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Original Article

Synthesis and Evaluation of Triphenylphosphonium-Pluronic F127 Conjugate for Enhanced Mitochondrial Targeting in Drug Delivery Systems

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ABSTRACT

Background: Mitochondria, often termed the "powerhouse of the cell", have increasingly become a focal point for the targeted delivery of therapeutic agents in the treatment of various metabolic disorders. This emerging field, known as mitochondrial medicine, emphasizes recognition of mitochondria as a critical target for the delivery of therapeutic agents aimed at addressing metabolic dysfunctions. Triphenylphosphonium (TPP) is the most utilized mitochondrial targeting moiety. It is a delocalized cationic lipid that efficiently accumulates within and traverses the mitochondrial membrane, driven by the strongly negative mitochondrial membrane potential. On the other hand, Pluronic F127 (PF127), a triblock copolymer known for its high Hydrophilic-Lipophilic Balance (HLB) value, demonstrates limited affinity for cellular membrane interactions. **Materials and Methods:** The conjugation of TPP with PF127 alters the physicochemical properties of the resulting polymeric micelles, reducing the HLB value and enhancing the interaction with cellular membranes. This modification enables the formation of micelles capable of delivering therapeutic moieties specifically to mitochondria, thereby significantly improving their bioavailability and therapeutic index. This study aims to synthesize the TPP-PF127 conjugate (TP) and subsequently characterize it using Fourier-Transform Infrared (FT-IR) Spectroscopy. **Results:** The characterization data confirmed the successful conjugation of TPP and PF127. **Conclusion:** Furthermore, the findings from confocal microscopy further support the efficacy of the conjugate in specifically targeting mitochondria, underscoring its promise in the development of mitochondrial-targeted therapeutic strategies.

Keywords: Mitochondrial Medicine, Triphenylphosphonium, Pluronic F127, Conjugate, Mitochondrial Targeting.

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INTRODUCTION

Mitochondria, often termed the "powerhouse of the cell", have increasingly become a focal point for the targeted delivery of therapeutic agents in the treatment of various disorders. This emerging field, known as mitochondrial medicine, emphasizes recognition of mitochondria as a critical target for the delivery of therapeutic agents aimed at addressing various dysfunctions as shown in Table 1. As an emerging field, mitochondria-targeted drug delivery faces several critical challenges. One of the primary obstacles is the selective delivery of therapeutic molecules to mitochondria without affecting other cellular organelles, owing to the unique double-membrane structure and selective permeability of mitochondria. Furthermore, many current delivery approaches depend on the mitochondrial membrane potential to facilitate accumulation; however, this potential is frequently disrupted in pathological conditions, thereby limiting the efficiency of such strategies in diseased cells.

Mitochondria maintain a substantial transmembrane potential ranging from 140 to 180 mV, with the interior being negatively charged. This potential can be exploited for the transport of positively charged molecules into the mitochondria. Among the most used mitochondrial targeting agents is Triphenylphosphonium (TPP), a lipophilic cation that efficiently traverses and accumulates within the mitochondrial membrane due to the highly negative membrane potential. The concentration of TPP within negatively charged membrane compartments increases exponentially, approximately tenfold, for every 60 mV of negative membrane potential. Given that the plasma membrane generally maintains

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a potential ranging from −30 to −60 mV, this gradient facilitates the significant accumulation of TPP within cells, reaching up to a 10-fold increase in concentration. In contrast, mitochondria, with their membrane potential of approximately −180 mV, enable a substantially greater accumulation of TPP, up to 1000-fold, within the mitochondrial matrix.[10]

Conversely, Pluronic F127 (PF127), a triblock copolymer categorized under Poloxamers, is commercially recognized as an ABA-type triblock copolymer comprising Polyethylene Oxide (PEO) and Polypropylene Oxide (PPO) units in a PEO-PPO-PEO configuration as shown in Figure 1. This structure is characterized by a high Hydrophilic-Lipophilic Balance (HLB) value, which imparts minimal interaction with cellular membranes.[11] The conjugation of TPP with PF127 alters the physicochemical properties of the resulting polymeric micelles, reducing the HLB value and enhancing the interaction with cellular membranes. This modification is hypothesized to exploit the mitochondrial membrane potential for selective accumulation within the mitochondrial matrix, thereby enhancing the bioavailability and therapeutic efficacy of loaded therapeutic drugs.

MATERIALS AND METHODS

Materials

PF127 and TPP-COOH [(5-carboxypentyl) triphenylphosphonium bromide] were procured from Central Drug Research Institute (CSIR-CDRI), Lucknow, India as a gift sample. Additionally, DCC (N,N'-dicyclohexylcarbodiimide), DMAP (4- dimethylaminopyridine) and anhydrous DMSO were procured from JSS College of Pharmacy, JSSAHER, Ooty, Tamil Nadu, India for this study.

Synthesis of Triphenylphosphonium-Pluronic F127 conjugate (TP)

TP was synthesized by first dissolving PF127 (1 mmol) in anhydrous DMSO within a round-bottom flask. Subsequently, TPP-COOH (2.5 mmol), DCC (1.5 mmol), and DMAP (1.5 mmol) were each dissolved in DMSO and added to the reaction. The resulting mixture was stirred at Room Temperature (RT) under a Nitrogen (N_2) atmosphere for 24 hr. Following the reaction, the mixture was dialyzed (1000 Da) against distilled water to remove unconjugated TPP. The final product was then flash-frozen, dried, and lyophilized to obtain the TP conjugate,[6] as depicted in Figure 2.

Characterization of Synthesized TP

The synthesized conjugate, TP was characterized using Fourier-Transform Infrared (FT-IR) spectroscopy (Agilent Technologies, Cary 600 Series).

Evaluation of Synthesized TP

Confocal Microscopy

Ultraclean coverslips were placed in a 6-well plate, treated with 200 µL (3-Aminopropyl) Triethoxysilane (APTES), a silane coupling agent for enhancing particle uptake, and incubated for 1 hr. Excess APTES was collected, and wells were rinsed twice with complete medium. Cells at ~90% confluency were seeded onto the treated coverslips (5,000-10,000 cells per well) and incubated for a day (24 hr) at 37°C with 5% Carbon Dioxide (CO_2). HeLa cells were selected for confocal imaging due to their well-characterized morphology, ease of culture, and frequent use in mitochondrial targeting studies. Their high metabolic activity and clearly distinguishable mitochondria make them an ideal *in vitro* model to evaluate intracellular localization and mitochondrial uptake of nanocarrier systems.[12] The medium was then exchanged with fresh medium containing the test compound (10 µg/mL) and Fluorescein Isothiocyanate (FITC) (1 µL/mL) for another 24 hr incubation. FITC was employed as a fluorescent probe because of its strong fluorescence, good photostability, and compatibility with confocal laser scanning microscopy. Its successful conjugation and emission properties enable clear visualization of the micelle distribution and mitochondrial localization.[13,14]

Cells were fixed with 3% paraformaldehyde for 20 min, rinsed with PBS, stained with DAPI (4',6-diamidino-2-phenylindole) (1 µg/mL), and washed again. Coverslips were mounted onto slides and imaged using a Laser Scanning Confocal Microscope (ZEISS, LSM 900) under DAPI, FITC, and merged channels.[15] Image analysis was performed using ImageJ (v1.54d).

RESULTS

FT-IR Spectroscopy

Yield 48%; FT-IR (KBr, cm -1) 3452.78, 1638.14, 502.46, 455.00.

The results obtained in this study as shown in Figure 3, are consistent with previously reported findings (He *et al.*, 2020), thereby supporting the reliability and reproducibility of the process.

Confocal Microscopy

In cellular uptake assays, FITC is commonly employed to label target molecules, enabling the tracking of their internalization into cells. Following labelling, cells can be analysed through flow cytometry or microscopy to quantify the extent of molecular uptake. Similarly, DAPI is a widely used fluorescent dye for staining cellular DNA. By binding to the minor groove of double-stranded DNA, DAPI produces blue fluorescence under ultraviolet light, facilitating the visualization of cell nuclei. In cellular uptake studies, DAPI often serves as a counterstain to identify nuclei and assess the internalization of specific molecules. The intensity of DAPI fluorescence can also be used as

Table 1: Mitochondrial Targeted Drug Delivery System for Various Diseases.

Sl. No.	Disease	Drug Delivery System	Drug Loaded	Mechanism of Action
1.	Diabetes	Pectin Nanoparticles	Metformin	When insulin binds to its receptor, it triggers uptake of glucose by activating a signalling cascade. The activated Insulin Receptor (IR) phosphorylates the Insulin Receptor Substrate 1 (IRS1), which subsequently activates Phosphoinositide 3-Kinase (PI3K). This, in turn, activates Akt, facilitating the uptake of glucose into cells. However, in the case of mitochondrial dysfunction, excessive reactive oxygen species are produced, which activate serine/threonine kinases. These kinases increase serine phosphorylation of IRS1, inhibiting PI3K activity and ultimately blocking glucose uptake. The uptake of glucose by RBCs was enhanced using these nanoparticles, likely attributable to the antioxidant properties of pectin. Pectin's antioxidant activity mitigated lipid peroxidation and oxidative damage within RBCs. Additionally, metformin regulates blood glucose levels by promoting increased glucose absorption at the cellular level.[1]
		Dequalinium liposomes (DQAsomes)	Resveratrol	These nanoparticles have been demonstrated to undergo cellular uptake via endocytosis, subsequently fusing with the mitochondrial outer membrane. This fusion facilitates their translocation into the mitochondrial matrix through the protein import machinery, enabling targeted intracellular delivery.[2]
		Nanoparticles	Epigallocatechin-3-gallate	The nanoparticles were found to reduce oxidative stress within skeletal muscle tissues and enhance the regulation of mitochondrial fusion machinery, thereby promoting improved mitochondrial dynamics.[3]
2.	Cancer	Gold Nanostars	Doxorubicin	The chemotherapy agent doxorubicin was co-encapsulated within a hyaluronic acid shell to enable tumor-specific photothermal chemotherapy. Furthermore, Triphenylphosphonium (TPP) was functionalized with a pro-apoptotic peptide to achieve selective mitochondrial targeting and induce mitochondria-mediated apoptosis in cancer cells. This approach demonstrated that the integration of mitochondrial targeting with the pro-apoptotic peptide markedly enhanced the efficacy of cancer cell apoptosis.[4]
		Liposomes	Doxorubicin	Liposomes were formulated by conjugating glycyrrhetinic acid, TPP, and doxorubicin, encapsulating them within cationic liposomes, and coating the liposomes with hyaluronic acid. These nanoparticles accumulate in tumor tissue via the enhanced permeability and retention effect and facilitate tumor-specific endocytosis through CD44 receptor-mediated uptake. The combined action of glycyrrhetinic acid and TPP enables efficient mitochondrial delivery of DOX.[5]
		Nanomicelles	Paclitaxel	Paclitaxel exhibited potent antitumor activity, likely due to the efficient internalization of nanomicelles. Hyaluronic acid acted as an active targeting ligand, selectively binding to CD44 receptors, which are overexpressed in many tumor cells, while also facilitating mitochondrial targeting for enhanced therapeutic efficacy.[6]

Sl. No.	Disease	Drug Delivery System	Drug Loaded	Mechanism of Action
3.	Alzheimer	Nanoparticles	Curcumin	Curcumin loaded nanoparticles enhances mitochondrial biogenesis while mitigating apoptotic signalling by preventing DNA fragmentation, suppressing reactive oxygen species production, and inhibiting caspase-3 activation. Additionally, curcumin helps maintain mitochondrial membrane potential and stabilizes ATP levels, thereby reducing mitochondrial dysfunction and contributing to neuroprotection in Alzheimer's pathology.[7]
		Liposomes	α- tocopherol and donepezil hydrochloride	Intranasal delivery of liposomes demonstrated efficacy in mitigating memory deficits and modulating the progression of Alzheimer's disease by decelerating the accumulation of Amyloid-beta (Aβ) plaques.[8]
4.	Atherosclerosis	Nanoparticles	Luteolin	Luteolin mitigates mitochondrial dysfunction by inhibiting intracellular calcium elevation and modulating mitochondrial membrane potential. It also influences apoptosis by regulating p53 phosphorylation, and promoting cytochrome C release, which activates caspase 3. Additionally, luteolin's antioxidant properties enhance endothelial function, making it a promising candidate for atherosclerosis therapy.[9]

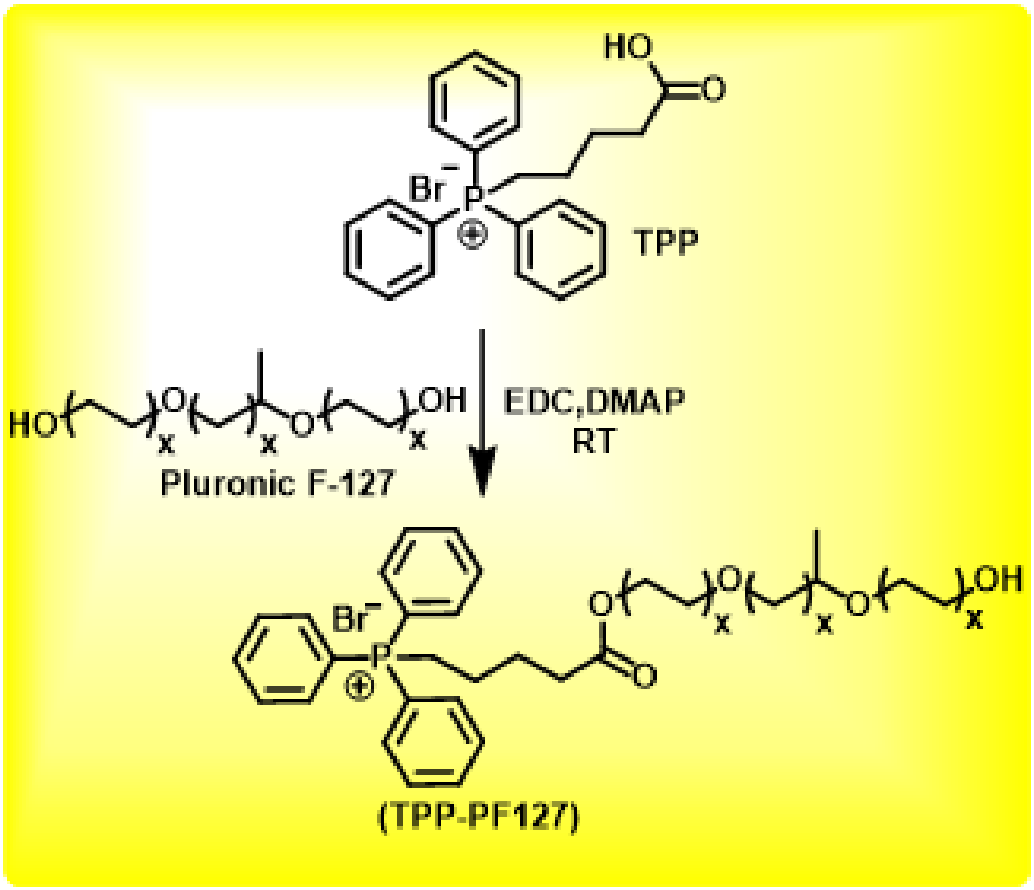
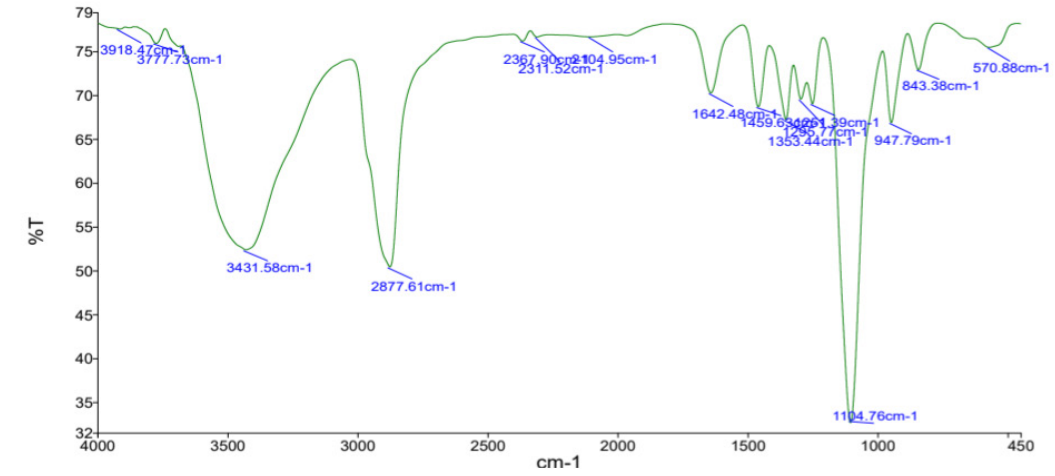
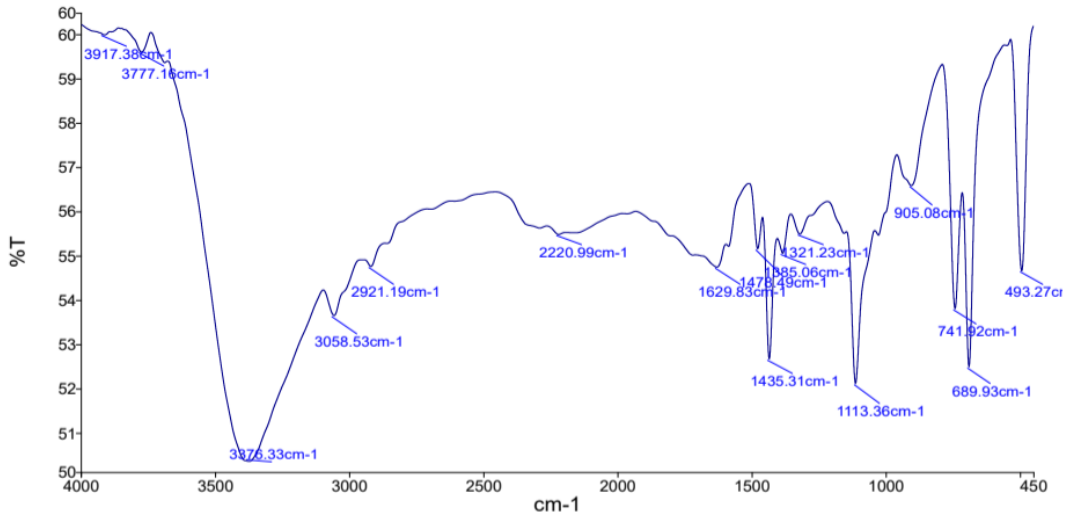


Figure 1: Chemical Structure of TPP, PF127 and Conjugate.

(2a) (2b) (2c)

Figure 2: Synthesis of TP: 2a: Reaction mixture containing PF127, TPP-COOH, DMSO, DCC and DMAP; 2b: Stirring of reaction mixture in N_2 atmosphere; 2c: Lyophilized product.





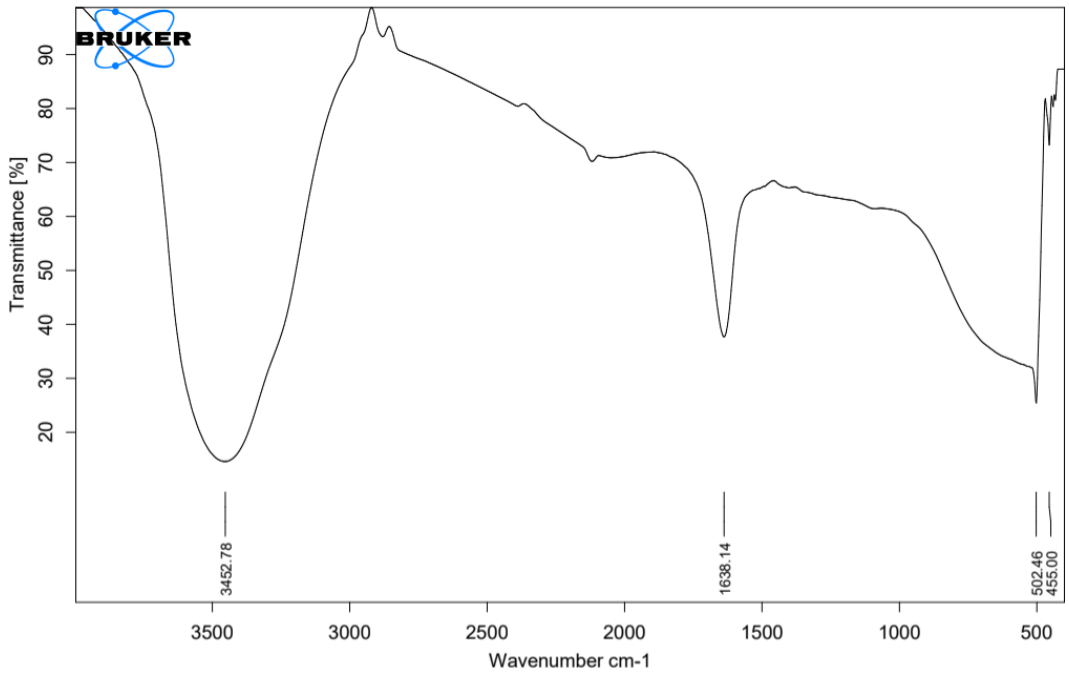


Figure 3: 3a: FT-IR of Pluronic F127; 3b: FT-IR of TPP-COOH [(5-carboxypentyl) triphenylphosphonium bromide]; 3c: FT-IR of Triphenylphosphonium-Pluronic F127 conjugate (TP).

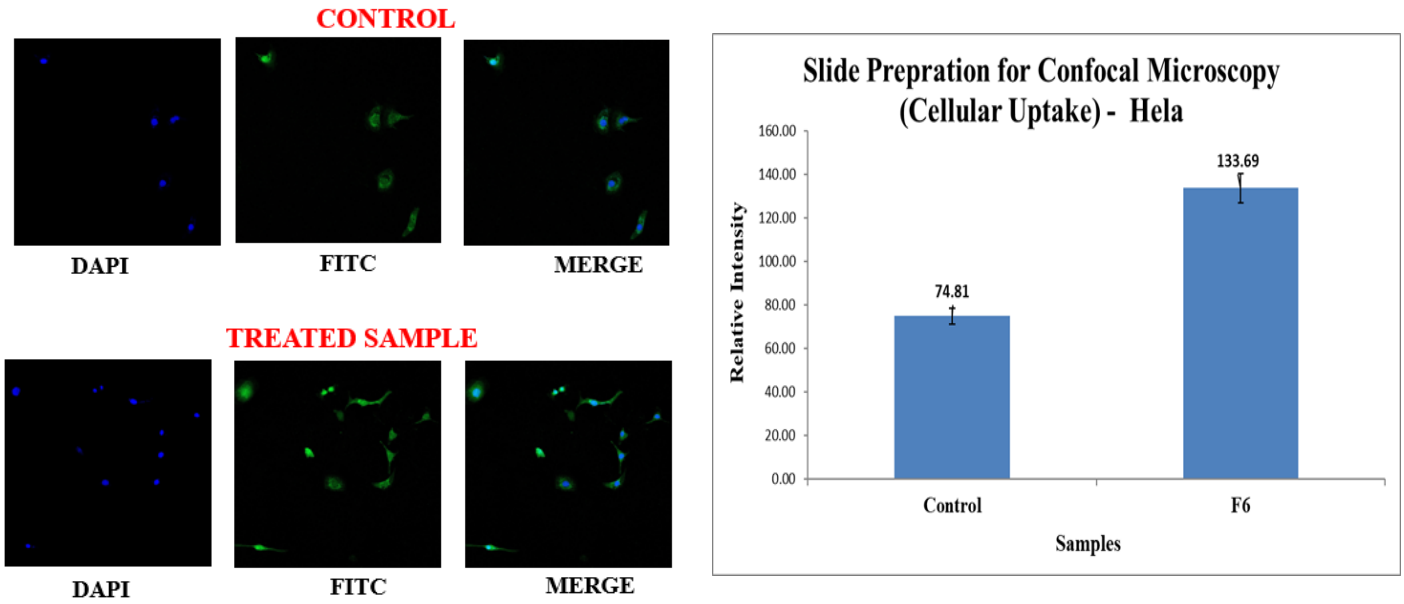


Figure 4: 4a: Confocal Microscopy Images- HeLa Cell Lines; 4b: Cellular Uptake Using HeLa Cell Lines.

an indirect measure of the number of molecules internalized by cells. Statistical analyses (20.87±3.76) along with *p*-values (0.03) is used to assess the significance of differences in cellular uptake. The cellular uptake was significantly higher in the treated group (133.69%±5.42%) compared to the control group (74.81% ± 4.31%, $p \geq 0.01$). Statistical significance was determined using an unpaired Student's *t*-test (two-tailed) as shown in Figure 4.

DISCUSSION

Mitochondria-targeting moieties are broadly classified based on their targeting mechanisms, physicochemical properties (such as charge, hydrophobicity, and molecular size), and structural attributes. Among the most prominent are lipophilic cations like TPP, which facilitate mitochondrial accumulation via

electrophoretic attraction to the highly negative mitochondrial membrane potential. In the current study, we synthesized a TPP-PF127 conjugate that harnesses the targeting efficiency of TPP, while incorporating PF127, a biocompatible triblock copolymer known for its amphiphilicity and high aqueous solubility to enhance the micellar system's stability and dispersibility.[16] Conventional mitochondrial-targeting approaches, including rhodamine derivatives, rely on similar membrane potential-driven mechanisms but are limited by phototoxicity and off-target effects. Alternatively, peptide-based systems, such as Szeto-Schiller peptides, target cardiolipin-enriched mitochondrial membranes to restore bioenergetics but often suffer from poor systemic stability and short *in vivo* half-lives.[17] The TPP-PF127 system developed here addresses these limitations by integrating efficient mitochondrial targeting with enhanced structural integrity and bioavailability. The micellar formulation improves drug encapsulation and intracellular delivery, while minimizing degradation and clearance. The successful synthesis and characterization of the TPP-PF127 conjugate demonstrates its potential as a mitochondria-targeted drug delivery platform. FTIR analysis confirmed the covalent linkage between TPP and PF127, validating the structural integrity of the conjugate. Confocal microscopy revealed clear mitochondrial localization, indicating that the conjugate retained the targeting ability of TPP, likely due to its affinity for the mitochondrial membrane potential. Moreover, the conjugate exhibited efficient cellular uptake, suggesting that the amphiphilic nature of PF127 enhances membrane permeation. This dual functionality-robust internalization and precise mitochondrial targeting positions the TPP-PF127 system as a promising tool for delivering therapeutics to mitochondria.

CONCLUSION

While TPP has been previously utilized as a mitochondrial targeting moiety, its successful conjugation with PF127; a polymer with inherently poor membrane interaction represents a novel strategy. This conjugation not only reduces the HLB of PF127 but also enables the formation of micelles with enhanced mitochondrial affinity, a property not previously reported to this extent. Our approach addresses a critical limitation in current mitochondrial delivery systems, namely the challenge of achieving both membrane interaction and mitochondrial specificity without compromising cell viability.

The characterization results provided clear evidence of the successful conjugation of TPP and PF127, thereby confirming the accuracy and reliability of the synthesis process. Analytical techniques, such as FTIR, likely identified the specific chemical bonds formed between TPP and PF127, validating the structural integrity of the conjugate. Confocal microscopy analysis further revealed that the synthesized conjugate effectively localized to the mitochondria within cells. This observation suggests that the conjugate retains the mitochondrial targeting properties of TPP, leveraging the negative membrane potential of mitochondria to achieve selective accumulation. The fluorescent signals captured by confocal microscopy provided visual confirmation of the conjugate's precise subcellular localization. In addition to targeting capability, the conjugate demonstrated effective cellular uptake, indicating that it can efficiently traverse cellular membranes and reach intracellular compartments. This dual functionality, efficient mitochondrial targeting and robust cellular uptake, underscores the potential of the TPP-PF127 conjugate for advancing therapeutic strategies aimed at mitochondria. Such targeted delivery systems could enhance the specificity and efficacy of treatments, particularly in diseases where mitochondrial dysfunction plays a critical role, such as cancer or neurodegenerative disorders.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ABBREVIATIONS

APTES: (3-Aminopropyl) Triethoxysilane; **Aβ:** Amyloid-beta **DAPI:** 4',6-diamidino-2-phenylindole; **DCC:** N,N'-Dicyclohexylcarbodiimide; **DMAP:** 4- Dimethylaminopyridine; **DOX:** Doxrubicin; **FITC:** Fluorescein Isothiocyanate; **FT-IR:** Fourier-Transform Infrared Spectroscopy; **HLB:** Hydrophilic-Lipophilic Balance; **IR:** Insulin Receptor; **IRS1:** Insulin Receptor Substrate 1; **N_2:** Nitrogen; **PBS:** Phosphate Buffer Solution; **PEO:** Polyethylene Oxide; **PF127:** Pluronic F127; **PI3K:** Phosphoinositide 3-kinase; **PPO:** Polypropylene Oxide; **RT:** Room Temperature; **TP:** Triphenylphosphonium- Pluronic F127 Conjugate; **TPP:** Triphenylphosphonium; **TPP-COOH:** (5-carboxypentyl) Triphenylphosphonium Bromide.

FUTURE ASPECTS

Future research will focus on the strategic modification of the TPP-PF127 conjugate with therapeutic agents to evaluate its potential for drug delivery applications. The drug loading capacity, release profiles under physiological conditions, and stability of the conjugate also will be investigated to determine

its suitability for clinical use. Additionally, *in vivo* studies will be done to assess its biodistribution, therapeutic efficacy, and biocompatibility. Collectively, these studies will be critical for advancing this mitochondrial-targeted delivery system toward clinical translation.

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