Journal of Drug Targeting

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/idrt20

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To cite this article: Lissette Sanchez-Aranguren, Mohamad Anas Al Tahan, Muhammad Uppal, Parag Juvale & Mandeep Kaur Marwah (08 Dec 2024): Mitochondrial-targeted liposome-based drug delivery – therapeutic potential and challenges, Journal of Drug Targeting, DOI: 10.1080/1061186X.2024.2437440

To link to this article: https://doi.org/10.1080/1061186X.2024.2437440

Published online: 08 Dec 2024.

JOURNAL OF DRUG TARGETING
https://doi.org/10.1080/1061186X.2024.2437440

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Mitochondrial-targeted liposome-based drug delivery – therapeutic potential and challenges

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ABSTRACT

Liposomes, as nanocarriers for therapeutics, are a prominent focus in translational medicine. Given their biocompatibility, liposomes are suitable drug delivery systems rendering highly efficient therapeutic outcomes with minimal off-site toxicity. In different scenarios of human disease, it is essential not only to maintain therapeutic drug levels but also to target them to the appropriate intracellular compartment. Mitochondria regulate cellular signalling, calcium balance, and energy production, playing a crucial role in various human diseases. The notion of focusing on mitochondria for targeted drug delivery was proposed several decades ago, yet the practical application of this idea and its translation to clinical use is still in development. Mitochondrial-targeted liposomes offer an alternative to standard drug delivery systems, potentially reducing off-target interactions, side effects, and drug dosage or frequency. To advance this field, it is imperative to integrate various disciplines such as efficient chemical design, pharmacology, pharmaceutics, and cell biology. This review summarises scientific advances in the design, development and characterisation of novel liposome-based drug delivery systems targeting the mitochondria while revisiting their translational potential.

PERSPECTIVES

- Several mitochondrial targeting moieties such as lipophilic cations, plant-based and peptide-based targeting approaches have been explored for the functionalisation of liposomal formulations allowing the delivery of hydrophobic drugs while improving their *in vivo* half-life.
- Amongst these targeting moieties, lipophilic cations, triphenyl phosphonium (TPP+) and dequalinium (DQA) are the most explored. However, recently developed mito-porters have gained traction in translational medicine beyond gene therapy.
- Research is still required to meticulously design a liposome formulation capable of targeting mitochondria as well as homing in on the specific organ of interest overcoming the challenge of crossing human barriers such as the blood-brain barrier.

ARTICLE HISTORY
Received 24 September 2024
Revised 22 November 2024
Accepted 26 November 2024

KEYWORDS
Nanocarriers; liposomes; mitochondria; targeted drug delivery; mitochondrial dysfunction

Introduction

Mitochondria are energy-producing organelles with emerging roles as cell signalling mediators [1]. Other biological functions include the regulation of calcium signalling, apoptosis, cell proliferation and cell cycle regulation [2]. Dysregulation in one or more of these functions is often found in a myriad of human conditions, including ageing, cardiovascular disease, cancer, neurodegenerative diseases and genetic conditions [3–6]. These human conditions are varied in signs and symptoms, common mitochondrial-related pathophysiological mechanisms have been widely described in the literature. These research efforts have provided compelling evidence on molecular mechanisms related to mitochondrial dysfunction, nonetheless, it is still imperative to identify approaches that can efficiently and safely address mitochondrial dysfunction.

In recent decades, there has been a growing focus on leveraging mitochondria for therapeutic and diagnostic purposes, aiming to selectively deliver and accumulate specific compounds of interest [7]. Nevertheless, this field of research has extensively focused on treating conditions such as cancer [8,9] and neurodegeneration [10,11] meanwhile, less familiar conditions, such as those related to cardiovascular issues and mitochondrial genetic disorders, are only now starting to garner attention. The direct and indirect covalent modifications of compounds have been explored to modify well-known compounds of mitochondrial interest, including metformin [12,13], vitamin E [14] and Coenzyme Q [15,16]. Most of these approaches, although promising, are still in the pipeline to clinical use. Many reasons hindering their translation to the clinic can be considered, including (i) their inherent lack of solubility in plasma/serum leading to toxicity and off-target effects [17]; (ii) limited drug stability due to degradation of the mitochondrial-targeting moiety [18] and (iii) rapid clearance leading to decreased biodistribution *in vivo* due to the biochemical modifications with cationic structures that provide the mitochondrial targeting properties [18].

Emerging fields of interdisciplinary research combining biomaterials, pharmaceutics and medicine led to the exploration of

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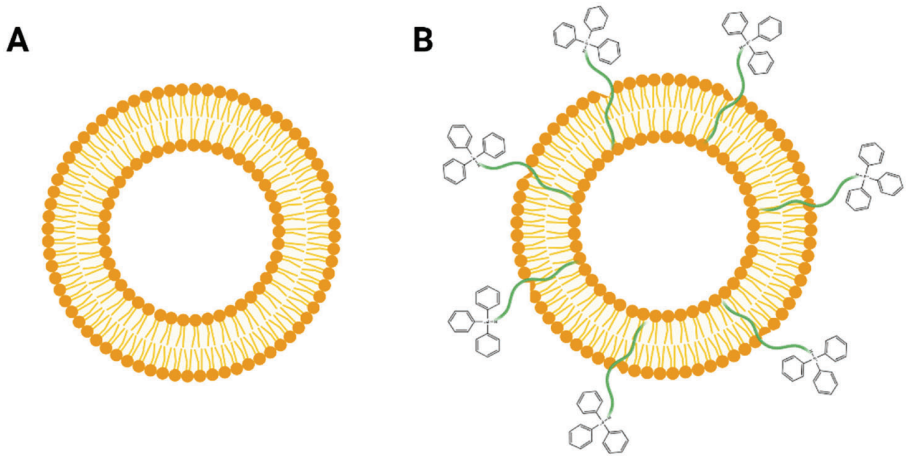

Figure 1. Schematic representation of liposomes depicting the functionalisation of the outer lipid bilayer to target the mitochondria. (A) Schematic representation of a conventional liposome formulated from phospholipids and (B) graphical representation of a liposome incorporating a targeting moiety (such as DSPE-PEG-TPP, TPGS or DQA-DSPE-PEG2000).

nanocarriers and their targeting to the mitochondria [19]. Nanoparticles offer a solution to these drawbacks as they can be used to deliver therapeutic reagents to mitochondria in a targeted and sustained manner [20]. Moreover, nanoparticles can be conjugated with traditional mitochondrially active drugs to create multifunctional nanoplatforms. Liposomes in particular, provide an alternative biocompatible approach, limiting the challenges posed by drugs with low solubility and rapid clearance that can be targeted to intracellular compartments through the functionalisation of their outer lipid layer [21] (Figure 1). Although in early stages of discovery, liposomes targeted to the mitochondria provide an alternative to standard drug delivery systems with the potential to overcome drug resistance, side effects and reduced drug administration regimes [22]. Moreover, liposomes can be administered through varied routes, which is particularly relevant in the development of patient-friendly delivery system [23].

This review aims to summarise scientific advances in the design, development and characterisation of novel liposome-based drug delivery systems targeting the mitochondria for the administration of active drugs/metabolites in a broad area of human conditions that share common mitochondrial dysfunction mechanisms and critically discuss their potential benefit in managing human disease in their route to the clinic.

Mitochondria in health and disease

Dysfunction of mitochondria in human disease is often accompanied by a reduction in ATP production, dysregulation in mitochondrial dynamics, excessive mitochondrial-derived reactive oxygen species generation and impaired mitochondrial-mediated apoptosis. These molecular mechanisms have been broadly explored in substantial published literature [4,24–27].

Mitochondria have different roles in supporting health; the molecular and biochemical events involved in energy metabolism is termed bioenergetics. Oxidative phosphorylation (OXPHOS) coupled to the electron transport chain (ETC) are responsible for generating ATP in mitochondria [24]. The reduced expression, inhibition and/or uncoupling of ETC complexes are well-studied mechanisms [24,26] often targeted with conventional mitochondrial-targeted therapeutics [28,29] for the improvement of mitochondrial health.

In order to effectively produce ATP, new mitochondria are generated in a process termed mitochondrial biogenesis. Mitochondrial factors play a key role in this process; however, the transcription and translation of nuclear respiratory factors (NRF1 and NRF2) are also involved in the regulation of mitochondrial biogenesis [25]. PGC-1α, a co-transcriptional regulation factor, promotes the expression of Tfam by activating NRF1 and NRF2, resulting in the transcription and replication of mitochondrial DNA (mtDNA) [30]. Reduction in PGC1-α expression and activity occurs in several human conditions from cardiovascular disease to neurodegeneration [30,31].

Mitochondria continuously undergo coordinated fusion and fission cycles in order to maintain the shape, distribution and size [25]. Alterations in these dynamic events leads to either a fragmented network of disconnected, small pieces of mitochondria or a fused network of connected and large mitochondrial structures [32]. Several proteins participate in mitochondrial dynamics. Fission is supported by dynamin-related/-like protein 1 (Drp1), dynamin 2 (Dnm2), and Fis1 while fusion is maintained by mitofusin 1 (Mfn-1), mitofusin 2 (Mfn-2), and optic atrophy 1 (OPA-1) [25]. Increased mitochondrial fission has been reported in cancer cells including melanoma, ovarian and breast cancer [33]. Moreover, existing reports suggest that increased mitochondrial fusion is linked to chemoresistance of tumour cells [34].

As part of the ETC and in association to the bioenergetic role of mitochondria, reactive oxygen species (ROS) are produced as by-product to the physiological metabolism of oxygen [35]. As well as in the mitochondria, other cellular structures (such as NADPH oxidase) generate these species, including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) [36]. There are several comprehensive publications detailing the mechanistic role of mitochondria in producing ROS [35–37]. Although ROS are generated physiologically, an imbalance between the generation of ROS and the activity of the endogenous antioxidant machinery capable to scavenge excessive ROS results in oxidative stress which extensively damage cellular components (proteins, DNA and lipids) leading to mitochondrial dysfunction [35]. The use of exogenous antioxidants has been largely explored to overcome oxidative stress; however, these have been widely proven unsuccessful in humans [38]. A potential explanation to this lack of effects may be explained by the inability of antioxidants to

reach and accumulate in cellular compartments such as the mitochondria.

Liposome-based nanocarriers targeting the mitochondrion

Nanocarriers are nanoscale materials or devices designed to transport and deliver cargo to specific target sites in the body [39]. Within the realm of nanocarriers, nanoparticles denote ultrafine particles with size ranging from 1 to 100 nm in diameter, though sizes up to 1000 nm can be included depending on the context and discipline [40,41]. A comprehensive understanding of nanoparticle uptake, accumulation and translocation is indispensable in order to use larger (>100 nm) particles as drug carriers.

Nanoparticles are able to improve drug bioavailability, increase their half-life, offer controlled release properties allowing reduced therapeutic doses, reduce off-target side effects, and target drug delivery to specific tissues or organs [39,42]. However, formulating systems with these qualities remains a considerable challenge in nanomedicine. Amongst these, to be able to deliver a payload to the target organ, a nanoparticle system needs to be able to encapsulate the therapeutic cargo, survive destruction or elimination when it enters circulation, enter the target organ through the endothelium and extracellular matrix, penetrate the desired cell type, evade endosomal or lysosomal destruction, and undergo disassembly to release its contents, without inducing immune activation or toxicity [43].

Liposomes

Liposomes are established carrier systems for therapeutically active agents owing to their unique beneficial characteristics, including biodegradability, biocompatibility, lack of immune system activation as well as having potential for controlled release and targeting abilities [44]. Liposomes consist of amphoteric lipid molecules (phospholipids) that form spherical, self-closed structures in aqueous media to minimise the entropically unfavourable interaction between hydrophobic chains and aqueous medium (Figure 1). They may incorporate either hydrophobic or hydrophilic drugs. Liposomal complexes are generally stable enough to protect a varied range of cargo from degradation and are competent to enter cells usually by endocytosis [45]. The suitability of liposomes as drug carriers depends on the physicochemical properties of the membrane, the nature of their components, size and surface charge. In the design of liposomes, an anchor amphoteric lipid such as phosphatidylcholine (PC) is used while cholesterol (Chol) is often included in the formulation for its stabilising properties. Liposome size is a critical physical aspect that determines the clinical success of the nanocarriers [39,44]. In average, liposomes applied in a clinical setting range between 50 and 450 nm in diameter [39].

Lipids may carry a positive or negative charge and are termed cationic and anionic lipids respectively. Cationic Lipids, capable to incorporate hydrophilic and hydrophobic drugs are useful in gene delivery (transfection), cell targeting, antimicrobial properties, vaccine adjuvants due to their ability to reduce toxicity of the compounds and lack of activation of the immune system. Anionic lipids are used in the stabilisation of emulsions, intracellular targeting, biocompatibility, cell signalling. Importantly, the specific applications and benefits of cationic and anionic lipids depend on their chemical structure, the context of use, and the desired outcome in a particular application.

Liposomes interact with cells through different molecular mechanisms including the interaction with cell membranes through endocytosis [46]. Endocytosis is a process by which cells are able to uptake substances such as macromolecules, fluids and large particles, including liposomes [46]. Moreover, the interaction between cells and liposomes is affected by the charge of the liposome. In this regard, cationic liposomes often show enhanced uptake by cells because they can better interact with the negatively charged cell membrane, promoting fusion or endocytosis [47]. Liposomes are also able to interact with intracellular compartments, including the mitochondrion. The functionalisation of the outer bilayer of the liposomes with mitochondrial-targeting moieties (lipophilic cations) allows the preferential uptake and delivery of compounds to the mitochondria because these lipophilic cations can pass through the mitochondrial membrane potential and accumulate selectively within mitochondria [48] (Figure 2).

Approaches to target liposomes to the mitochondria

The mitochondrial membrane potential (ΔΨm) is maintained at −150 to −180 mV (negative to the cytosol) allowing the influx of protons and calcium into the mitochondria, thus determining its biological functions [49] (Figure 2). Targeting mitochondria poses a challenge due to their complex structure with a double membrane and a highly negative potential. Penetrating these organelles with drugs is challenging, and unless positively charged, it is difficult to achieve effective accumulation and subsequent impact [17,50]. Nonetheless, the targeting of liposomes to reach subcellular compartments is emerging as drug delivery system in chronic conditions. Although targeted liposomes are a current topic of interest in mitochondrial-related conditions, there are still concerns related to the toxicity of the targeting motifs and the chemistry used to achieve the targeted properties. The use of lipophilic cations as targeting motifs for mitochondrial-specific drug delivery may be associated to increased cellular toxicity. It has been previously reported that cationic compounds such as triphenyl phosphonium (TPP+) are considered not to exhibit any significant biological effect, nonetheless, their high affinity for phospholipid membranes has shown to significantly disrupt the mitochondrial membrane integrity leading to impairment of the respiratory chain and ATP synthesis [51]. In order to assess the effect of mitochondrial

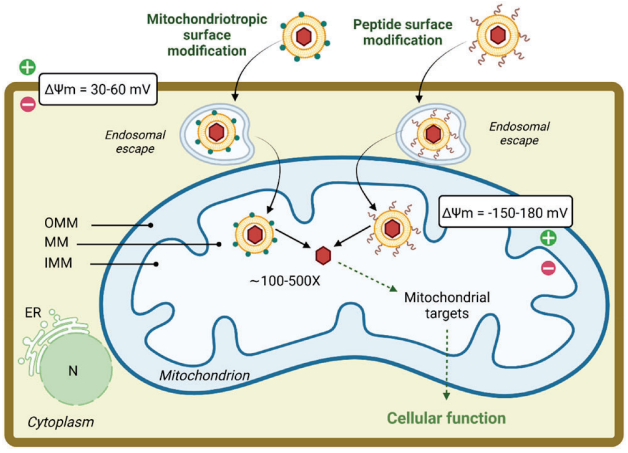

Figure 2. Schematic representation of different approaches employed to target liposomes to the mitochondria including mitochondriotropic functionalisation of the outer lipid bilayer and peptide surface modifications.

targeting motifs on cellular viability, the content of these targeting compounds used for liposomal preparations should be critically analysed against toxicity parameters while confirming the liposomal formulation retains the intended targeting properties.

Mitochondrial-targeted liposomes have been investigated in different scenarios of human disease as novel carriers for drug delivery targeted to the mitochondria. Table 1 summarises mitochondrial-targeting approaches employed in the preparation of liposomal formulations in different models of human disease.

Mitochondriotropic modification

In the past two decades, several cationic compounds, including triphenyl phosphonium (TPP+), rhodamine, 1,10-Decamethylene bis-4-aminoquinaldinium chloride (Dequalinium, DQA), guanidinium and pyridinium cations and berberine (a benzylisoquinoline alkaloid) have been identified as efficient mitochondrial-targeting motifs due to their ability to accumulate in the mitochondrial matrix given the electrostatic interactions with the negatively charged inner mitochondrial membrane [7] (Figure 3).

Triphenyl phosphonium (TPP+). TPP+, a well-characterised and vastly employed lipophilic cation targeting compounds of interest to the mitochondria [7,49], is composed of a delocalised positive charge over three phenyl groups, stabilised by resonance (Figure 3). The charge and hydrophobicity associated with TPP+ enables interactions with the hydrophobic inner mitochondrial membrane and allows penetration. TPP+ is by far the lipophilic cation of choice in the targeting of liposomes, due to inherent properties including: stability, low chemical reactivity to cell components and simplicity in production and purification [83]. At least two different approaches based on TPP+-targeting properties has been explored so far. First, the conjugation of the TPP+ group to a polymer matrix incorporated into liposomes and second, the non-covalent introduction of TPP+ into phospholipids (when TPP+ is conjugated to an amphiphilic derivative). Although still in exploration, both approaches seem to enhance mitochondrial uptake and accumulation of active compounds. However, there is still controversy on which approach has better targeting properties and less toxicity to cells.

TPP+-polymer conjugates. DSPE-PEG (1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol) and polyethylene glycol- phosphatidylethanolamine (PEG-PE) are well-known phospholipid-polymer conjugates used to impart stealth properties and increase circulation half-life in liposome formulations [84,85]. Both polymers can be functionalised with various molecules, including TPP+ to generate mitochondriotropic TPP+ polymer conjugates (Figure 4).

In 2012, Biswas et al. conjugated TPP+ to PEG-PE polymer to generate a TPP-PEG-PE conjugate and incorporated it into liposomes to encapsulate paclitaxel (Table 1). In this report, it was observed that the anti-tumour efficacy of paclitaxel was enhanced when delivered in these mitochondrial-targeted liposomes with a significant reduction in tumour volume with minimum toxicity effects in a murine model of breast adenocarcinoma [86]. Other authors have used DSPE-PEG to generate a TPP-DSPE-PEG polymer conjugate to incorporate into liposomes observing increased compound accumulation within the mitochondria thus enhanced *in-situ* effects [53–56].

It is not clear whether the polymer-based structure makes a significant effect into the targeting properties of TPP+-polymer conjugates. However, lack of stability in serum and potentially low tumour selectivity have been reported in both. To overcome these concerns, Wang et al., designed a TPP+-tocopheryl polyethylene glycol succinate (TT) polymer conjugate incorporated into liposomes coated with hyaluronic acid *via* electrostatic interactions, to encapsulate and deliver paclitaxel in the treatment of multidrug-resistant lung cancer. They observed greater stability in serum of the hyaluronic acid -coated liposomes when compared to preparations lacking the hyaluronic acid coating [57]. The authors suggested that hyaluronic acid may protect against the interaction between the positively targeted liposomes and negatively charged plasma proteins. This assumption could not be more appropriate when proposing the translation of these novel liposome formulations to the clinic. It is imperative to identify effective routes to sustain liposome stability.

TPP+-phospholipid conjugates. Over a decade ago, Boddapati et al. conjugated TPP+ to a non-polar stearyl residue to produce stearyl triphenyl phosphonium (Stearyl TPP+) (Figure 4). This amphiphilic molecule was incorporated into the liposomal lipid bilayer to produce Stearyl TPP+-modified nanocarriers (Table 1). Using this delivery approach, Boddapati showed the efficient mitochondrial-targeted delivery of ceramide to colon cancer cells followed by a robust apoptotic response [87].

Meanwhile, efforts have been made to not only target drugs to the mitochondria, but to enhance the accumulation of drugs of interest in cancer cells, using "mitocancerotropic liposomes" [88]. Thus, liposomes using stearyl TPP+ as the amphiphilic anchoring structure were incorporated with a cancerotropic ligand to encapsulate doxorubicin (Table 1). The authors reported a synergistic effect between stearyl TPP+ and folic acid conjugates in the delivery of doxorubicin to cancer cells [88]. These approaches show that the dual functionalisation of liposomes allows better anti-cancer efficacy.

Other approaches using Stearyl TPP+ have focused on developing mitochondrial-targeted vegetable oil-based nano-emulsions encapsulating cyclosporine A for the prevention of doxorubicin-induced cardiomyopathy. Nano-emulsions are colloidal dispersions of nanoscale droplets of one immiscible liquid within another immiscible liquid, stabilised by an emulsifying agent. Although not a liposome-based technology, the use of nano-emulsions is proposed to be a good carrier candidate for highly hydrophobic compounds [89]. The use of Stearyl TPP+ to target the mitochondria has been controversially reported as cytotoxic by some authors. This has led to further efforts to generate more biocompatible approaches with similar mitochondriotropic advantage that the TPP+ group provides. In this regard, Benien et al. reported novel TPP+-phospholipid conjugates (with dioleoyl, dimyristoyl or dipalmitoyl lipid moieties) observing that the phospholipid anchors employed stabilised cell and mitochondrial membrane [90].

Recently, other cationic liposomes non-covalently modified with TPP+ and Imidazolium were reported [66,91]. These liposomes were prepared by incorporating TPP+ and Imidazole with 10, 12, 14, and 16 carbon atoms (in hydrocarbon chain) into the lipid bilayer [67]. Moreover, the use of TPP+-functionalised liposomes has started to gain interest outside cancer research. Liposomes modified with TPP+ following sonication steps to entrap and delivery platinum and ruthenium nanozyme are novel approaches in the amelioration of retinal neovascularisation [58] and temporomandibular disorders [59] (Table 1).

Rhodamine. Another lipophilic cation, rhodamine, has been used mainly in the preparation of mitochondrial probes, including the fluorescent tetramethyl rhodamine methyl ester (TMRM) for the detection of changes in mitochondrial membrane potential [7].

Table 1. Therapeutic potential of mitochondrial-targeted liposomes as drug carriers.

Mitochondrial-targeting motif	Condition	Lipids	Model	Compound	Effect	References
Berberine	Glioma	SPC Chol BBR-Chol FA-PEG_{3350}-Hz-Chol Ratio = 65: 25: 9: 1	C6 cells C6-bearing *in situ* glioma model in mice	Paclitaxel	Enhanced blood-brain barrier penetration. Effective targeting of glioma.	[52]
TPP-DSPE-PEG2000	RIR injury model	Di-S-PC Lecithin Chol†	R28 retinal cells RIR model in rats (Sprague-Dawley)	Quercetin	Targeting of FOXO3A pathway. Reduction of oxidative stress and inflammation.	[53]
	Photodynamic therapy	DOTAP DOPC†	HCT116 cells	Verteporfin Gold nanoparticles	Enhanced mitochondrial-targeted effect when combined with X-ray.	[54]
TPP-DSPE-PEG2000 DQA-DSPE-PEG2000	Cancer	POPC Chol Ratio = 7:3	B16F10 cells	Resveratrol	Induced cytotoxicity of cancer cells.	[55]
TPP-DSPE-mPEG2000	Bladder cancer	SPC Chol†	MB49 cells MB49-bearing *in situ* bladder tumour in mice (C57BL/6)	Brequinar	Inactivation of dihydroorotate dehydrogenase. Induced mitochondrial lipid peroxidation. Infiltration of CD8+ T cells into tumour microenvironment.	[56]
TPGS	Non-small cell lung cancer	Cationic liposome†	A549/T cells	Paclitaxel	Higher apoptotic rate and stronger anticancer efficacy.	[57]
TPP+-nanozyme	Retinal neovascularisation	Lecithin DSPE-PEG-NH_2 Chol†	Oxygen-induced retinopathy in C57BL/6 J mice	Platinum (Pt) nanozyme	Reduced hypoxia-induced neovascularization.	[58]
	Temporomandibular joint disorders	Lecithin DSPE-PEG-NH2 Chol Ratio = 10:1:2	Model of temporomandibular joint disorder in mice	Ruthenium (Ru) nanozyme	Inhibition of ROS and alleviated TMD pain *via* the TNF-α/NF-κB/NEAT1 pathway.	[59]
TPP+	Glioma	SPC Chol Chol-TPG Ratio = 60:34:3	C6 cells C6-bearing *in situ* glioma model in mice	Doxorubicin and Ionidamine	Anti-glioma effects with reduced off-site effects.	[60]
TPP+	Colorectal cancer	S100 Chol DPPE DOPE DSPE-mPEG2000 Ratio = 4:1:1:2:0.4	HCT116 cells NCM460 cells HCT116-bearing BALB/c mice	Cyclopeptide RA-XII	Anti-tumour efficacy when combined with photodynamic therapies.	[61]
TPP+-Chol	Cancer	SPC Chol-TPP Ratio = 30:3	HepG2 cells HepG2-bearing BALB/c mice	Celastrol	Liposomes coated with hyaluronic acid enhanced drug uptake.	[62]
CTPP-Chol	Glioma	Chol SPC Chol-CTPP Ratio = 34:60:3	C6 cells C6-bearing *in situ* glioma model in mice (Kunming mice)	Doxorubicin and Ionidamine	Ability to cross the blood-brain barrier and selectively accumulate in tumour cells. Enhanced synergistic effect of doxorubicin and ionidamine.	[63]
	Breast cancer	DPPC: mPEG†	MCF-7	Ceramide	Generation of ROS and cell apoptosis.	[64]
TPPB-14	Alzheimer's disease	PC Chol†	Rat motoneurons Transgenic AD mice model – APP/PS1 (*in vivo*)	α-tocopherol and donepezil hydrochloride	Decrease memory impairment. Reduction in Aβ plaque formation.	[65]
TPPB-n	Not specified	DPPC†	PANC-1	Rhodamine B Metronidazole	Confirmed mitochondrial-targeted delivery.	[66]
TPPB-n IA-n(OH)	Not specified	PC Chol Ratio = 9:11	PANC-1 HuTu 80 WI-38	Rotenone	Reduced IC_{50} for rotenone. Selectivity for cancer cells. Dose-dependent decrease in mitochondrial membrane potential.	[67]
Stearyl TPP+	MIR	Egg yolk lecithin Chol†	H9C2 MIR model in rats	Astaxanthin	Reduction in hypoxia-reoxygenation-dependent production of ROS. Improved cardiac function in rat model.	[68]

(*Continued*)

Table 1. Continued.

Mitochondrial-targeting motif	Condition	Lipids	Model	Compound	Effect	References
Guanidinium	Not specified	SPC Chol DSPE-PEG2000 Ratio = 5: 1: 0.35	4T1 cells 4T1-bearing BALB/c mice	Indocyanine green	3.7-fold higher mitochondrion-targeted delivery efficacy compared to TPP+. Tumour eradication after combined photothermal and photodynamic therapies.	[69]
Mito-porter	Mitochondrial disease Heteroplasmic G625A $tRNA^{Phe}$ mutation of the mitochondrial DNA	DOPE SM STR-R8 Ratio = 9:2:1	G625A fibroblasts (skin biopsy from patient)	Wild-type mitochondrial pre-$tRNA^{Phe}$	Reduced mutant tRNA levels for up to eight days post-transfection. Improved mitochondrial respiratory activity.	[70]
	Cytochrome c oxidase subunit II	DOPE SM STR-R8 Chol-GALA Ratio = 9:1:1:0.2	HeLa cells	Antisense RNA oligonucleotide (ASO) targeting COX II	10-fold higher packaging efficiency. Reduction in target mRNA levels and ATP production.	[71]
	Mitochondrial disease A1555G point mutation coding mutant rRNA 12S	DOPE SM STR-R8 Ratio = 9:2:1	A1555G fibroblasts (skin biopsy from patient)	Wild-type-rRNA (12S)	Significant decrease in mutant rRNA content. Improved mitochondrial respiratory activity.	[72]
	Cancer	DOPE Chol STR-R8 Ratio = 9:2:1	SAS cells SAS-bearing tumour model in BALB/c mice	π-extended porphyrin-type photosensitiser	Tumour inhibition in combination with photodynamic therapy.	[73]
	Renal cancer	DOPE Chol STR-R8 Ratio = 9:2:1	OS-RC-2 cells	Doxorubicin	Increased cell death mediated by doxorubicin in doxorubicin-resistant cells.	[74]
	Neurodegenerative diseases	DOPE SM STR-R8 Ratio = 9:2:1	Neuro2a cells	Berberine (BRB)	Improved mitochondrial respiratory activity. Increased intracellular ATP levels. Increased expression of mitochondrial ubiquitin ligase.	[75]
KALA peptide	Skeletal muscles with systemic disease	DOPE SM Ratio = 9:2 DC-Chol EPC SM Ratio = 3:4:3	C2C12 cells	pDNA: pCMV-mtLuc (CGG)	Efficient mitochondrial targeting by KALA peptide. Mitochondrial transgene expression.	[76]
	Cancer	DSPE-PEG†	MDA-MB-231 (*in vitro*) MDA-MB-231 tumour-bearing mouse model (*in vivo*)	5-fluorouracil and paclitaxel	No *in vivo* toxicity. Mitochondrial-targeted apoptosis.	[77]
KLA peptide	Breast cancer	SPC Chol DSPE-PEG2000†	HUVECs MDA-MB-231 MDA-MB-453 MDA-MB-436 human breast carcinoma cells MDA-MB-231 tumour-bearing mice (*in vivo*)	5-fluorouracil Paclitaxel	No systemic toxicity. Anti-tumour activity.	[77]
ALD5 peptide	Photodynamic therapy for rheumatoid arthritis	SPC Chol DSPE-mPEG2000†	RAW264.7 cells HUVEC Adjuvant-Induced Arthritis model in rats	Chlorine 6	Reduced pro-inflammatory macrophage infiltration and pro-inflammatory cytokine secretion.	[78]
SS31 peptide	Glioma	HSPC Chol DSPE-PEG2000†	U87 cells Bend.3 cells Brain glioma model in BALB/c mice (*in vivo*)	Doxorubicin	Liposomes effectively crossed the blood-brain barrier. Effective targeting of glioma No obvious toxicity or side effects.	[79]
Dimethylmaleic anhydride	Cancer	SPC Chol DSPE-mPEG2000 Ratio = 8:2:5	CBRH-7919 cells CBRH-7919 cells-bearing BALB/c mice (*in vivo*)	Hyperoside	Tumour growth inhibition Low toxicity.	[80]

(*Continued*)

Table 1. Continued.

Mitochondrial-targeting motif	Condition	Lipids	Model	Compound	Effect	References
Mitochondria-fusogenic liposomes	Cardiovascular diseases	DC-Chol EPC SM Ratio = 3:4:3	H9C2 cells	Resveratrol	Significantly activated cellular maximal respiratory capacity. Low toxicity	[81]
Pheophorbide a–quinolinium conjugate	Glioma	EPC Chol DSPE-mPEG2000†	GL261 cells U251 cells U118 cells IMR-90 cells (*in vitro*) Glioma orthotopic model (GL261-bearing C57BL/6 mice)	Pheophorbide a	Low systemic toxicity. Enhanced potency of photodynamic therapy. Visualisation of drug biodistribution and tumour imaging. Prolonged survival rates.	[82]

† = molar ratio of lipid not available.

Abbreviations: 4T1 cells: mammary carcinoma cell line, A549/T cells: Alveolar Type II pulmonary epithelium, B16F10 cells: Murine melanoma cell line, Bend.3: mouse brain microvascular endothelial cell lines, BRB: Berberine, C2C12: myoblasts, C6 cells: Rat glioma cell line, CBRH-7919 cells: Rat hepatoma cells, Chol: Cholesterol, Chol-TPG: pH-responsive ligand Chol-TPG, DC-Chol: Cholesterol hydrochloride, Di-S-PC: ROS-responsive lipids, DOPC: 1,2-Dioleoyl-sn-glycero-3-phosphocholine, DOPE: 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine, DOTAP: N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl, DPPC: Dipalmitoylphosphatidylcholine, DPPE: Phosphatidylethanolamine, DQA: DQAsome, DSPE-PEG: distearoyl phosphatidyl ethanolamine-polyethylene glycol, EPC: egg yolk phosphatidylcholine, EPC: l-α-phosphatidylcholine, FA: Folic acid, GALA: Cholesteryl (Chol)-GALA known as endosome escape device, GL261: murine glioma cells, H9C2 cells: Rat cardiomyocytes, HCT116 cells: Cancer cell line derived from colon cancer, HSPC: Hydrogenated Soybean Phosphatidylcholine, HuTu80 cells: Cell line isolated from small intestine, HUVEC: Human umbilical vein endothelial cells, IA-n(OH): 3-alkyl-1-(2-hydroxyethyl)imidazolium bromides, ICG: indocyanine green, IMR-90 cells: Cell line derived from lung tissue, MB49 cells: Mouse bladder carcinoma cell line, MIR: Myocardial Ischaemia-Reperfusion injury, NCM460 cells: human normal intestinal epithelial cell line, Neuro2a: murine neuronal cell line, OS-RC-2 cells: DOX-resistant cancer cell, PANC-1: Carcinoma cell line from pancreatic duct human cells, PEG: polyethylene glycol, POCP: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, PQC: pheophorbide a–quinolinium conjugate, R28 cells: Retinal cells, RIR: Retinal-ischaemia reperfusion, S100: Lipoid S100, SAS cells: human tongue cancer cells, SM: sphingomyelin, SPC: Soybean phospholipid, Stearyl TPP+: stearic acid triphenylphosphine, STR-R8: Stearylated R8, TMD: Temporomandibular joint disorders, TPGS: triphenylphosphine (TPP)-tocopheryl polyethylene glycol succinate, TPP: Triphenyl phosphonium, TPPB-n: alkyltriphenylphosphonium bromides, U118 cells: glioblastoma cells, U251: glioma cell line, U87 cells: human astroblastoma cell line, WI-38 cells: human diploid cell line.

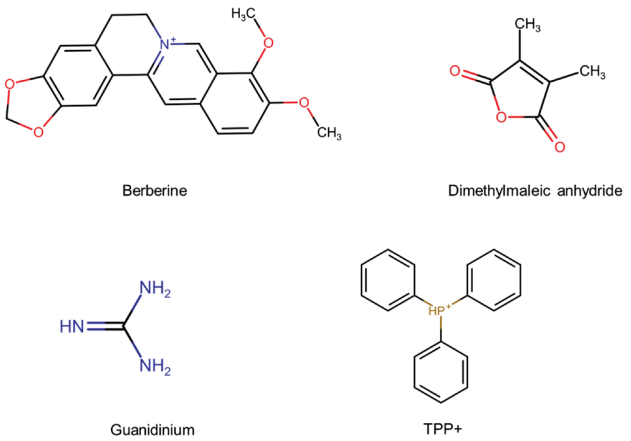

Figure 3. Chemical structures of mitochondrial targeting compounds offering a mitochondrial-targeting moiety.

Given the mitochondriotropic characteristics of rhodamine-123, it has been explored as an alternative to TPP+ in the functionalisation of liposomes. In 2011, Biswas. et al. synthesised a novel amphiphilic polymer by conjugating PEG-PE with rhodamine-123 incorporated to liposomes to produce Rhodamine-modified liposomes loaded with paclitaxel. The authors observed greater mitochondrial uptake and accumulation when paclitaxel was delivered in rhodamine-modified liposomes along with increased apoptotic effects of paclitaxel suggesting the greater accumulation in the mitochondria rendered better drug effects [92] (Table 1).

The use of lipophilic cationic modifications of liposomes targeting the mitochondria is still in its early steps. So far, there is no existent comparison between TPP+ and rhodamine-conjugated to liposomes in terms of their toxic effects and targeting capabilities. To demonstrate the viability of each of these approaches for clinical application, further research is necessary.

Berberine. Berberine is a benzylisoquinoline alkaloid derived from plants of the *Berberidaceae* family that serves as a delocalised lipophilic cation (Figure 3). The use of Berberine to target the mitochondria is still novel. It was first reported in 2011 by Lyamzaev et al., in where Berberine was intended as an alternative for chemical mitochondrial targeting. Since, Berberine has been proposed as a mitochondrial targeting compound given its membrane potential-driven accumulation. Although Berberine has been a choice in the exploration of different carcinomas, just recently Yang et al., investigated its mitochondrial-targeting properties for the treatment of glioma. The authors observed this approach successfully crossed the blood-brain barrier maintaining high tumour specificity [52]. In other scenarios, Berberine has been explored as cargo in mito-porter mediated drug delivery in where showed beneficial effects in modulating mitochondrial dysfunction in neuronal cell Neuro2a [75] (Table 1).

Dequalinium (DQA). DQA is a cationic bolaamphiphile that can be localised within the mitochondria, making it a good candidate for mitochondria-based delivery (Figure 4). DQA can form liposome-like aggregates called DQAsomes, which were first reported in 1998 by Weissig et al. [93,94]. This ability is attributed to DQA's amphiphilic structure, which contains two cationic centres separated by a long hydrophobic chain, forming cationic liposome-like structures in aqueous environments [95]. Furthermore, DQAsomes are DNA-specific targeting moieties for mitochondria since they facilitate the plasmid DNA's cellular uptake, condensation, and protection against DNAse digestion [95]. The functionalisation and modification of DQAsomes can take many forms. The use of DQA to target liposomes to the mitochondria is relatively novel with applications often aimed to address drug-resistant cancer [55,96]. In this regard, research has focused in elevating the DQAsomes complexity using peptides such as HER-2 peptide-PEG-Schiff base-cholesterol [97] and additional functionalisation of the liposomes using hyaluronic acid

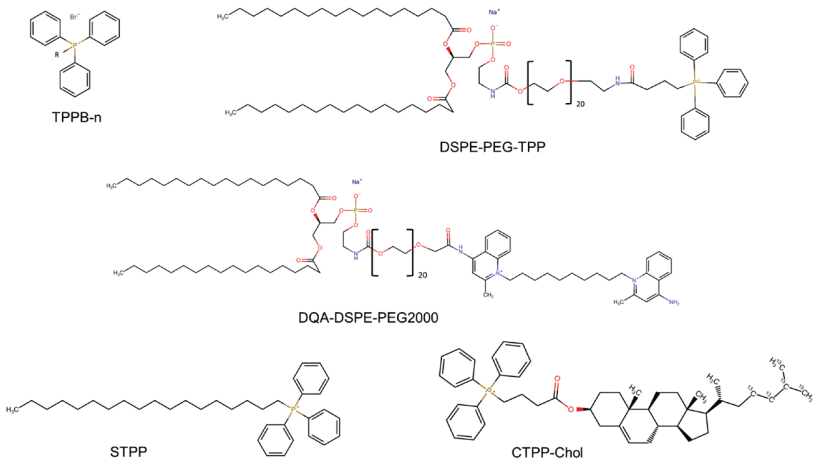

Figure 4. Chemical structures depicting mitochondrial-targeting moiety conjugates.

and DQA [98] to achieve better results in drug-resistant cancer using conventional chemotherapeutics (Table 1).

A study by Kang and Tag Ko in 2019 explored the subcellular trafficking of resveratrol delivered in TPP+ and DQA targeted liposomes. This study observed similar mitochondrial targeting properties when the two mitochondrial-targeting moieties where use and similar cytotoxicity profiles in comparison to resveratrol delivered in not targeted liposomes [55]. Nonetheless, more studies are necessary to evidence which choice of targeting improve mitochondrial accumulation of drugs.

Peptide modifications

The mitochondrial precursor proteins (commonly known as mitochondrial penetrating peptides, MPP) have been critical factors for generating targeting signals to the mitochondria often characterised by an arginine-based amino acid sequence and amphiphilic N-terminal groups [69]. Peptide decorated liposomes offer advantages over the use of lipophilic cations due to their ability to target the mitochondria irrespective of the mitochondrial membrane potential [99] (Figure 2).

KALA, KLA and SS31 peptides. The KALA peptide (WEAKLAKALAKALAKHLAKALAKALKA) is an amphiphilic peptide depicting an α-helical structure at physiological pH [100]. Katayama at al. modified liposomes by linking lipids with KALA peptide, where they presented a high uptake into C2C12 myoblast cells and the ability to transfect genes into the mitochondria [76]. Other examples of peptide modification include the KLA peptide (*D*-$[KLAKLAK]_2$). KLA is a proapoptotic peptide that disrupts the mitochondria membrane [77]. In addition, Cen et al. reported the functionalisation of doxorubicin-loaded liposomes with SS31, a small peptide that can cross the blood-brain barrier and target the mitochondria [79]. In other studies, masking the charge of cationic liposomes was proposed by Shueng et al. as a possible strategy to enhance targeting efficiency [64]. This approach used charge conversion polymers that mask the positive charge of liposomes and become neutral at certain pH levels. Another example of shielding the charge is the inclusion of PEG, which protects the carrier and acts as a redox-detachment substrate to ensure targeting efficiency [101]. Using peptide modifications offers a wealth of opportunities not only for mitochondrial targeting but specific organ delivery. Caution must be taken when attempting to combine both organ and organelle specific delivery, owing to increased liposomal size, which may initiate an immune response.

Mito-porter. Mito-porters are an emerging liposome-based nanocarrier strategy first reported by Yamada et al. in 2008 consisting of a liposome carrying octaarginine, a cell penetrating peptide, that fused with the mitochondrial membrane allowing the release of its cargo to the intra-mitochondrial compartment in living cells [102]. Since, the mito-porter strategy has been explored in mitochondrial-targeted gene therapy for the delivery of tRNA and rRNA targeting the mtDNA [70–72,103]. Mito-porters have been explored in the delivery of doxorubicin [74], berberine [75] and photothermal drugs [73] (Table 1). Data suggests mito-porters are an interesting nanocarrier approach not only for cancer and mitochondrial transplantation therapy but might also be an important alternative to conventional chemical mitochondrial-targeting strategies, including lipophilic cations. However, their application in other areas of human health remains pending.

Conclusions

Liposome-based formulations represent an innovative drug delivery system at the forefront of translational medicine. Given their biological compatibility they represent a feasible strategy to deliver drugs and improve their *in vivo* half-life and targeting potential whilst reducing drug toxicity. These characteristics highlight the potential for liposome-based formulations in improving therapeutic outcomes in a wide range of human conditions. Mitochondria are probably one of the most studied cellular organelles in health and disease, due to their responsibility in mediating cell signalling, energy production and oxidative stress.

Nonetheless, the mitochondria are difficult organelles to target, their double membrane and highly negative potential make it difficult for drugs to penetrate and unless charged positively, difficult to accumulate and produce effects. Liposomal modifications to deliver mitochondrial active compounds challenges these precepts. From biochemical liposome modification using positively charged liposomes to decorating them with peptides, mitochondria have emerged as a promising therapeutic target. Moreover, research has advanced the translational potential of mitochondrial-targeted liposomes to overcome the challenges presented by the physiological nature of mitochondria. In this regard, recent studies employing hyaluronic acid-coated liposomes suggest better protection against the interaction between the positively targeted liposomes and negatively charged plasma proteins.

The choice of targeting approach, its biocompatibility and increased mediated effects is still not clear. Literature shows that

lipophilic cations such as TPP+ and DQA are probably the most explored approach so far. However, there is no clear evidence of which choice of mitochondrial targeting motif offers better mitochondrial accumulation of drugs. Other approaches are gaining increased recognition and may offer an alternative for translational medicine, from plant-based targeting approaches (such as berberine) to peptide-based approaches such as mito-porter. Research outcomes underscore the bright future of these nanocarriers and certainly more research is necessary to validate the use of these liposomes to target mitochondrial-related conditions.

Future perspectives

We envisage the future of liposomes as nanocarriers to be designed to be able to overcome biological barriers (such as the blood-brain barrier) as well as offering alternative solutions to the undesired barrier passage (such as transplacental passage of drugs). Polymer conjugates as well as additional liposome coating with compounds such as hyaluronic acid may offer better results avoiding challenges pose by the nature of mitochondria as a two-membrane organelle. Additional research is required to meticulously design a liposome capable of not only targeting mitochondria but also homing on the specific organ of interest. We foresee that by achieving not only mitochondrial targeting but specific tissue accumulation these nanocarriers will achieve greater therapeutic potential. As of now, there is a notable research gap in elucidating the potential side effects of mitochondrial-targeted liposomes on non-target cells. Future research is required to resolving this outstanding question which is imperative for the effective translation of these methodologies into clinical practice.

Author contributions

LSA, MKM and MAAT prepared sections for the manuscript, edited, prepared figures and approved final manuscript. MU and PJ prepared sections, edited and approved the final manuscript. LSA secured funding.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was funded the Royal Society Grant-Round 1 2021 (RGS/R1/221169) awarded to LSA.

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