Chapter 9
Accessing Mitochondrial Targets Using NanoCargos

Ru Wen, Afoma C. Umeano, and Shanta Dhar

Abstract Mitochondria are membrane bound organelles that play essential roles for cell life, including energy production, apoptosis, redox balance, and regulation of calcium. Mitochondrial dysfunction is a hallmark for various diseases ranging from well-known diseases like cancer to rare genetic disorders like Barth's syndrome. Accordingly, mitochondria have been identified as key targets for therapeutic intervention. Mitochondria targeting strategies using nanocargos are rapidly growing tools for delivery of therapeutic and/or diagnostic payloads to mitochondria. In this chapter, we will highlight specific mitochondrial targets for nanotechnology-based delivery vehicles, NanoCargos, and discuss intracellular uptake mechanisms for NanoCargos, as well as technological methods for investigating mechanism for NanoCargo internalization into mitochondria.

Keywords NanoCargo • Mitochondria targeting • Mitochondrial uptake • Technological methods

Abbreviations

AFM	Atomic force microscopy
ANT	Adenosine nucleotide translocator
ATP	Adenosine triphosphate
CPP	Cell-penetrating peptide
CDs	Carbon dots
CPZ	Chlorpromazine
DOX	Doxorubicin
FCCP	Carbonylcyanide-p-(trifluoromethoxy)phenylhydrazone
FRET	Förster Resonance Energy Transfer
GQDs	Graphene quantum dots

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HDL	High density lipoprotein
HGC	Hydrophobic modified glycol chitosan
HK	Hexokinase
ICP-MS	Inductively coupled plasma mass spectrometry
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IVIS	In vivo image system
JC-1	5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide
LDH	Layered double hydroxide
MM	Mitochondrial matrix
mPTPC	Mitochondrial permeability transition pore complex
mtDNA	Mitochondrial DNA
MTS	Mitochondria targeting sequence
nDNA	Nuclear DNA
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PEG	Polyethyleneglycol
QDs	Quantum dots
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman scattering
TEM	Transmission electron microscopy
TPP	Triphenyphosphonium cation
TMRM	Tetramethylrhodamine methyl ester
TMRE	Tetramethylrhodamine ethyl ester
VDAC	Voltage dependent anion channel

9.1 Introduction

Mitochondria were the first subcellular organelles to be isolated from a eukaryotic cell (Ernster and Schatz 1981). As membrane bound organelles, mitochondria exist in almost all living organisms with eukaryotic cells. In humans, each cell contains hundreds of mitochondria. However, the exact number varies regarding to cell type, tissue and species. For example, muscle fibers have more mitochondria than adipocytes. Mitochondria range from 0.5 to 1.0 μm in diameter but provide most of a cell's energy as adenosine triphosphate (ATP), a source of chemical energy, *via* oxidative phosphorylation (OXPHOS) (Ernster and Schatz 1981). Specially, mitochondria differentiate themselves from other organelles by accommodating DNA, so called "mitochondrial DNA" (mtDNA). Because each mitochondrion contains dozens of copies of mtDNA, all associated with unique mutations, each cell contains thousands of unique copies of mtDNA. Located in the mitochondrial matrix,

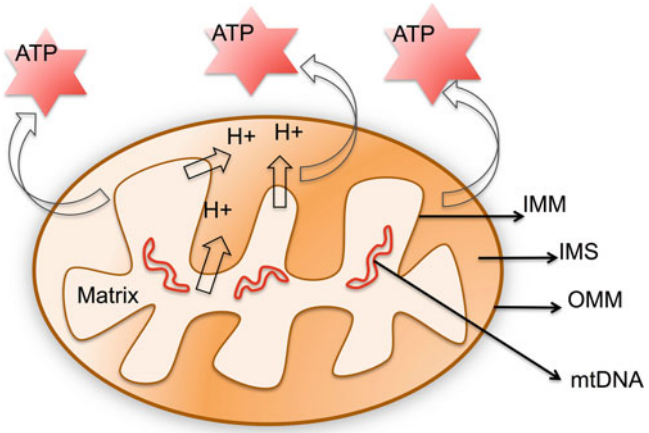

Fig. 9.1 The structure of the mitochondrion. *IMS* intermembrane space, *IMM* inner mitochondrial membrane, *OMM* outer mitochondrial membrane

mtDNA is made of 16,569 base pairs and 37 genes encoding 13 proteins, 2 rRNAs, and 22 tRNA associated with their own code for unique amino acids (Dimauro and Davidzon 2005). Mitochondria are heteroplasmic; mtDNA is much more likely to be mutated than nuclear DNA (nDNA) and variations in mtDNA occur among mitochondria in a cell, as well as, within a single mitochondrion. Unlike nDNA, mtDNA is exclusively maternally inherited, meaning offspring have no redundancy for protection against genetic mutation (DiMauro et al. 2001). Vulnerability of mtDNA is further conferred through its proximity to damaging agents. Because mtDNA lies in close proximity to reactive oxygen species (ROS) formed during various mitochondrial functions such as OXPHOS and mitochondria lack efficient DNA repair mechanisms, mtDNA undergoes frequent mutations, which may be passed down from mother to offspring (DiMauro et al. 2001). Thus, mitochondria play a major role in disease and disease progression. Mitochondria dysfunction has been associated with a vast array of diseases, ranging from rare hereditary diseases—like Barth's disease, myoclonic epilepsy with ragged-red fibers, and epilepsy—to common idiopathic diseases—like cancer, diabetes, and Alzheimer's diseases (Dimauro and Davidzon 2005; Federico et al. 2012; Pathak et al. 2015; Vasquez-Trincado et al. 2016; Wen et al. 2016). In addition to its unique genetic characteristics, mitochondria also possess unique membrane phospholipids, cardiolipin (CL), which contribute to a large array of mitochondrial processes, including, initiation of apoptosis, redox balance, and calcium homeostasis (Houtkooper and Vaz 2008; Santucci et al. 2014).

The structure of the mitochondrion is also complex: two lipid membranes, the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM), separated by an intermembrane space (IMS), which together enclose a mitochondrial matrix (MM) (Fig. 9.1). The OMM contains 8–10 % of the total proteins, mostly protein translocators, pore forming proteins, and mitochondrial fusion and

fission proteins encoded by the nucleus and synthesized using cytosolic ribosomes (Walther and Rapaport 2009). Small low molecular weight compounds can diffuse across OMM and large high molecular weight compounds use protein translocators and pores (Endo and Yamano 2009; Stojanovski et al. 2012; Tatsuta et al. 2014). IMM is densely packed with a complex structure of unusually high protein to phospholipid ratio and provides a restrictive environment for entrance of chemical entities into the MM (Friedman and Nunnari 2014). Furthermore, a mitochondrial membrane potential ($\Delta\Psi_m$) of ~ −160 to −180 mV that prevails across the membranes imposes an additional level of difficulty for foreign molecules to cross into the matrix (Perry et al. 2011). The expanse of mitochondrial and mitochondrial-related diseases stems directly from the various biological pathways and functions that are dependent on mitochondria. These four parts: OMM, IMS, IMM, and MM participate in various important cellular activities carried out by mitochondria, such as, glucose metabolism, fatty-acid oxidation, heme biosynthesis, cell signaling, differentiation, senescence, homeostasis, and cell growth.

The vast scope of mitochondrial and mitochondrial-related diseases stems directly from the various biological pathways and functions that are dependent on mitochondria. Mitochondria are essential participants in all cellular and biologic function, including energy production, blood sugar modulation, and immune protection. Mitochondrial drug targets have been identified in the four major components towards addressing several diseases that feature mitochondrial damage or dysfunction, such as, cancer and aging, metabolic syndromes, cardiovascular disease, and neurological diseases (Pathak et al. 2015; Wen et al. 2016). Currently, techniques and therapeutics that reverse, modify and/or mitigate existing mitochondrial dysfunction to improve overall health are being developed (Marrache and Dhar 2012; Marrache and Dhar 2013; Marrache et al. 2013; Marrache et al. 2014; Pathak et al. 2014; Feldhaeusser et al. 2015; Marrache and Dhar 2015; Pathak and Dhar 2015; Pathak et al. 2015; Kalathil et al. 2016; Wen et al. 2016). Several protein targets have been identified for mitochondrial regulation in the context of disease and are listed in Table 9.1 (Milane et al. 2015). Here, we are expanding on the therapeutic potential of certain targets and how they relate to cellular and mitochondrial functions.

Although mitochondria undergo replication using various associated fusion/fission proteins, mitochondrial fusion/fission processes and associated proteins are used in various other functions, such as trafficking, mitophagy, etc. Mitochondria are degraded through autophagy or, more specifically "mitophagy." Mitophagy involves trafficking of healthy substituents away from damaged areas of mitochondria, or compartmentalizing damaged areas, and discarding these damages compartments for degradation, while keeping the functional section of mitochondria intact. Mitophagy ensures mitochondria quality control and clearance of ROS. Damaged portions of a mitochondrion inefficiently produce ATP and, thus, have significantly increased ROS production. Separation and destruction of damaged areas, protects remaining healthy mitochondrion and cell from damaging ROS and protects mtDNA from mutation. In a therapeutic context, mitophagy plays a critical role in important immunologic systems, especially in the presentation of antigens to antigen presenting

Table 9.1 Mitochondrial proteins as possible targets for various disease, including cancer, cardiovascular disease, and other mitochondria-related diseases

Mitochondrial targets for therapeutic intervention		
Outer mitochondrial membrane (OMM)	Inner mitochondrial membrane (IMM)	Mitochondrial Matrix (MM)
Fusion and fission proteins	Electron Transport Proteins (complexes I-V, etc.)	TCA cycle proteins
Hexokinase	Adenine nucleotide translocators (ANTs) or ADP/ATP carriers	Fatty acid oxidation
K-voltage dependent anion channel (VDAC)	Mitochondrial potassium channels (K_{ATP} channels)	
Anti-apoptotic proteins (e.g. Bcl-2)	Uncoupling proteins	mtDNA
Peripheral benzodiazepine receptor		Cyclophilin D
Mitochondrial P-gp drug efflux pump		
Mitochondrial p53		
Mitochondrial migratory proteins		
Mitochondrial transporters, importers and pores		

cells via major histocompatibility complex II for Th1/2-mediated immune response. Fusion and fission proteins are also significant in MM maintenance and turn over, as well as in the delivery of proteins from the endoplasmic reticulum.

Targeting fusion and fission proteins involved in mitophagy could have profound therapeutic implications (Kroemer 2006; Maximo et al. 2009; Scatena 2012; Wallace 2012; Verschoor et al. 2013). For example, PTEN-induced kinase 1, a protein involved in mitophagy in OMM is able to recruit Parkin, a protein subunit of a ubiquitin ligase and the causative gene for autosomal recessive Parkinson's disease, for damaged mitochondria selection (Mizuno et al., Greenamyre and Hastings 2004; Chen and Dorn 2013; Matsuda et al. 2013; Han et al. 2015; Kazlauskaite and Muqit 2015; Pathak et al. 2015). Pink1 and Parkin may be targeted for pharmaceutical gain by engineering therapeutics that alter mitochondrial contacts to the endoplasmic reticulum involved in biosynthesis. Altering aberrant mitochondria of cancer cell via fusion pathways could be a possible strategy for efficient chemotherapy by driving cancer cells towards intrinsic apoptotic processes. On the other hand, increasing fission can sensitize cancer cells to extrinsic apoptotic pathways, such as those involved in immunologic derived cancer protection (e.g. cancer vaccines), and alter bioenergetics and host-response to improve cancer therapy (Milane et al. 2015).

Proteins involved in signal transduction, biosynthetic and OXPHOS have been associated with high-impact diseases, like diabetes, cardiovascular disease,

mitochondrial dysfunction, and neurological disease (DiMauro et al. 2001; Roberts and Sindhu 2009; Milane et al. 2015; Vasquez-Trincado et al. 2016), and provide interesting and challenging targets for metabolic control of cells using NanoCargos (Pathak et al. 2014; Marrache and Dhar 2015). The first step of glycolysis—ATP mediated phosphorylation of glucose—is initiated by hexokinase (HK) catalyzation. Located in OMM, HK-II is related to ATP acquisition from potassium voltage dependent anion channels (VDAC)) and, interestingly, when HK-II binds to the OMM through VDAC, the interaction spatially inhibits pro-apoptotic proteins of Bcl-2 family and protects against cell death *via* mitochondrial apoptotic pathways. Recently, metabolic reprogramming of cancer cells using 3-bromopyruvate, a HKII inhibitor, was accomplished by mitochondria-targeted gold nanoparticles (Au-NPs) coated with triphenyphosphonium cation (TPP) as a mitochondrial targeting moiety (Marrache and Dhar 2015). Furthermore, these metabolic reprogramming Au-NPs displayed therapeutic efficacy in cancer cell killing (Marrache and Dhar 2015). Mitochondrial permeability transition pore complex (mPTPC) consists of VDAC, adenine nucleotide translocators (ANT) and cyclophilin-D (CyP-D) which expand between IMM and OMM (Mathupala et al. 2006). The mPTPC is formed under conditions associated with mitochondrial death and apoptosis and regulates permeabilization of both IMM and OMM (Vieira et al. 2000). Transporting pores, biosynthetic pathways, and channels are especially desirable targets in concerns to cancer because they destabilize cell bioenergetics and facilitate apoptosis. Mitochondrial P-gp drug efflux pump is an especially important target for cancer drug resistance. P-gp is responsible for pumping chemotherapeutics out of the mitochondria and cell. Any cellular stress, such as hypoxia, causes increased expression of P-gp. Furthermore, multiple-drug resistant cancer cells have shown high levels of P-gp in OMM (Solazzo et al. 2006).

There are various and important mitochondrial proteins. The challenge for research is in designing reliable methods and tools for targeting and mitigating aberrant mitochondrial functions. One solution is the use of nanotechnology-based delivery vehicles, “NanoCargos”. NanoCargos provide a means of targeting mitochondrial membranes and delivering payloads (Yousif et al. 2009; Marrache et al. 2013; Sakhrani and Padh 2013; Pathak et al. 2015; Weinberg and Chandel 2015; Wen et al. 2016) (Fig. 9.2). Research in nanotechnology has been extremely promising towards answering the challenges of cancer, cardiovascular disease, diabetes, mitochondrial dysfunction related diseases, and neurological disease (Wen et al. 2016).

9.2 Mitochondrial Dysfunctions in Various Diseases

Mitochondria generate ROS as a byproduct of their role in energy production via OXPHOS (Cooper 2000). ROS production causes somatic mtDNA mutations which can produce an amplifying loop of increased respiration dysregulation resulting in accumulation of more ROS generated mutations. ROS production causes tissue aging by interrupting normal cellular metabolism, increasing cell death, and decreasing

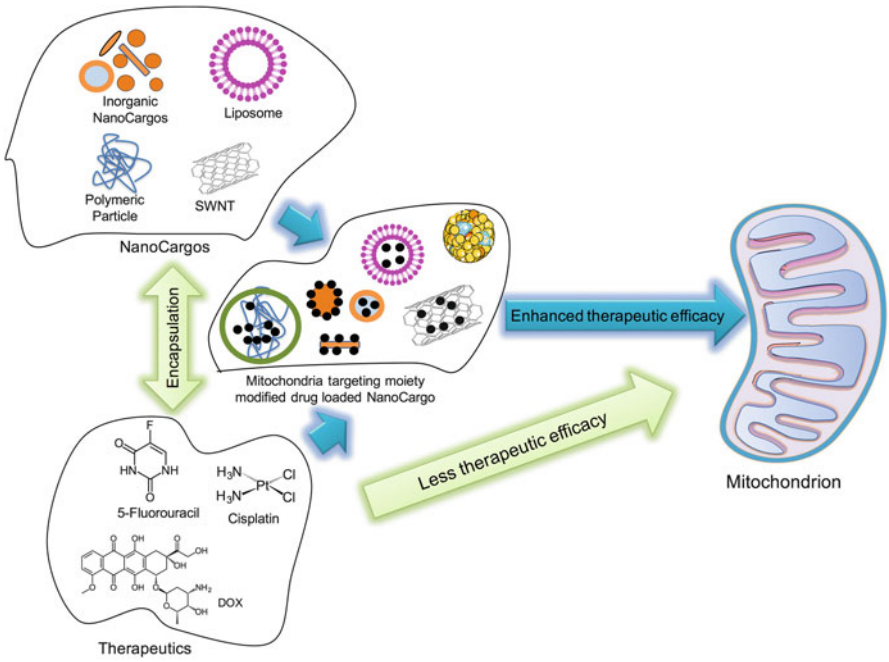

Fig. 9.2 A schematic diagram for NanoCargo and mitochondrial association

cellular capacity to replicate the genome (Roberts and Sindhu 2009; Federico et al. 2012; Milane et al. 2015). Our body's regenerative capacity—which underlies youthful features—is inherently dependent on our cells' replicative and functional capacity. Commercially, this is why most "age-defying" cosmetics and skincare products feature high levels of antioxidants such as tocopherols and ascorbic acid. ROS damage is not exclusive to aging. Currently, a number of NanoCargos incorporate antioxidant moieties in order to treat diseases like cancer and neurodegeneration (Yamada et al. 2015b).

In 2011, D. Hanahan and R. Weinberg expanded on the six hallmarks of cancer: (1) resistance against cell death, (2) sustained proliferation, (3) enhanced replicative endurance, (4) ability to escape from growth suppressors, (5) inducing metastasis, and (6) angiogenesis development by adding two additional hallmarks: reprogrammed energy metabolism and evasion of tumor destruction and two enabling features: genome instability and inflammation (Hanahan and Weinberg 2011). All these hallmarks are either directly or indirectly associated with mitochondria.

The dysfunctional mitochondria in cancer cells is attributed to mtDNA mutations, ROS production, and enhanced glycolysis for ATP generation (Armstrong 2006; Fadeel et al. 2008). Unlike normal mitochondrial genomes that are *heteroplasmic*, indicative of random ROS damage producing random mutations in a given mitochondrial genome, cancer cell mtDNA mutations are *homoplasmic*, meaning

that all mitochondrial genomes are *identically* mutated. Several studies have highlighted associations between various cancers and specific mitochondrial mtDNA mutations. However, the mechanism of how mtDNA relates to a cancer cell's evolutionary gain has not yet been fully understood (Brandon et al. 2006; Chatterjee et al. 2006; van Gisbergen et al. 2015).

Dysfunction in mitochondria-mediated apoptosis inherently makes cells resistant to death. Generally, cancers cells are resistant to intrinsic and/or extrinsic apoptotic stimulators. The intrinsic pathway for programmed death is mediated primarily through cellular or mitochondrial damage (an "apoptotic signal") that triggers an increase in p53, a tumor suppressor protein (Wickramasekera and Das 2014). Ultimately, the p53-mediated effect is the reduction of Bcl-2 and other anti-apoptotic protein levels and an increase in Bid, Bax, Bim and other pro-apoptotic proteins on the mitochondrial membrane. Overexpression of Bcl-2 is another mechanism that confers resistance to apoptosis. Leakage of cytochrome c through mitochondrial pores triggers a series of caspase-mediated interactions that creates an apoptosome, a destroyer of cells (Cooper 2000). In comparison, the extrinsic pathway for apoptosis is relatively simple. A pro-apoptotic ligand binds to death receptors, which directly trigger caspase-mediated interactions that create apoptosomes (Cooper 2000). Many cancer nanotherapeutic methods focus on commonalities and interactions between intrinsic and extrinsic apoptotic pathways (Mkandawire et al. 2015; Zhang et al. 2015). Several drugs such as 5-fluorouracil, cisplatin were encapsulated into NanoCargos for altering cancer metabolism in order to destabilize cancers and induce apoptosis (Sakhrani and Padh 2013; Pathak et al. 2014; Rin Jean et al. 2014; Marrache and Dhar 2015; Milane et al. 2015; Pathak et al. 2015; Weinberg and Chandel 2015; Wen et al. 2016). Furthermore, mitochondria-targeting NanoCargos demonstrate significant therapeutic effect (Marrache et al. 2014; Pathak et al. 2014; Marrache and Dhar 2015).

Type 2 diabetes mellitus is associated with dysfunctional mitochondria which results in deregulated glucose pathway and excessive ROS accumulation in cells (e.g. pancreatic beta cells and hepatocytes) *via* OXPHOS (Ernster and Schatz 1981). Together, these traits produce systemic insulin resistance. Mitochondrial dysfunction is most overtly displayed in glucose-stimulated insulin secretion processes. For instance, low activity of NADH shuttles in beta cells of type 2 diabetic models results in impaired K_{ATP}-dependent and -independent GSIS glucose-sensing (Weksler-Zangen et al. 2008; Halperin et al. 2012). Such mechanisms provide insight into clinical manifestations of hyperglycemia and poor glucose control. Systemic ROS accumulation coincides with the inflammatory state associated with diabetes, characterized by macrophage recruitment in adipose and hepatic cells and increased inflammatory markers in the blood. Inflammatory markers in the liver, especially TNF-α, induce gluconeogenesis and further lead to elevated blood glucose levels (Flemming et al. 2015; Sharma 2015). NanoCargos have shown potential *via* encapsulation of therapeutics, such as plasmid DNA (Basarkar and Singh 2009), insulin (Damgé et al. 2010), and antioxidants (Ratnam et al. 2009). NanoCargo-based delivery of therapeutics to the mitochondria is a more precise targeting and promising strategy to improve uptake and therapeutic effect.

Neurological diseases have increasingly attracted great attention in the field. Several NanoCargo formulations have been made to target oxidative damage in neurological diseases (Wen et al. 2016). Liposomes, polymeric nanoparticles, solid nanoparticles and metal nanoparticles have been used as NanoCargos to deliver anticonvulsants and antioxidants. Furthermore, studies of mitochondria targeting NPs demonstrated significantly enhanced therapeutic effects such as mitochondria targeted ceria NanoCargos (Kwon et al. 2016) and aspirin containing NanoCargos (Kalathil et al. 2016). Intensive studies of mitochondria targeting NanoCargos are needed in order to potentiate clinical applications.

Apoptosis often occurs due to ROS mediated oxidation of proteins, membrane phospholipids and mtDNA, and eventual release of cytochrome c and apoptotic inducing factor (AIF). High density lipoprotein (HDL) mimicking NanoCargos were developed by our group for potential application in atherosclerosis. We developed mimics of HDL which can functionally perform reverse cholesterol transport (Marrache and Dhar 2013). These polymer-based HDL NanoCargos contained encapsulated contrast agents such as quantum dots (QDs) for detection of vulnerable plaques, apolipoprotein (apo)A-1 mimentic 4 F peptide, cholesteryl oleate for preventative treatment, and triphenylphosphonium cations as mitochondria targeting moieties (Marrache and Dhar 2013). HDL mimic NanoCargos were non-immunogenic and demonstrated promising therapeutic properties for combating atherosclerosis.

9.3 NanoCargo Intracellular Uptake Mechanisms for Targeting Mitochondria

Endocytosis is a common energy-dependent pathway used by cells to communicate with biological surroundings and for the uptake of ions, biomolecules, and/or nutrients. Endocytosis can be classified into: (i) clathrin mediated endocytosis, (ii) caveolae dependent endocytosis, (iii) phagocytosis, pinocytosis, (iv) macropinocytosis, (v) flotillin dependent pathway, (vi) circular dorsal ruffles, (vii) CLIC/GEEC-type, (viii) entosis pathway and so forth (Doherty and McMahon 2009).

Clathrin-mediated pathway is characterized by trafficking molecules from the plasma membrane to early endosomes using clathrin-coated vesicles (~100 nm in diameter). Adaptor proteins initiate clathrin-coated pits by promoting clathrin assembly, which transforms the plasma membrane into a deeply bended clathrin-coated pit for molecule recruitment (Ungewickell and Hinrichsen 2007). Caveolae are glycolipid raft invaginations of plasma membrane that are sensitive to cholesterol depletion and dynamin inhibition. Cholesterol depleting agents (e.g. filipin, nystatin, or methyl-cyclodextrin) and dynamin inhibitors can be used to investigate caveolae-mediated endocytosis (Nabi and Le 2003). Endocytosis inhibitors are commonly used for mechanistic uptake studies of NP internalization. NPs are internalized mainly through clathrin- and caveolae-mediated endocytosis (Qaddoumi et al. 2003; Harush-Frenkel et al. 2007; Wang et al. 2009; Voigt et al. 2014), which

are receptor-mediated pathways such as mannose complement/Fcγ/scavenger receptor with proteins directly in contact with NanoCargos (Oh and Park 2014). Oh *et al.* reported that layered double hydroxide (LDH) NanoCargos were internalized through clathrin-mediated endocytosis (Oh et al. 2006). The colocalization of LDH NPs with dynamin, eps15, clathrin, and Tf further validated clathrin-mediated endocytosis of LDH NPs.

Macropinocytosis is a signal-dependent process, involving the formation of macropinosomes (>200 nm in diameter) and frequently occurs in antigen presenting cells (Andersen et al. 2014). Macropinosomes are larger than clathrin-coated vesicles and allow for non-specific internalization of large quantities of solute, membrane, nutrients, and antigens (Lim and Gleeson 2011). Iversen et al. reported a macropinocytosis-like mechanism for the uptake of ricinB QD NPs in HeLa cells (Iversen et al. 2012). RicinB-QD NPs uptake was inhibited in dynamin-negative HeLa cells in the presence of macropinocytosis inhibitor amiloride analog EIPA. The depletion of cholesterol by methyl-β-cyclodextrin and use of cytochalasin D (inhibitors of actin polymerization) reduced the uptake of RicinB-QD NPs. The colocalization of RicinB-QD NPs and dextran, a marker of fluid-phase uptake by macropinocytosis, confirmed a macropinocytosis-like mechanism for NP internalization. Fernando et al. reported that PFBT NPs were taken up through a macropinocytosis pathway, demonstrated by colocalization of NPs with Texas Red dextran and inhibition of uptake in cells treated with phosphoinositide 3-kinase inhibitors (Fernando et al. 2010).

Phagocytosis is an internalization process characterized by formation of particles-loaded invaginations, phagosomes, in the plasma membrane (Doherty and McMahon 2009). Fontana et al. reported phagocytic uptake of amoxicillin-loaded polyethylcyanoacrylate NPs (200–300 nm) using surface modifications (Fontana et al. 2001). Polyethyleneglycol (PEG) is an FDA-approved polymer. Coating NPs with PEG can yield a 50-70 % reduction in phagocytosis.

NPs may also be taken up through several different endocytotic pathways simultaneously. For example, Nam et al. reported that hydrophobic modified glycol chitosan (HGC) NPs were internalized through clathrin-mediated, caveolae-based and macropinocytosis pathways simultaneously (Nam et al. 2009). HGC NP uptake was decreased with chlorpromazine (CPZ), filipin III, and amiloride by 20, 40, and 30 %, respectively. Synergistic inhibitory effect was observed by CPZ and filipin III with 60 % reduction of HGC NPs internalization. Some HGC NPs were observed in late endosomes, lysosomes, and endoplasmic reticulum by TEM in colocalization studies. Dausend et al. reported modulation of uptake pathway *via* surface charge in polymeric NPs (Dausend et al. 2008). Polymeric NanoCargos with positive charge were internalized *via* macropinocytosis and partially clathrin-mediated pathways, microtubules, and cyclooxygenases. The uptake of negatively charged NPs, which may involve an unidentified dynamin-independent process, was inhibited to a lesser extent by dynamin than NPs with positive charge.

Direct penetration of the plasma membrane is another mechanism for NP uptake and is associated with NPs modified with cationic cell-penetrating peptide (CPP) or CPP-like molecules. It is a passive and energy independent process that involves the

formation of a pore and/or inverted micelle, membrane thinning models, and/or carpet-like models (Herce et al. 2009; Madani et al. 2011). CPPs can be used for modification of NPs carriers for intracellular delivery of therapeutic agents (Lindgren et al. 2000; Deshayes et al. 2005; Wang and Melosh 2012). Verma *et al.* compared the plasma membrane penetration of NPs with similar composition (Verma et al. 2008). NanoCargos with highly ordered functional groups on their surfaces could penetrate plasma membrane with the bilayer intact, where as NanoCargos with accidental distribution of the same functional groups were recruited in endosomes.

Once NanoCargos enter cells, they are usually trapped into endosomes and trafficked through lysosomes into mitochondria *via* various mechanisms, including potential-mediated mechanisms, protein machinery pathways, and/or membrane fusion processes. A summary of NP internalization pathways is shown in Fig. 9.3. NPs modified with a cationic groups, like TPP (Marrache and Dhar 2012; Marrache and Dhar 2013; Pathak et al. 2014), and methyl-TPP (Smith et al. 2003), mainly undergo potential mediated pathways for mitochondrial uptake. Owing to the presence of $\Delta\Psi_m$, lipophilic cation decorated NanoCargos are able to have mitochondria association properties. Lipid soluble cations adsorb to the mitochondrial surface and take advantage of mitochondrial membrane potential for their association (Ross et al. 2005).

Our group has designed several mitochondria targeted NP systems exploiting this mechanism (Marrache and Dhar 2012; Marrache and Dhar 2013). Targeted NPs were able to undergo endosomal escape, and then associate with mitochondria *via* a potential mediated pathway (Marrache and Dhar 2012). QD-TPPs is reported to internalize in mitochondria *via* a $\Delta\Psi_m$ mediated process (Chakraborty and Jana 2015) upon entering the cell *via* lipid raft or caveolae-mediated endocytosis.

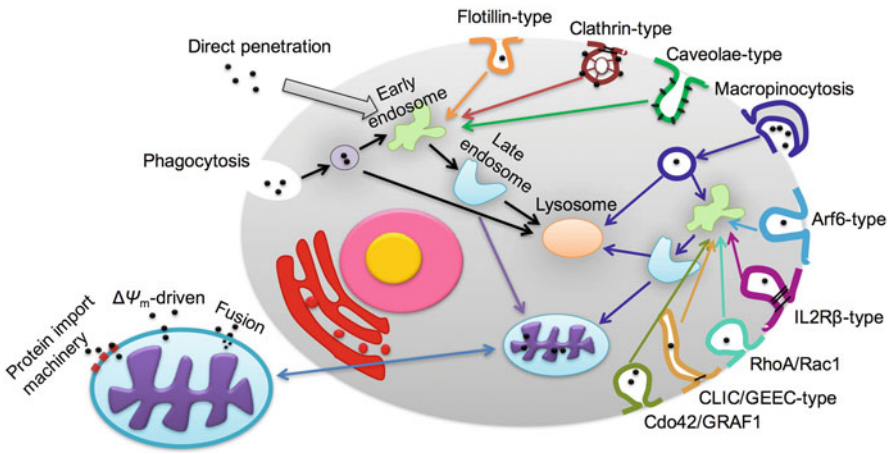

Fig. 9.3 Possible intracellular pathway of NP association with mitochondria

Protein import machinery was identified by the existence of mitochondria-penetrating peptides and/or mitochondria targeting sequences (MTSs), which consists of about 20–40 amino acids (Zhang et al. 2011). MTSs can be used for mitochondria-targeted NP design. Amyloid β-peptide is reported to import to mitochondria through translocation of OMM machinery (Petersen et al. 2008). Membrane fusion process of NanoCargo internalization in mitochondria can be achieved by a liposome based carrier MITO-Porter (Yamada et al. 2008). MITO-Porter based NanoCargos were brought into cells *via* macropinocytosis and underwent macropinosomal escape before binding to mitochondria thourgh electrostatic interactions. The membrane fusion was studied using sphingomyelin in MITO-Porter for delivery of macromolecules to the IMS, IMM, and MM.

9.4 Detection Techniques for Nanoparticle Internalization into Mitochondria

The most common methods for tracking NanoCargo internalization is achieved by labeling these nanomaterials and mitochondrial components with different labels. Figure 9.4 depicts techniques which are used for mitochondrial trafficking of NPs. A comparison of these techniques used in mitochondrial uptake studies for NanoCargos in terms of advantages and disadvantages is highlighted in Table 9.2.

9.4.1 Fluorescence Based Detection

Labels are commonly fluorophores that have measurable fluorescence in response to specific stimulation. Small organic molecules can be used in mitochondria labeling to visualize dynamic cellular or organismic behaviors. Organic fluorophores like tetramethylrhodamine methyl ester (TMRM), rhodamine 123, tetramethylrhodamine ethyl ester (TMRE), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), MitoTracker®, are sensitive to $\Delta\Psi_m$ (Fig. 9.5) (Cossarizza et al. 1993; Gilmore and Wilson 1999; Keij et al. 2000; Nicholls and Ward 2000; Pendergrass et al. 2004). Thus, functional mitochondria with intact $\Delta\Psi_m$ attract and preserve these dyes. Loss or presence of dye may be used to indicate $\Delta\Psi_m$ integrity. Rhodamine 123 is specific for mitochondria labeling, but TMRE, TMRM, and JC-1 dyes labels endoplasmic reticulum to some extent (Chazotte 2009). MitoTracker CMTMRos and MitoTracker Green, are $\Delta\Psi_m$-insensitive and able to remain in mitochondria with decreased $\Delta\Psi_m$ (Pendergrass et al. 2004; Agnello et al. 2008). MitoTracker®CMTMRos chemically reacts with thiol moieties and can remain in the mitochondria after cell fixation (Chazotte 2009). MitoTracker Green is one of the most widely used labeling agents for mitochondria. MitoTracker Orange is reported to induce mitochondrial permeability transition and inhibit complex I, causing depolarization in liver mitochondria (Scorrano et al. 1999).

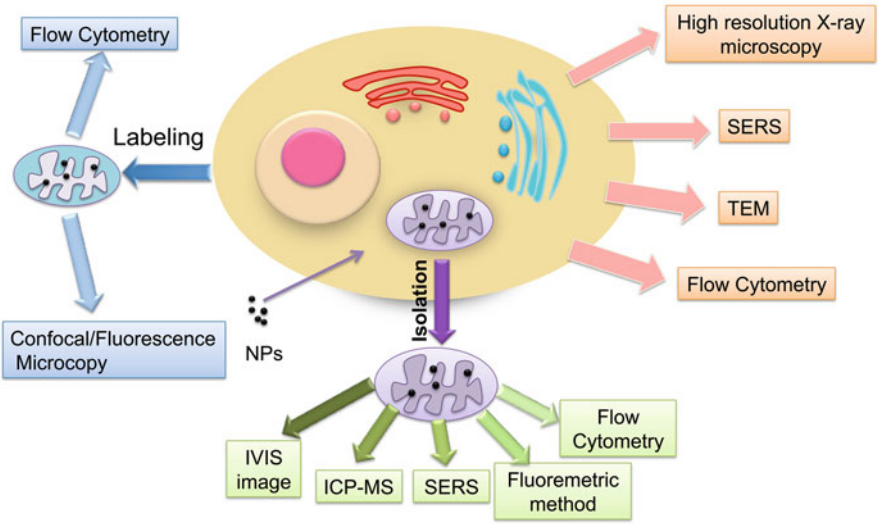

Fig. 9.4 Diagram of techniques used for intracellular trafficking of NPs into mitochondria. *ICP-MS* inductively coupled plasma mass spectrometry, *IVIS* in vivo image system, *SERS* surface-enhanced Raman scattering, *TEM* transmission electron microscopy

Table 9.2 A summary of advantages and disadvantages for techniques used in mitochondrial uptake studies for NanoCargos

Techniques	Advantages	Disadvantages	Comments
Confocal microscopy	Provides 3-D structures of sample	Limited resolution attributed to diffraction	Widely used in NP uptake studies
	Can monitor dynamic information in living organism	Can not provide detailed information at the subcellular level	Sample needs to be fluorescently stained
		Fluorophore may suffer from photobleaching and autofluorescence	
		Fixation of samples may cause artifacts	
Fluorescence microscopy	Provides the pattern/ location of NPs in a specific component in living/fixed cell	Suffers from photobleaching and autofluorescence	Sample needs to be fluorescently stained
		Fixation of samples may cause artifacts	Less widely used compared to confocal microscopy
		Can not provide detailed information at subcellular level	

(continued)

Table 9.2 (continued)

Techniques	Advantages	Disadvantages	Comments
TEM	Provides high accuracy of NPs status (e.g. aggregation, shape)	Complicated sample preparation.	Mostly used for metallic NPs
	High resolution and can distinguish cellular components	Time-consuming process.	
		Interpretation may be difficult.	
		Can not visualize the live cells.	
		Expensive	
		Only provides 2D images.	
SEM	Provide surface details of sample	Dehydration of samples may create artifacts	Limited application on NP uptake studies
	High resolution	Surface coating of sample is required	
ICP-MS	Superior detection limits of low concentration of elements	High cost	Widely used for *in vivo* tracing studies and need metallic component
		Can not quantify the elements in live samples.	
	Efficient and fast analysis of multiple elements	Expensive	
SERS	Non-destructive	Low sensitivity	A few studies has been made for cellular uptake
	Non-invasive	The fluorescence of samples may affect the results	
	No need of complicated sample preparation	Spectra interpretation may be difficulty	
	Can analyze samples both *in vivo* and *in vitro*		
	High spatial resolution.		
High resolution X-ray microscopy	High resolution	Unable to visualize relatively thick samples	A few studies was made for cellular uptake
	Can analyze small NP status in the cell samples	Complicated sample preparation and time-consuming process	
IVIS imaging	Non-invasive	Provides only 2D image	Widely used *in vivo*
	Easy operation	Same position is required for a group of samples	
	Non irradiative	Image saturation may be susceptible to inappropriate background setup	
	Enable both fluorescence and bioluminescence detection		

(continued)

Table 9.2 (continued)

Techniques	Advantages	Disadvantages	Comments
Fluorescence analyses	Relatively simple	Less sensitive and require high concentration of sample	Sample needs to be fluorescently stained
	Low cost		Widely used
FRET assay	Provides real-time monitoring of live cell dynamics	The properties of fluorescent probes are subjected to any physical changes	Has been used for vesicle-mitochondria fusion studies
	Good sample penetration	The process often causes protein fusion, mutation and/or alternation, which may affect the properties of molecules	
	Extremely high sensitivity		
	Can provide molecular distances		
Flow cytometry	Multiparameter data acquisition at the same time	Expensive	Widely used
	Relatively fast	User operation may be critical to obtain reliable data	
	Quantitative	Individual cell behavior may be difficult	
	Accurate and reproducible	Low cell throughput rate	
AFM	Provides 3D image	Limited area size for image	Some studies have been made for cellular uptake
	Simple sample preparation	Low scanning speed	
	High resolution	Subject to image artifacts and piezoelectric material	
		Expensive cantilevers	

The uptake of MitoTracker Red is controlled by $\Delta\Psi_m$, and its retention is associated with thiol groups of mitochondrial proteins after cell fixation, thus MitoTracker Red can be used for mitochondria-specific observation (Poot and Pierce 1999). Buckman *et al*. reported several MitoTracker dyes for mitochondria labeling in the central nervous system (Buckman et al. 2001). Mitochondria targeted GFP (MitoGFP) labeling is $\Delta\Psi_m$-independent and remains in the mitochondria. MitoGFP can easily be detected by confocal or fluorescence microscopy, and can be used for investigating mitochondrial dynamics in living cells (Hill et al. 2014).

NanoCargos can be fluorescently labeled by encapsulating a fluorescent probe. Yamada *et al*. reported the use of NBD and rhodamine for delivery vehicle internalization studies (Yamada and Harashima 2013). The liposome MITO-Porter was modified by MTS and labeled with NBD-DOPE and rhodamine-DOPE for Förster Resonance Energy Transfer (FRET) analysis. The modified MITO-Porter liposomes displayed efficient delivery of bioactive macromolecules to mitochondria.

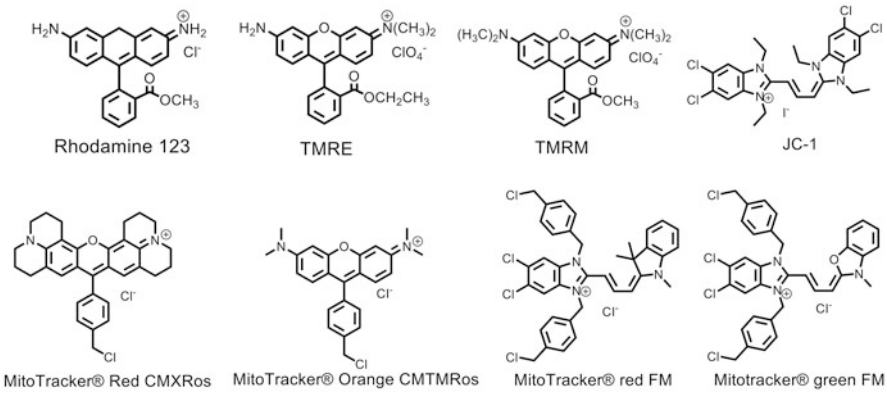

Fig. 9.5 Structure of $\Delta\Psi_m$-dependent fluorescent labelling reagents for mitochondria. TMRM-tetramethylrhodamine methyl ester, TMRE-tetramethylrhodamine ethyl ester, JC-1-5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1)

Several therapeutics are fluorescent such as doxorubicin (DOX) and can be directly used for delivery vehicle tracking. Qu *et al.* reported delivery of DOX loaded silica NanoCargos to mitochondria for cancer therapy (Qu et al. 2015). The fluorescence of DOX and MitoTracker green was used for NanoCargo and mitochondria tracking, respectively. These NanoCargos were successfully delivered to the mitochondria by surface functionalization with TPP, evidenced by colocalization of DOX and MitoTracker green fluorescence using both fluorescence analysis and confocal microscopy.

Autofluorescence can come from biological molecules. Extracellular matrix may also contribute to autofluorescence in tissues (Monici 2005). Hence, fluorescent probes that avoid or minimize autofluorescence should be selected (Mosiman et al. 1997). Reduction of autofluorescence can also be accomplished by adding a reducing agent. $NaBH_4$ can decrease the interference of cellular autofluorescence upon cell fixation (Clancy and Cauller 1998). Schnell reported a solution composed of $CuSO_4$ (1–10 mM) and ammonium acetate (50 mM) buffer at pH 5 or Sudan Black B (1 %) in 70 % ethanol reduced autofluorescence from lipofuscin in tissue sections (Schnell et al. 1999). Photobleaching is attributed to unstable properties inherent in fluorophore and/or non-specific binding molecules. Prolong exposure to stimulus light can damage dye and reduce its capability for fluorescence (Bernas et al. 2005). Minimizing intensity and exposure during preparation and measurement is a key step towards solving this problem. By labeling NPs and mitochondria, NP intercellular trafficking can be investigated using instruments such as confocal microscopy, fluorescence microscopy, *in vivo* image system (IVIS), fluorescence analysis, FRET, and flow cytometry. Confocal microcopy is an optical imaging technique that reduces noise and enhances resolution compared to conventional fluorescence microscopy (White et al. 1987), and is most commonly used in NP intracellular uptake studies. Table 9.3 summarizes current mitochondria labeling strategies by

Table 9.3 Summary of mitochondria labeling strategies for fluorescence imaging

Label	Examples	Model	NanoCargo label	Comments	Reference
Mitotracker green	HDL mimicking NanoCargo	Live cell	Quantum dots (QDs)	QDs were well encapsulated into the NPs	Marrache and Dhar (2013)
MitoFluor Green	Stearyltriphenyl phosphonium (STPP)-modified NPs	Fixed cell	Rhodamine (Rh)	Rhodamine labeled phosphatidylethanolamine was encapsulated	Boddapati et al. (2008)
MitoTracker orange	Fe_2O_3-loaded NPs	Live cell	QDs	QD-TPP and Fe_2O_3-TPP NPs were toxic at high concentration	Chakraborty and Jana (2015)
	QD-TPP				
MitoTracker Red	SWNT-PL-PEG-FITC	Live cell	Fluorescein isothiocyanate (FITC)	SWNTs located in mitochondria of tumor and normal cells	Zhou et al. (2010)
MitoFluor Red 589	MITO-Porter	Live cell	NBD, GFP	MITO-Porter incorporated with GFP or NBD–DOPE	Yamada et al. (2008)
MitoTracker Deep Red	Iridium (III) complexes	Live cell, Zebrafish model	Iridium (III)	Iridium complexes exhibited green to red emission under irradiation	Li et al. (2013)
MitoTracker Red, MitoTracker green	Fe_3O_4@mSiO_2-TPP/ Carbon dots (CDs)	Fixed cell	CDs	Fe_3O_4@mSiO_2-TPP/CDs	Zhang et al. (2015)

confocal microscopy technique. Our group reported mitochondrial association of targeted PLGA-*b*-PEG-TPP/PLGA-*b*-PEG-QD NPs in HeLa cells *via* confocal microscopy (Marrache and Dhar 2012). Targeted NPs were able to access mitochondria after efficient endosomal escape. Non-targeted NPs remained in the lysosomes and cytoplasm. By using the same labeling method, we reported mitochondrial internalization of polymer-lipid hybrid NPs (Marrache and Dhar 2013). The internalization of targeted HDL NPs was confirmed by confocal microscopy in healthy RAW 264.7 cells. When the cells were treated with carbonylcyanide-p-(trifluoromethoxy)phenylhydrazone (FCCP) to induce depolarization of $\Delta\Psi_m$, targeted HDL NPs did not accumulate in mitochondria, as seen by IVIS analyses of mitochondrial and cytosolic fractions. Chakraborty *et al.* investigated internalization mechanisms for QD-TPP in HeLa cells using fluorescence microscopy (Chakraborty and Jana 2015). QD-TPP showed strong subcellular uptake with TPP functionalization, whereas, QD only demonstrated internalization on a cellular level. Colocalization of QD-TPP and mitochondria was confirmed by confocal microscopy using MitoTracker Orange. The cellular uptake mechanism was elucidated by flow cytometry studies using pathway-specific inhibitors to block the various endosytosis pathways. QD-TPP accessed cells mainly *via* caveolae or lipid raft mediated endocytosis in both CHO and HeLa cells. Qu *et al.* reported the colocalization of MSNP-PPh_3-DOX and mitochondria by confocal microscopy (Qu et al. 2015). The delivery of DOX to mitochondria was confirmed by fluorescence analysis of FITC and DOX in isolated mitochondria. Yamada *et al.* reported FRET analysis for investigating liposome NP fusion activity with mitochondria, in which two different organic dyes, NBD and rhodamine, were incorporated into NPs (Yamada et al. 2015a). The energy transfer in isolated mitochondria was evaluated by relative fluorescence intensity. The capability of nucleic acid delivery to MM was quantified by fluorescence analysis of Cy-5 RNA oligomer in mitochondrial subfractionation.

9.4.2 Mass Spectrometry Based Detection

Quantification of small amount of NPs in the mitochondria requires highly sensitive techniques like inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS procures concentration of NPs by an elemental analysis, such as Cd concentration determination for Cd-Se QDs. Another strategy is to replace fluorescent probes with inorganic nanocrystals like quantum dots (QDs), carbon dots (CDs), iron oxide, and gold (Au), with unique optical, physical, and chemical properties. CdSe and CdTe are the most commonly used NP tracking tools in biological imaging applications (Resch-Genger et al. 2008). QDs may cause cytotoxicity to some degree since they consist of toxic metals (Smith and Nie 2009). Our group has demonstrated successful application of QDs for NPs tracking both *in vitro* and *in vivo* (Marrache and Dhar 2012, 2013; Marrache et al. 2014; Pathak and Dhar 2015; Pathak et al. 2015; Wen et al. 2016), as well as the use of Au-NPs (Marrache and Dhar 2015). Recently developed, CDs are carbon NPs with tunable fluorescence emissions and potential

competitors to conventional QDs (Lim et al. 2015). Yang *et al.* reported successful *in vivo* studies for use of CDs as an imaging agent (Yang et al. 2009). Graphene quantum dots (GQDs) are being explored for NP labeling. Recently, Chong *et al.* calculated *in vivo* and *in vitro* safety profiles for GQDs (Chong et al. 2014).

9.4.3 Transmission Electron Microscopy (TEM) Based Methods

Transmission electron microscopy (TEM) is widely utilized for internalization studies of NPs as it is of extremely high resolution providing high accuracy of NPs status (e.g. shape, aggregation) and allows for distinguishing cellular organelles (Klein et al. 2015). Sample preparation is complicated compared to fluorescence imaging. Inorganic nanocrystals, such as QDs, iron oxide, Au, silver (Ag), are possible labeling agents for NPs. Our group reported the application of TEM for mitochondrial localization of AuNP based NanoCargos (Marrache and Dhar 2015). Mitochondria targeted Au-NPs were localized in MM and non targeted Au-NPs were found outside the mitochondria of PC3 cells. Karataş *et al.* depicted the interaction of Au NPs with mitochondrial membrane using TEM (Karataş et al. 2009).

9.4.4 Miscellaneous Methods

Karataş *et al.* also introduced surface-enhanced Raman scattering (SERS) techniques for further detailing interactions between Au-NPs and mitochondria (Karataş et al. 2009). Lung cancer cells and corresponding isolated mitochondria were treated with Au colloidal suspension. Atomic force microscopy (AFM) showed intact mitochondria interacting with Au NPs. Bressan *et al.* reported the absence of Ag NPs inside the mitochondria and, instead, the close association of Ag NPs to OMM in patient-derived human cells using TEM analysis (Bressan et al. 2013). A relative abundance of Ag NPs were aggregated in the perinuclear zone of cytoplasm and around mitochondria. Peckys et al. was able to trace Au NPs uptake in live cells by liquid scanning TEM (STEM) (Peckys and de Jonge 2011). Au NPs with round or oval shapes were bound to the 67 % of vesicles' membrane surface area and remained aggregated after 24 h. Important to note, STEM did not produce any significant damage to the cells. Chen et al. evaluated the distribution of NPs in cells by high resolution x-ray microscopy (Chen et al. 2011). High-resolution x-ray microscopy generates x-ray micro-images through phase and absorption contrast with two-dimensional (2-D) projection and 3-D tomographic reconstructions.

9.5 Conclusions

Mitochondria provide promising targets for new therapeutics aimed towards treating mitochondrial dysfunction associated diseases such as aging, cancer, heart disease, and neurodegeneration. Mitochondria targeted NanoCargos have shown to enhance therapeutic efficacy by directing biomolecules to mitochondria *in vitro* and *in vivo*. Such NanoCargos are able to enter cells through endocytosis and/or direct penetration, and internalize in mitochondria *via* $\Delta\Psi_m$-mediated mechanisms, protein machinery pathways, and/or membrane fusion processes. Endocytosis inhibitors and localization of NanoCargos to specific subcellular compartments like, mitochondria are widely investigated using many techniques, such as confocal microscopy, IVIS imaging, fluorescence analysis, flow cytometry, mass spectrometry, and electron microscopy, for identifying the mechanism of NanoCargo uptake. Real time imaging and tracking of NanoCargos in subfractions of mitochondria are still in need for intensive investigation. The comprehensive understanding of these uptake pathways will help to design efficient mitochondria-targeting drug delivery systems for manipulating conventional or pathological processes occurring in subcellular compartments and developing therapies for mitochondrial dysfunctions related diseases.

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