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Mitochondria Penetrating Peptide-Conjugated TAMRA for Live-Cell Long-Term Tracking

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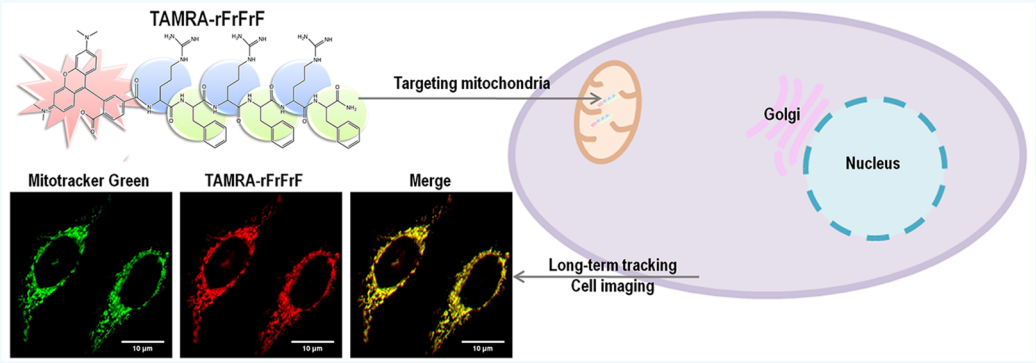
Supporting Information



ABSTRACT: Mitochondria are essential targets for treatment of diseases with mitochondrial disorders such as diabetes, cancer, and cardiovascular and neurodegenerative diseases. Mitochondria penetrating peptides (MPPs) are composed of cationic and hydrophobic amino acids that can target and permeate the mitochondrial membrane. Herein, a novel D-argine-phenylalanine-D-argine-phenylalanine-D-argine-phenylalanine-NH_2 (rFrFrF) was tagged with a rhodamine-based fluorescent chromophore (TAMRA). This probe (TAMRA-rFrFrF) exhibited advantageous properties for long-term mitochondria tracking as demonstrated by fluorescence microscopy. Cell viability assays and oxygen consumption rates indicate low cytotoxicity and high biocompatibility of the new contrast agent. Colocalization studies suggest that TAMRA-rFrFrF is a promising candidate for continuous mitochondrial tracking for up to 3 days.

Mitochondria are essential organelles as the site of respiration in eukaryotic cells and are involved in multiple functions in cell life, such as energy supply, cell cycle and growth, cell signaling pathways and apoptosis, cell proliferation, and metabolism.[1,2] Meanwhile, these organelles are dynamic and continually undergo fusion, fission, transport, and degradation. These processes are critical for maintaining a healthy functional mitochondrial network.[3,4] Dysfunctions of mitochondrial metabolism and/or morphologies have been reported in human cancers as well as cardiovascular and neurodegenerative diseases. Thus, the capability of monitoring mitochondrial morphology is important in both scientific and clinical research.[5−7] However, due to the continuously moving and changing morphology, monitoring mitochondria morphological changes and dynamics over prolonged periods of time is still difficult, and so far the longest reported time for live cell mitochondria imaging is 24 h.[8,9] Therefore, a long-term tracking strategy would be helpful for studying mitochondria morphology and prospective drug delivery systems.

Over the last few decades, a variety of mitochondria targeting modules have been developed. Most of those reported are delocalized lipophilic cations, which exhibit relatively high toxicity to mitochondria.[10,11] Mitochondria penetrating peptides (MPPs) represent a relatively new direction to develop mitochondria targeting vectors and are short peptides with high mitochondria uptake. The structure of MPPs is generally cationic and hydrophobic, which facilitates permeation through the hydrophobic mitochondrial membrane and accumulation in the mitochondria matrix.[12−14] Due to their primary peptide structures and biocompatible properties, MPPs with short amino acid sequences (fewer

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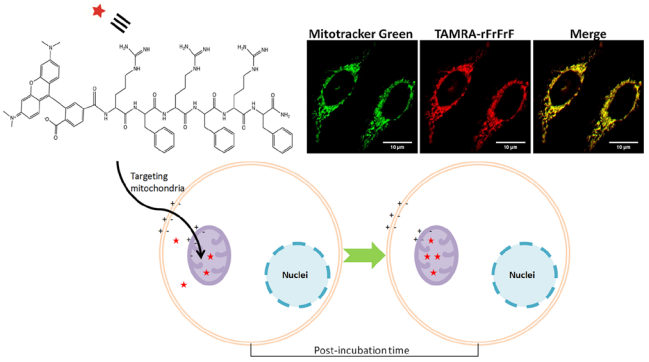
than 10 amino acids) are expected to exhibit low cytotoxicity, making them promising candidates for the development of new probes for investigation of mitochondria.[15−17]

Recently, MPPs were employed for targeting and delivering therapeutic cargos into mitochondria with high efficiency.[10,12,14−18] Peptides such as (FxrFxrFxr) (r is D-argine and Fx is cyclohexylalanine) exhibited low cytotoxicity and high mitochondria targeting.[16] Although numerous MPPs have been developed to deliver fluorescent dyes, drugs, and other cargos to mitochondria, there are no reports for long-term mitochondrial tracking thus far, possibly due to their low serum stability.[19]

Herein, we report a peptide containing six amino acid residues (Figure S1a), D-argine-phenylalanine-D-argine-phenylalanine-D-argine-phenylalanine-NH_2 (rFrFrF), conjugated to a rhodamine-derived fluorescent probe (TAMRA)[20] (Scheme 1 and Figure S1b), and studies of the resulting probe, TAMRA-rFrFrF, in time-dependent mitochondria targeting and imaging. Unnatural D-arginine (r) in the first and third positions was selected to provide positive charges, minimize amino peptidase degradation, and increase serum-stability.[21] The natural amino acid phenylalanine (F) residue, which has a hydrophobic side group, was selected to tune the hydrophobicity and impart low cytotoxicity. This prospective mitochondria penetrating peptide (MPP) sequence possesses three positive charges with a balance of hydrophilicity and hydrophobicity designed to reduce potential disruptions to mitochondrial activity and maintain normal mitochondrial functions.[22,23] Moreover, the amidated C-terminus was designed to increase structural stability and attenuate hydrolysis.[14,24]

Scheme 1. Molecular Structure of TAMRA-rFrFrF and Mitochondria Penetration



The cytotoxicity and biocompatibility of rFrFrF and TAMRA-rFrFrF were evaluated in HeLa and pig kidney (LLC-PK1) cells via an MTS assay (Figures S2 and S3).[25] The peptide rFrFrF itself did not show any acute toxic effects over a range of concentrations; even at the highest concentration employed of 80 μM, cell viability remained at 100% after 24 h incubation.The TAMRA-rFrFrF probe also exhibited very good cell viability with a 90% survival rate at concentrations up to 80 μM after 24 h. Meanwhile, commercial MitoTracker Green and Red were evaluated in HeLa cells to compare the cytotoxicity with rFrFrF and TAMRA-rFrFrF (Figure S4). MitoTracker Green appears to be more toxic at concentrations over 8 μM, while MitoTracker Red exhibits high cytotoxicity at 4 μM with only 30% of cells alive after 24 h incubation.

The use of the new MPP probe, TAMRA-rFrFrF, was then investigated as a mitochondrial-targeting agent by fluorescence imaging and colocalization studies. Three experimental groups were conducted, including TAMRA-rFrFrF, TAMRA, and TAMRA physically mixed with unconjugated rFrFrF. After 1.5 h incubation with one of the aforementioned reagents with either HeLa or LLC-PK1 cells, the culture media was changed to pure Dulbecco's Modified Eagle Medium (DMEM) and the HeLa cells were further incubated for 1 d.

As shown in Figure 1, bright fluorescence was observed for cells incubated with TAMRA-rFrFrF. By contrast, no fluorescence was detected for the other two groups (TAMRA and TAMRA mixed with unconjugated rFrFrF). Furthermore, TAMRA-rFrFrF displayed a high level of selectivity for mitochondria targeting, with a Pearson's correlation coefficient value of 0.88 relative to MitoTracker Green, a commercial mitochondrial staining agent, while unconjugated TAMRA itself and the physical mixture of TAMRA with rFrFrF exhibited no mitochondria targeting ability nor the capability of being uptaken by cells.[26] To further evaluate mitochondrial selectivity, TAMRA-rFrFrF colocalization studies with Lysotracker Green at short incubation times and endoplasmic reticulum (ER-tracker Green) were also performed with HeLa cells. Poor colocalization was observed between TAMRA-rFrFrF with Lysotracker Green or ER-tracker Green, in which Pearson's values were less than 0.4 (Figures S5 and S6). Thus, the designed rFrFrF sequence is attributed for successful cell uptake and high selectivity for mitochondrial targeting.

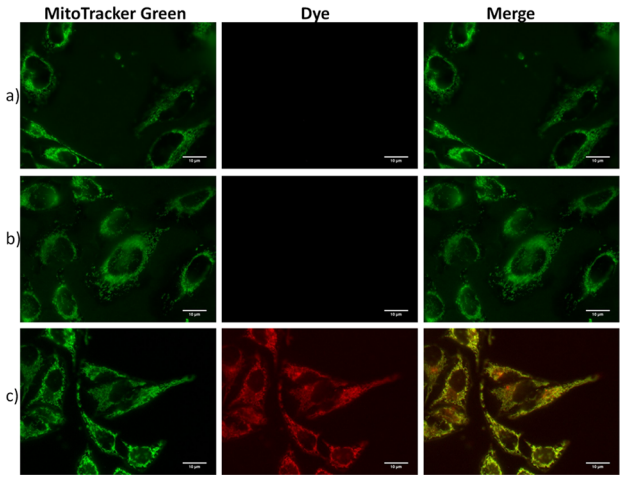


Figure 1. HeLa cells were incubated with 5 μM of TAMRA, TAMRA mixed with unconjugated rFrFrF, and TAMRA-rFrFrF separately for 1.5 h, and after washing, additional incubation of the cells at 37 °C for 1 d (post-incubation time). (a) HeLa cells treated with TAMRA, (b) HeLa cells treated with TAMRA mixed with unconjugated rFrFrF, (c) HeLa cells treated with TAMRA-rFrFrF.

To better understand the time dependent mitochondrial uptake of TAMRA-rFrFrF, time-dependent cell imaging experiments were conducted. In general, after incubation with TAMRA-rFrFrF for 1.5 h, and after washing, HeLa or LLC-PK1 cells were then incubated in cell medium alone for various times (post-incubation time) before cell imaging for long-term mitochondria tracking was performed. Cells that underwent 0 to 4 h post-incubation (Figures S8 and S9) displayed strong fluorescence; however, mitochondria targeting

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was less efficient at these time points, which may be related to the charge and hydrophobicity of TAMRA-rFrFrF. The mitochondrial membrane exhibits hydrophobicity and a potential difference in which the inner mitochondrial membrane is negatively charged and the outer membrane is positively charged.[12,13,27] MPPs that possess positive charges and hydrophobicity tend to target and penetrate the mitochondria membrane more easily.[15] However, increasing the peptide charge or hydrophobicity may increase mitochondrial disruptive activity and lead to unwanted side effects and toxicity.[22,23]

We discovered that our MPP could slowly but effectively target mitochondria. As shown in Figures 2a, S8, S9, and S10, TAMRA-rFrFrF largely permeated the mitochondria after 6 h of post-incubation time. Moreover, cells still exhibited

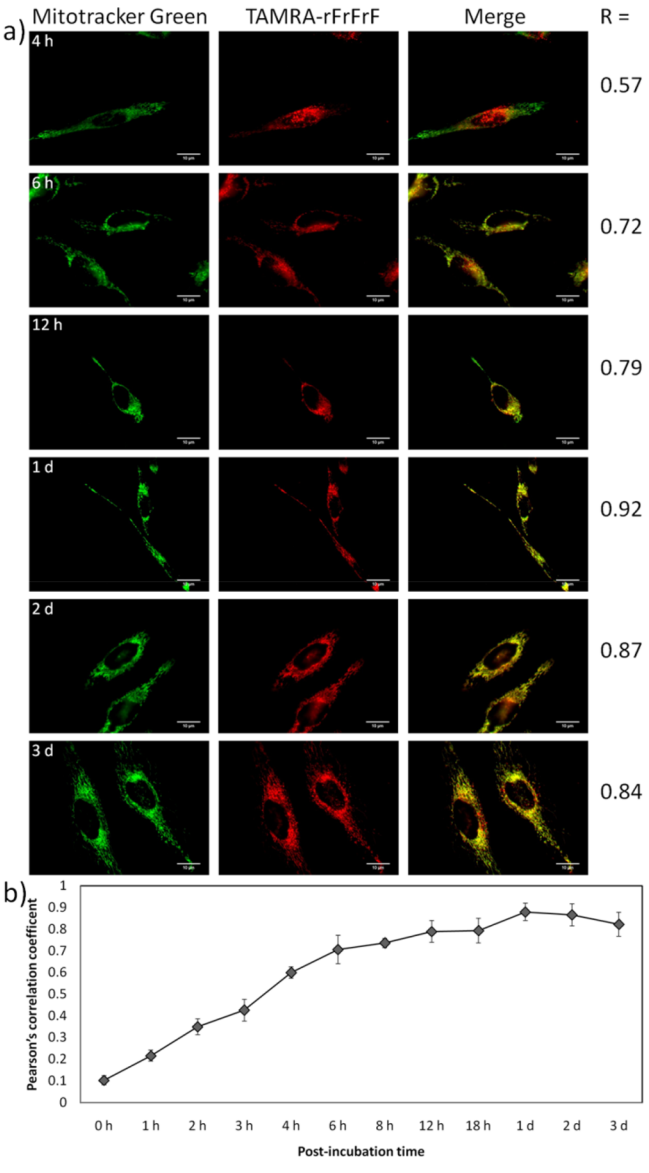

Figure 2. (a) HeLa cells were incubated with 5 μM of TAMRA-rFrFrF for 1.5 h, and after washing out the TAMRA-rFrFrF, an extra incubation of the cells at 37 °C was carried out for various times (post-incubation time). Then, cells were incubated with MitoTracker Green for 15 min and washed before cell imaging. Pearson's value is labeled as R. (b) Post-incubation time-dependent Pearson's correlation coefficient of HeLa cells.

significant fluorescence in mitochondria even at 3 d post-incubation. By comparison, MitoTracker Green was not capable of tracking mitochondria reliably much longer than 6 h (Figure S7). These results clearly demonstrated long-term mitochondria tracking ability of the new MPP probe.

Mitochondria are highly dynamic organelles that constantly undergo fission, fusion and degradation,[28] e.g., HeLa cells undergo 1.44 events/min of fission.[29] These constant changes cause asymmetrical distribution of matrix proteins in the daughter mitochondria.[30,31] In our study, TAMRA-rFrFrF exhibited persistent localized mitochondria staining ability without significant depletion or leakage over the 3 d post-incubation period, an assertion that was quantitatively confirmed by the Pearson's correlation coefficient values (Figures 2b), indicating TAMRA-rFrFrF is capable of mitochondria targeting under frequent fission and fusion events.To further confirm the mitochondria targeting ability of TAMRA-rFrFrF, mitochondria were isolated from TAMRA-rFrFrF treated cells and fluorescence intensities were recorded with a fluorescence spectrometer.[32] The fluorescence intensities of isolated mitochondria with 6 h, 12 h, 1 d, 2 d, and 3 d post-incubation were unquestionably much more intense than those of the blank—isolated untreated mitochondria (Figures 3 and S10). These results clearly demonstrate that the uptake

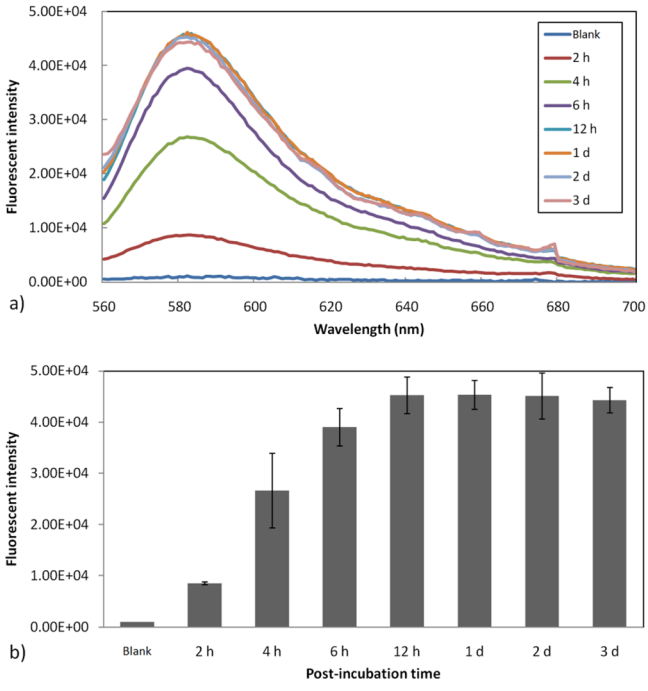

Figure 3. 5 μM TAMRA-rFrFrF was preincubated with HeLa cells for 1.5 h, followed by washing out TAMRA-rFrFrF and post-incubation of the HeLa cells for 2 h, 4 h, 6 h, 12 h, 1 d, 2 d, and 3 d. Isolated mitochondria from untreated cells were the blank (N = 3).

of TAMRA-rFrFrF is time-dependent and appears rather efficient. There was no intensity decrease of TAMRA-rFrFrF in mitochondria upon prolonged incubation. In fact, the post-incubation time was a key factor for TAMRA-rFrFrF to accumulate in the mitochondria in HeLa cells.

Further, the mitochondria present a whole set of complex proteolytic enzyme systems that regulate mitochondrial function and activities. Especially for mitochondrial degradation,[33,34] endogenous proteases are responsible for the

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degradation of their internal proteins, which are eventually shuttled back to the cytoplasm by a specific peptide transporter.[35,36] The fluorescence intensity of TAMRA-rFrFrF did not decrease from 12 h to 3 d, which indicated TAMRA-rFrFrF underwent no apparent release to the cytoplasm and was proteolytically stable. Overall, TAMRA-rFrFrF is capable of long-term mitochondrial tracking up to 3 d, with high stability and selectivity.

To probe mitochondria function of cells incubated with TAMRA-rFrFrF, oxygen consumption rates were measured in HeLa cells using a Seahorse analyzer.[37] The results demonstrated that the basal respiratory rate and maximal respiratory capacity (Figure 4) decreased somewhat in the

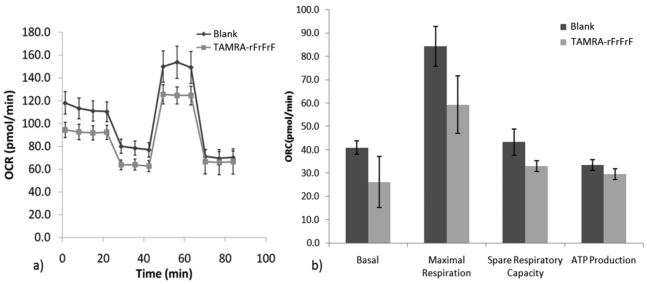

Figure 4. (a) Oxygen consumption rates (OCR) in TAMRA-rFrFrF treated vs untreated HeLa cells (blank, n = 5). OCR was measured approximately every 8 min using an XF-96 analyzer as described in the Supporting Information Methods section. (b) The rates of basal respiration, maximal respiratory capacity, spare respiratory capacity, and ATP-linked respiration (ATP production).

TAMRA-rFrFrF treated vs untreated HeLa cells. Although decreases in maximum respiratory capacity and basal respiratory rate are strong indicators of potential mitochondrial dysfunction,[38] in this situation, the decreases are most likely a result of a change in the potential difference of the outer and inner mitochondrial membranes caused by the cationic rFrFrF rather than mitochondrial dysfunction.[38,39] The drop of mitochondrial membrane potential difference will inhibit the activity of the electron transport chain, which would decrease the maximum respiratory capacity and the basal respiratory rate.[40,41] Furthermore, spare respiratory capacity exhibited only a slight decrease while ATP production underwent no significant reduction when compared with a control, indicating that the TAMRA-rFrFrF probe has a minimal influence on mitochondrial respiration.[42] In fact, the results indicated that mitochondrial functions were mostly unchanged.

In conclusion, a novel MPP probe, TAMRA-rFrFrF, was designed that contained six amino acid residues. Our investigations demonstrated the new MPP probe's utility in long-term in vivo mitochondrial tracking for up to 3 d, which is superior to commercial MitoTracker Green and Red. Importantly, the TAMRA-rFrFrF probe can undergo mitochondria distribution, activities, and fission or fusion events with no degradation or transportation by mitochondria proteolysis, which fulfills major criteria for long-term mitochondria tracking. In addition, we have shown that the TAMRA-rFrFrF was essentially benign and biocompatible from cell viability and oxygen consumption rate experiments. There is an important link between the mitochondrial morphology change and mitochondrial dysfunction,[6,43−45] in which mitochondrial morphology serves as a marker to study and treat mitochondria dysfunction-related disease. Therefore, this TAMRA-rFrFrF MPP probe is a potential candidate for live-cell mitochondrial morphological and mitochondrial dysfunction studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.9b00465.

Materials and methods, stuctures, cell viability, cell imaging using HeLa and LLC-PK1 cells (PDF)

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Notes

The authors declare no competing financial interest.

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