence between the signals of the TPP^+-electrode in the presence of mitoFluo and concomitant changes in the respiration rate.

Bright fluorescence of mitoFluo (Fig. S1, ESI†) enabled us to supplement the data on the macroscopic energy-dependent uptake of mitoFluo from the medium into mitochondria (Fig. S4, ESI†) by fluorescence correlation spectroscopy (FCS) measurements of mitoFluo accumulation inside single mitochondria (Fig. 2C). Energization of mitochondria with succinate resulted in the appearance of high-amplitude peaks in the fluorescence intensity traces recorded using an FCS set-up which reflected mitoFluo binding to mitochondria. Subsequent addition of CCCP leading to complete deenergization of mitochondria markedly (but not completely) suppressed the high-amplitude fluorescence bursts, thereby showing a partial release of mitoFluo from mitochondria. A similar energy-dependent behavior of the fraction of high-amplitude peaks was observed in our previous FCS study with tetramethylrhodamine ethyl ester (TMRE),[12] the widely used mitochondrial marker.[13]

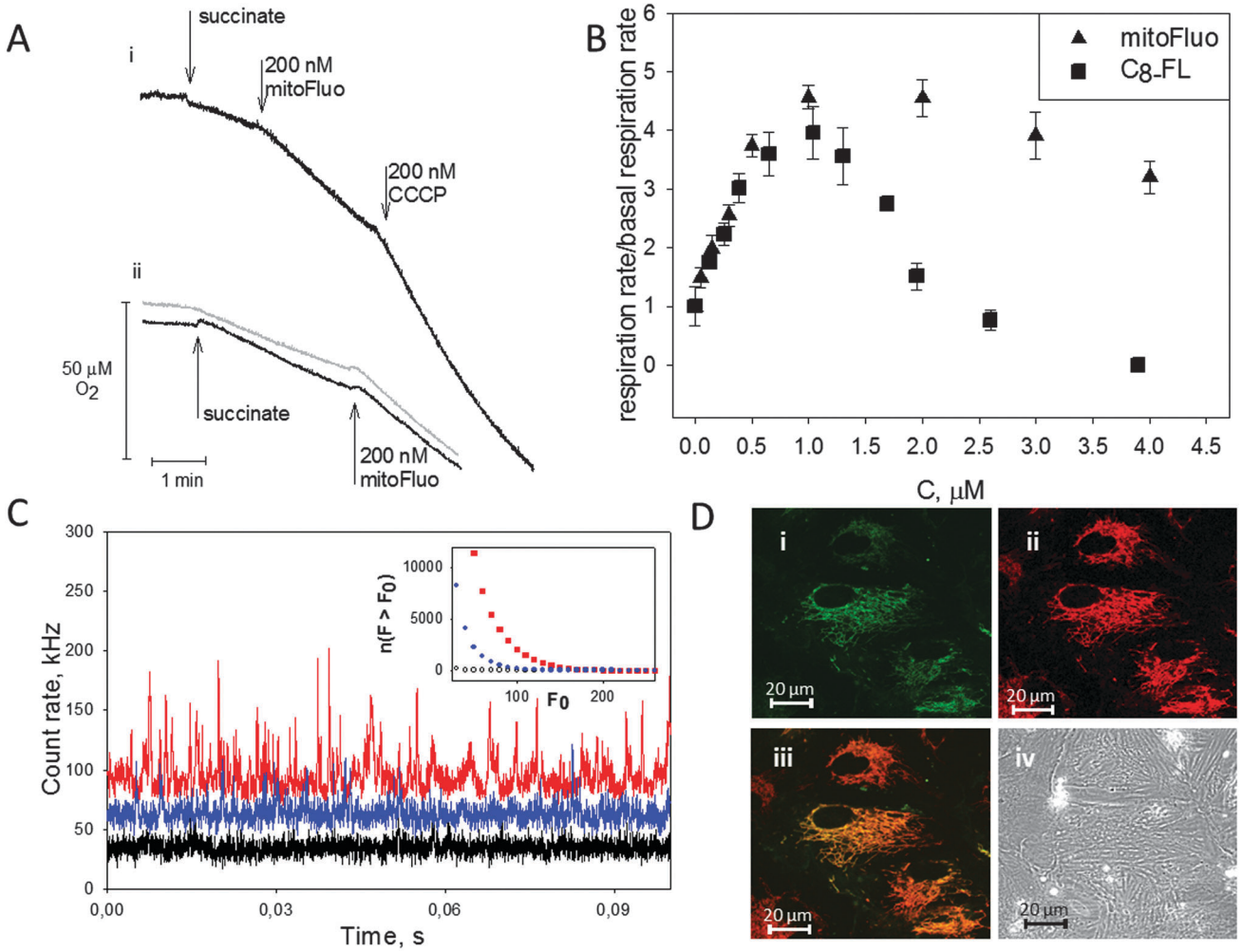


Fig. 2 Responses of isolated rat liver mitochondria to mitoFluo. (A) Stimulation of RLM respiration by mitoFluo, as evidenced by the mitoFluo-induced increase in the rate of succinate-driven mitochondrial oxygen consumption both in the absence (i, ii – black curves) and presence (ii – grey curve) of 20 μM atractyloside. (B) Dependence of the mitochondrial respiration rate on the concentration of mitoFluo and C8-FL. (C) Energy-dependent accumulation of mitoFluo in RLM, as monitored by mitoFluo fluorescence intensity traces recorded using the FCS set-up in the presence of RLM (black curve), successively supplemented with 5 mM succinate (red curve) and 1 μM FCCP (blue curve). Inset: corresponding dependencies of the number of peaks with the fluorescence intensity F exceeding the threshold F_0, $n(F > F_0)$, on the value of F_0. (D) Confocal fluorescence microscopy of primary kidney tubule cells loaded with mitoFluo (i, green), TMRE (ii, red) and overlay showing co-localization as integral yellow color (iii). Phase contrast microscopy of the cells loaded with mitoFluo (iv).

Confocal fluorescence microscopy of primary kidney tubule cell culture revealed significant colocalization of mitoFluo and TMRE fluorescence, thus demonstrating the accumulation of mitoFluo in mitochondria (Fig. 2D). By contrast, according to ref. 14 the conjugate of TPP with 6-carboxyfluorescein, being predominantly anionic at physiological pH, failed to accumulate in mitochondria. Measurements of mitoFluo cytotoxicity on L929 fibrosarcoma cells showed a decrease in cell viability starting from 2 μM mitoFluo (Fig. S6, ESI†).

In summary, based on the widely used ability of lipophilic cations to accumulate in mitochondria due to their inside-negative membrane potential[15] and the recently found protonophoric activity of alkyl fluoresceins,[8] we designed and tested a new small-molecule fluorescent probe that proved to be an effective mitochondria-targeted protonophore, promising for use both in uncoupling and fluorescence imaging[16] of mitochondria in cells.

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