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# Rhodamine-based sensor for real-time imaging of mitochondrial ATP in living fibroblasts

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ABSTRACT

Mitochondria are essential for the production and maintenance of ATP in the eukaryotic cell. To image and monitor intracellular ATP level without cell breakage, biological and chemical sensors were developed in the last years. Here, we have internalized a rhodamine-based sensor $RSL^+$ into living cells and monitored the mitochondrial ATP levels in cultured mouse embryonic fibroblasts. To evaluate the robustness of the sensor we imaged the changes of the mitochondrial ATP levels under non-physiological conditions upon incubation with FCCP, oligomycin, azide, deoxyglucose or phosphoenolpyruvate; all compounds that interfere with ATP homeostasis of the cell. The ATP sensor allowed us to determine the mitochondrial ATP levels in human skin fibroblasts where we observe a similar amount of ATP compared to mouse embryonic fibroblasts. We propose the $RSL^+$ to be a valuable tool for the assessment of mitochondrial dysfunction in human cells derived from mitochondrial OXPHOS patients and for basic studies on bioenergetics metabolism.

## 1. Introduction

In eukaryotic cells, the energetic requirements are satisfied through the synthesis of ATP inside mitochondria, where the protein complex V (ATP synthase or CV) exploits the presence of a membrane potential across the mitochondrial inner membrane established during oxidative phosphorylation (OXPHOS) [1]. An alteration of the membrane potential in the eukaryotic cell leads to mitochondrial dysfunction and appears as a common phenotype in mitochondrial diseases (MD) [2]. To evaluate and diagnose mitochondrial dysfunction, fluorescent chemical probes that surveys mitochondrial membrane potential have been developed over the past decade [3].

The majority of the probes are fluorescent lipophilic dyes based on the cationic rhodamine molecule that accumulate upon incubation within functional (negatively charged) mitochondria [4–6]. In this way, eukaryotic cells with a mitochondrial dysfunction are quickly diagnosed and identified by fluorescence microscopy [4]. Alternatively, a series of chemical sensors that detect ATP have been synthetized using zinc-based complexes such as dinuclear polyamino-phenolic-Zn(II) [7,8], Zn(II)-dipicolylamine [9,10], Zn-pyridine [11] or [Zn2L]$(ClO_4)_4$ [12] that selectively recognize phosphates but do not discriminate mono-(XMP) or di-(XDP) from triphosphates (XTP) nor distinguish purines from pyrimidines or nicotinamides. Non-homogeneous distribution of these sensors inside the cells due variable uptake efficiencies reflects a high variability of the cellular ATP concentrations.

Currently, ATP quantification is based on conventional bioluminescent assays based on the luciferin-luciferase reaction [13]. In this assay, ATP fuels the luciferase-mediated conversion of luciferin into oxyluciferin that produces chemiluminescent light that is proportional to the concentration of ATP. However, this assay requires the physical extraction of the cellular ATP and the literature reflects a large variability of the cellular ATP concentration as accurate measurements depend on the ATP extraction yield that itself depends on factors as cell culture harvesting, cell lysis, and separation steps [14].

Several strategies focus on sensors that directly image the ATP concentrations inside living cells. One strategy is to internalize a variant

*Abbreviations:* 2-DG, 2-deoxyglucose; ATP, adenosine triphosphate; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; MEF, mouse embryonic fibroblasts; HSF, human skin fibroblasts; MD, mitochondrial diseases; NMR, Nuclear Magnetic Resonance; PEP, phosphoenolpyruvate; Rho123, rhodamine 123; OXPHOS, oxidative phosphorylation; ETC, electron transport chain

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of the luciferase protein fused to a protein transduction domain (PTD-Luc), to ensure a controlled and homogenous cellular uptake of the sensor [15]. Another strategy is the development of bioluminescent sensors based on genetically encoded fluorescent reporter proteins fused to ATP binding proteins as Parceval (GFP-GlnK1) [16,17] or A-Team (CFP-$F_1$-Epsilon-YFP) [18]. These chimeric proteins are heterologously produced inside the eukaryotic cells and allow the specific real-time the measurement of the ATP concentration within an *ex vivo* tissue cell culture. Nonetheless, gene expression is particularly challenging in primary cells as it might cause their direct translation to pathological cells [19].

In this paper we present the synthesis and application of the ammonium salt of the previously reported non-charged rhodamine-based spirolactam ATP sensor (Herein: rhodamine-based spirolactam; *RSL*) [20]. The accumulation properties of cationic rhodamine-based dyes into the mitochondrial matrix will allow the real-time detection of ATP in cellular tissue cultures as mouse embryonic fibroblasts (MEF). Confocal microscopy demonstrates that $RSL^+$, the protonated form of the chemical parent, permeates the plasma membrane and senses variations of the mitochondrial ATP concentrations inside *ex vivo* tissue cultures. Ultimately, we also used $RSL^+$ to monitor ATP levels in human skin fibroblasts (HSF).

## 2. Material and methods

### 2.1. Synthesis of the $RSL^+$

Compound *RSL* was synthesized according existing literature [20]. Briefly, rhodamine B (2.00 g; 4.18 mmol) and diethylenetriamine (10 mL; 92 mmol) were dissolved in ethanol and refluxed during 24 h. After solvent evaporation under reduced pressure, the crude product was purified by silica gel column chromatography using dichloromethane:ethanol (10:1, v/v; ThermoFisher, Waltham, MA USA) as eluent solution to obtain a light pink solid product (*RSL*; Yield: 450 mg). Compound $RSL^+$ was obtained after acid-wash of *RSL* dissolved in 20 mM of $CH_2Cl_2$ with aqueous HCl (pH 3) and $RSL^+$ was recovered from the organic phase by eliminating the solvent under reduced pressure. Both compounds have been characterized by $^1$H NMR spectroscopy (CAI of NMR; Universidad Complutense Madrid) (see supplemental material).

### 2.2. UV-IR spectroscopy

UV-VIS-NIR absorption using spectra were recorded at 20 °C (Genesis 10 spectrophotometer, Fisher scientific) with a spectral bandwidth of 1.0 nm and a scan rate of 200 nm/min. All experiments were carried out using plastic cuvettes with a 1 cm optical path.

### 2.3. Fluorescence spectroscopy

Fluorescence emission spectra of compounds $RSL^+$ and *RSL* were recorded at 20 °C on a single photon counting ISS-PC1 photon counting spectrofluorimeter equipped with Glan-Taylor polarizers. Excitation wavelength was fixes at 510 nm with a slit width of 4 nm using quartz Ultra-Microcells 105.251-QS (Hellma Analytics, Germany) with a 3 × 3 cm optical path.

### 2.4. Electroformation of giant unilamellar vesicles

Giant unilamellar vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids doped with fluorescent 1-palmitoyl-2-6-[(7-nitro-2-1,3-benzoxa diazol-4-yl) amino] hexanoyl-*sn*-glycero-3-phosphocholine (NBD-PC) were produced by electroformation [21] (Lipids were purchased from Avanti Polar Lipids, AL, USA). Briefly, 10 μL of POPC/NBD-PC (99:1 mol, 0.5 mg/mL dissolved in chloroform was spread over two conductor indium tin oxide (ITO)-coated glass slides (15–25 Ω/sq surface resistivity, Sigma Aldrich, St. Louis, MO, USA) separated 0.5 mm by a Teflon/Bytac spacer. After solvent evaporation, the fabrication chamber was sealed using Vitrex® putty (Vitrex Medical A/S, Copenhagen, DK) and the lipid films were rehydrated with a K-Buffer (30 mM KCl, 100 mM sucrose, 20 mM Mg-ATP, 5 mM $MgCl_2$) and in the presence of an AC electric field (8 Hz, 1.1 V/mm) for 2 h. Experiments were performed with 25 μL of POPC GUVs that were diluted into 50 μL of isoosmolar buffer solution (250 mM glucose, 20 mM HEPES, 1 mM KCl, pH 7,4). Prior to the addition of 15 μM valinomycin, sedimented GUVs were supplemented 35 μM of $RSL^+$. In the presence of valinomycin (Sigma Aldrich, St. Louis, MO, USA), $K^+$ ions from the lumen ($[K^+]_{in}$ = 30 mM) is transported to the external medium ($[K^+]_{out}$ = 1 mM) giving rise to an negative inside electrical transmembrane potential ($\Delta\Psi \sim log\frac{[K]_{out}}{[K]_{in}}$) [22], which triggers $RSL^+$ internalization across the lipid bilayer.

### 2.5. Cell culture

The mouse embryonic fibroblasts (3T3NIH MEF; purchased from ATCC) and human skin fibroblasts (HSF; Hospital 12 de Octubre, Madrid) were cultured in complete DMEM (high glucose Dulbecco Modified Eagle Medium), 25 mM Glucose (Gibco) supplemented with 10% fetal bovine serum (South Africa S1300; Biowest, Nuallé, France), penicillin/streptomycin (final concentration 100 U/mL of penicillin and 100 μg/mL of streptomycin respectively) and 1% of non-essential amino acids (all Gibco). The cells were grown in a humidified incubator (Forma Steri-Cycle Themofisher; 5% $CO_2$) at 37 °C and maintained, with split ratio of 1:10, at 80% of confluence in T75 flasks (Nunc).

### 2.6. Confocal fluorescence imaging of living cells

MEF were adhered at 1 × $10^4$ cells per $cm^2$ in a four-chamber Lab-Tek® slide (Thermofisher) and incubated complete DMEM for 24 h at 37 °C. Prior to confocal fluorescence imaging, adhered Mouse embryonic fibroblasts (MEFs) were supplemented either with 50 nM rhodamine 123 (Rhod123) to monitor the presence of a membrane potential or 1 μM $RSL^+$ to monitor the ATP and incubated for 60 min at 37 °C. For the co-localization, 50 nM Rhod123 and 1 μM $RSL^+$ were added simultaneously to MEFs. In addition, cells were supplemented with 25 mM HEPES medium (pH 7.4) to buffer the pH during the observation. The Lab-Tek® slide was mounted on the temperature (37 °C) controlled stage of an inverted Nikon Ti-E microscope equipped with a Nikon point scanning confocal microscope module C2, Nikon Plan Apo 100 × NA 1.45 oil immersion objective and two lasers (488 nm and 561 nm). Image capture was performed with Nikon NIS-Elements software and further processed with ImageJ [23] and Adobe Creative Suite 6 software package (Adobe Systems Incorporated). To modulate ATP homeostasis of the MEFs (N > 20), after the addition of either Rho123 or $RSL^+$ cells were supplemented individually with 1 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), 1 μM oligomycin, 500 nM azide, 10 mM deoxyglucose (2-DG) or increasing concentrations (1 mM, 5 mM and 10 mM) of phosphoenolpyruvate. All compounds were purchased from Sigma-Aldrich St. Louis, MO, USA. Rho123 and the $RSL^+$ fluorescence were excited at 488 nm and 561 nm respectively. Collected fluorescence intensities from the confocal microscope images were corrected for laser-induced photobleaching. When appropriate, the time dependent $RSL^+$ fluorescence intensity signal decay was fitted to $I = I_0 e^{-kt + I_f}$, where $k$ is the decay rate and $I_0$ and $I_f$ account for initial and final fluorescent intensity respectively. The fluorescence decay of $RSL^+$ upon the addition of azide or deoxyglucose required a fit to a bi-exponential equation indicating two simultaneous processes responsible for the fluorescence decrease.