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Functionalized Nanosystems for Targeted Mitochondrial Delivery

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Abstract

Mitochondrial dysfunction including oxidative stress and DNA mutations underlies the pathology of various diseases including Alzheimer's disease and diabetes, necessitating the development of mitochondria targeted therapeutic agents. Nanotechnology offers unique tools and materials to target therapeutic agents to mitochondria. As discussed in this paper, a variety of functionalized nanosystems including polymeric and metallic nanoparticles as well as liposomes are more effective than plain drug and non-functionalized nanosystems in delivering therapeutic agents to mitochondria. Although the field is in its infancy, studies to date suggest the superior therapeutic activity of functionalized nanosystems for treating mitochondrial defects.

Keywords

Mitochondrial Targeted Delivery; Nanotechnology; Liposomes; Polymeric Nanoparticles; Metallic Nanoparticles

1.0 Introduction

Essential for cellular energy production and key metabolic processes, the mitochondrion has implications in disparate diseases. For instance, mitochondrial dysfunction wreaks havoc in cancerous cells by producing energy for cellular growth as well as inhibition of apoptosis pathways (Ferri et al., 2005). Oxidative stress plays a key role in many mitochondrial diseases and hence, the majority of mitochondrial targeted therapeutics exhibit anti-oxidant properties.

Several hurdles exist in the development of mitochondrial targeted therapeutics including, biological barriers and toxicity. Once the drug has reached the target cell and has entered the cytoplasm, it has additional barriers including intracellular diffusion/transport to the mitochondria and outer and inner mitochondrial membranes. Another concern is mitochondrial toxicity. Several therapeutics such as haloperidol and thiothixine exhibit mitochondrial toxicity due to inhibition of complex I (NADH dehydrogenase) within the electron transport chain (Burkhardt et al., 1993). Inhibition of complex I by these therapeutic

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agents resembles pathology similar to idiopathic Parkinson's disease and hence, constitutes a serious side effect. Membrane barriers as well as mitochondrial toxicity are significant hurdles in the development of effective mitochondrial therapeutics.

Nanotechnology, encompassing materials and methods at the nanoscale (10^{-9} m), is an attractive approach to design mitochondrial therapeutics that either target or avoid mitochondria. While nanosystems targeting mitochondria can be used for enhanced efficacy in treating mitochondrial diseases, those that avoid mitochondria might be useful in reducing mitochondrial toxicity. Several nanotechnology-based therapeutics have been approved by the FDA including Doxil™ (a liposomal formulation of doxorubicin), Abraxane™ (albumin nanoparticle formulation of paclitaxel), and Renagel™ (cross-linked poly(allylamine) resin encapsulating sevelamer), for treating non-mitochondrial diseases. By modifying the surface of nanosystems using materials that enhance cell or organelle delivery, functionalized nanosystems can be prepared. Such nanosystems are currently under investigation for various diseases including those associated with mitochondrial dysfunction.

The purpose of this paper is to describe a) the relationship between oxidative stress and mitochondrial dysfunction and the pathological role of mitochondria in Alzheimer's disease and diabetes, b) barriers for drug delivery to the mitochondria, and c) functionalized and non-functionalized nanosystems for mitochondrial drug delivery. The nanosystems discussed include mitochondrial targeted liposomes, poly(lactide-co-glycolide) (PLGA) nanoparticles, gold nanoparticles, titanium dioxide nanoparticles, platinum nanoparticles and bimetallic nanoparticles.

2.0 Oxidative Stress and Mitochondrial Dysfunction

Mitochondria have vital roles in nearly every human cell and function to provide cellular energy, adenosine triphosphate (ATP), by metabolizing biofuels glucose and pyruvate. In the mitochondrial matrix, tricarboxylic acid and glycolysis cycles reduce nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) to NADH and $FADH_2$. NADH and $FADH_2$ supply electrons to the electron transport chain in order to fuel ATP synthase. The electron transport chain contains five proteins: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome bc_1), complex IV (cytochrome c oxidase), and complex V (ATP synthase) (Figure 1B). The electron transport chain is responsible for the mitochondrial source of superoxide anion radicals due to the strong reduction potential between complex I and complex IV (−0.32V to +0.39V) (Turrens, 2003). Superoxide anion radicals are precursor molecules for production of reactive oxygen species (ROS). The major source of superoxide depends on the organ; for instance, heart and lung mitochondria generate superoxide radicals primarily from complex III (cytochrome bc_1). However, complex I is the major source of superoxide radicals in the brain. Decoupling of the electron transport chain by transfer of an electron to oxygen to form superoxide radical instead of transfer to ADP is harmful to the mitochondria and the cell. Superoxide anion and ROS released from mitochondria can have various cellular and mitochondrial consequences including lipid oxidation, DNA/RNA damage, protein oxidation, and activation of the Ca^{+2}-dependent mitochondrial permeability transition pore (Orrenius et al., 2007). Superoxide anion radicals can cause the release of cytochrome c, formation of the apoptosome, and ultimately, apoptosis.

3.0 Mitochondrial Dysfunction in Disease

Neurological disorders such as Alzheimer's, Parkinson's and Down syndrome are most associated with mitochondrial dysfunction. Although the importance of mitochondria is central to understanding the pathology of neurological disorders, mitochondria also play an essential role in the pathology of other diseases such as ischemic heart disease, non-

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alcoholic fatty liver disease, hyperthyroidism, phenylketonuria, and diabetes. The importance of mitochondria in the pathology of Alzheimer's disease and diabetes is further discussed below (Table 1).

3.1 Alzheimer's Disease

According to the Delphi consensus study, nearly 18 million people world-wide in 2000 were suffering from Alzheimer's disease (Ferri et al., 2005), characterized by phenotypic neurological changes associated with memory, thinking, behavior, and pathological changes characterized by abundant neurofibrillary tangles and senile plaque neuritis (Kidd, 1963; Masters et al., 1985; Tomlinson et al., 1970). Dr. R.D. Terry in 1963 was the first to describe mitochondrial involvement in Alzheimer's disease as unusually swollen, watery, and random distribution in the cytoplasm compared to healthy human brains, which had dense mitochondria in neuronal cells (Terry, 1963). He further hypothesized that the change in mitochondrial activity was due to insufficient glucose or oxygen. Several studies in the mid 1990's supported the involvement of oxidative abnormalities in the pathology of Alzheimer's disease. Free radical oxygen species were generated by the β-amyloid peptide upon incubation with neuronal hippocampal cultures, which correlated directly with neuronal toxicity (Harris et al., 1995). The level of radical formation and neuronal toxicity directly correlated with peptide pre-incubation time at 37°C. However, when the peptide was added directly to the neuronal cells without pre-incubation, no toxicity or radical generation was observed. These results support the involvement of an altered form of β-amyloid peptide (which occurs due to pre-incubation at 37°C). The association and aggregation of the natively unfolded β-amyloid peptide (active unfolded protein) due to instability was found to be the initiator in fibrillogenesis (Booth et al., 1997). The cause of fibrillogenesis is debatable; most research is focused on the association of several single point mutations and the risk of developing Alzheimer's disease including a mutation in the β-amyloid precursor protein and amyloid protein (Levy et al., 1990). Treatment of human neuroblastoma cells with 200 μM H_2O_2 markedly increased intracellular accumulation of the Aβ protein, which suggests there is a causal role for oxidative stress in the early stages of disease (Misonou et al., 2000). Oxidative stress appears to have a role in both the initiation and progression of disease since oxidative stress can cause β-amyloid peptide accumulation and can be induced by β-amyloid peptide fibrils.

In the early stages of disease, oxidative stress is a key modulator of disease as indicated by the presence of several forms of oxidative stress including 8-hydroxy-guanosine and 4-hydroxynonenal (HNE) as well as others. Brain tissue from an autopsy collected from 22 subjects, demonstrated that the content of 8-hydroxy-guanosine was significantly higher at 0–10 years of Alzheimer's diagnosis and inversely correlated with % β-amyloid peptide (Nunomura et al., 2001). At only 2.5 yrs of Alzheimer's diagnosis the average percentage of Aβ protein was only 2 % and increased to approximately 6 % at 15 years of diagnosis. The authors suggested that oxidative stress may play a causal role since the level of 8-hydroxy-guanosine was highest at the beginning of disease and decreased after 10 years of diagnosis. A second study quantified the levels of HNE in 6 mild cognitive impairment brains of early stage Alzheimer's and 6 non-MCI brains and found that the percentage of HNE modified proteins was approximately 30 % higher in MCI brains than non-mild cognitive impairment brains (Butterfield et al., 2006). HNE is a product of lipid peroxidation and is extremely reactive with thiol and amine groups of biological proteins to form HNE-adducts or HNE-modified proteins. HNE modification and accumulation in the cell can disrupt membrane structure and ionic homeostasis as well as induce cytotoxicity, genotoxicity, and gene expression (Esterbauer et al., 1991). Several other studies have confirmed the presence of oxidative stress in early stages of Alzheimer's in the presence of minimal or no Aβ protein accumulation (Reed et al., 2009; Sultana and Butterfield, 2010).

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An alternative view of the role of oxidative stress in Alzheimer's disease and the consensus among many researchers in this field is that oxidative stress is initiated by β-amyloid peptide fibrils and hence, oxidative stress is not the cause, but the result of fibrillogenesis. Addition of β-amyloid peptide (1-42) to neuronal cells induces protein oxidation and death, lipid peroxidation and production of ROS (Butterfield, 2002). The oxidative stress and neurotoxicity properties of β-amyloid peptide (1-42) are completely abolished by substitution of the sulfur atom of Met35 with a methylene group; this suggests that the sulfur atom is playing a key role in oxidative stress initiation in neuronal cells.

Other modes of mitochondrial dysfunction have also been presented in the brains of Alzheimer's patients. For instance, aggregated β-amyloid peptide is capable of binding cyclophilin D, an integral membrane pore in the inner mitochondrial membrane with nM specificity (Du et al., 2008). Binding of cyclophilin D, opens the pore to reduce the potential difference across the inner mitochondrial membrane, which leads to cell death. Mice deficient of cyclophilin D had significantly lower levels of ROS and Ca^{+2} imbalance as well as improved cognitive and synaptic function indicating that cyclophilin D is a major contributor to the initiation of oxidative stress and neuronal cell death. A mitochondrial therapeutic target may be cyclophilin D for Alzheimer's disease.

Earlier studies have confirmed other mitochondrial defects in the brains of Alzheimer's patients including a deficiency in complex IV, but normal levels of cytochromes b, c_1 and aa_3 (Parker et al., 1994). The distribution of amyloid plaques is not colocalized with neuronal cells that are deficient of complex IV which suggests amyloid plaques may not be responsible for complex IV deficiency (Cottrell et al., 2002). Further, neuronal cells that are complex IV deficient and have many mtDNA mutations do not cause neuronal cell death nor are they capable of increasing the amount of β-amyloid peptide. The neuronal cells from Alzheimer's patients also have an increased amount of mtDNA mutations, which may induce deficiencies in electron transport chain proteins as well as other proteins. For example, mutations in the mtDNA control region have been detected in approximately 63% of Alzheimer's disease patients and have implications for mitochondrial transcription and replication suppression (Coskun et al., 2004). The location of these mutations reduces L-strand ND6 mRNA levels, which would inhibit respiration complex I, since ND6 is necessary for protein assembly. Mutations in the control region can also reduce the copy number of mtDNA, which can result in depletion of mtDNA and consequently, reduced activity of complexes I, III, IV, and V since mtDNA encodes major subunits of these proteins. Ultimately, mutations in mtDNA can cause a significant deficiency in mitochondrial crucial proteins such as complex IV. Deficiency or malfunctioning electron transport chains of the mitochondrion are associated with higher levels of oxidative stress in Alzheimer's disease brains. For example, complex IV deficiency induced by β-amyloid precursor protein is directly correlated with increased H_2O_2 in mitochondria obtained from Alzheimer's disease brains (Devi et al., 2006).

Thus, Alzheimer's disease pathology directly correlates with mitochondrial dysfunction on multiple levels including mitochondrial induced oxidative stress, mitochondrial interaction with amyloid aggregates, and decreased electron transport chain protein levels. Mitochondrial targeted therapeutics for Alzheimer's could include inhibition of amyloid aggregates binding to cyclophilin D, repair of mtDNA, supplying cytochrome c to the inner mitochondrial membrane or anti-oxidant treatment to the mitochondrion.

3.2 Diabetes

Diabetes has become a worldwide pandemic effecting approximately 160 million individuals in the world in 2000 and is expected to rise to 370 million individuals in 2030, an estimated 50% increase over 30 years in the number of individuals suffering from

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diabetes (Wild et al., 2004). The disease results from the body's inability to regulate blood glucose levels with insulin, the hormone responsible for cell uptake and storing of glucose. Type 2 diabetes is a specific form of diabetes wherein the body is resistant to insulin or does not produce enough insulin. Type 1 diabetes is slightly different whereby β cells in the pancreas do not produce insulin due to autoimmune reactions against insulin. Common symptoms of diabetes include increased risk for various disorders including hypertension, dyslipidemia, coronary heart disease, and metabolic disorders among many others. Both environmental, diet, drug, genetic, and viral infection can cause type 1 diabetes; risk factors for type 2 diabetes also include lifestyle factors as well as genetic contributions. Therapeutic approaches for management of Diabetes include insulin injection to allow glucose cellular uptake from the blood and metformin, which suppresses glucose production in the liver. Recent efforts to better understand the pathology of the disease have led to novel therapeutic targets including mitochondrial specific therapeutics.

The general dogma for the cause of type 2 diabetes is central to mitochondrial dysfunction, which increases cellular glucose levels and consequently reduces insulin production (Lowell and Shulman, 2005). As early as 1963, it was shown that fatty acids in rat muscle can cause insulin resistance. Over 45 years later, the field is uncovering mechanisms of lipid oxidation and increased levels of acetyl CoA and citrate, which inhibit enzymes that utilize glucose (pyruvate dehydrogenase and phosphofructo kinase). Ultimately, reduced glucose consumption leads to increased intracellular levels of glucose and hence, insulin production is reduced. This has been validated by studies that demonstrate type 2 diabetes patients have lower activity of mitochondrial oxidative enzymes, smaller mitochondria and reduced bioenergetic capacity compared to healthy individuals. Further, hereditary type 1 diabetes individuals have genetic mitochondrial mutations, which are thought to cause β cell dysfunction and hence, β cell inability to sense blood glucose and production of insulin. Another cause of β cell dysfunction may be linked to lipid or glucose toxicity.

Research studies have identified reduced mitochondrial function and content in type 2 diabetes patients compared to healthy individuals, which directly correlated with poor insulin mediated glucose metabolism (Ritov et al., 2005). Reduced mitochondrial function correlated with reduced electron transport activity by approximately 4 times in type 2 diabetes patients compared to healthy individuals. Mitochondrial protein yield was significantly lower ($p < 0.001$) in type 2 diabetes compared to healthy individuals (approximately 1 mg/g compared to approximately 2 mg/g, respectively). Most importantly, reduced electron transport chain activity significantly correlated with reduced insulin stimulated glucose metabolism ($p < 0.001$). Further, type 2 diabetes patients were mitochondrial deficient compared to healthy individuals and mitochondrial thickness was 3 fold lower in type 2 diabetes patients (approximately 1 μm vs. 3 μm, respectively, $p < 0.01$). Reduced mitochondrial content and function may be attributed to the reduced mtDNA content in type 2 diabetic patients compared to healthy individuals (approximately 1, 850 vs. 2, 514 copy number, respectively, $p < 0.05$). Oxidative stress is well known to play a role in progression of Diabetes, but others hypothesize that oxidative stress may actually be the culprit as well.

An excellent manuscript in 2000 demonstrated that mitochondrial dysfunction can cause impaired insulin secretion and β cell loss (Silva et al., 2000). A mouse model was generated with a pancreatic tissue specific knockout of the mitochondrial transcription factor A (Tfam or mtTFA) to prevent expression of mitochondrial DNA. 7 weeks after birth, these mice had severe depletion of mtDNA in the islets and these mitochondria were deficient in complex IV and exhibited abnormal tubular cristae morphology. The pancreatic β cells exhibited deficiencies in respiratory chain activity as well at 7 weeks. At 5 weeks, these mice were considered diabetic with low levels of blood insulin (approximately half that of normal

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mice). The authors confirmed that the β cells were unable to secrete insulin at beginning stages of disease (7 weeks) and later in disease there was β cell loss (> 20 weeks). This study confirms that mtDNA mutations can cause mitochondrial respiration dysfunction, which leads to reduced blood insulin levels.

Central to the above studies that focus on mitochondrial dysfunction in the cause of diabetes, mitochondria can cause formation of lipid peroxidation by releasing ROS. Lipid peroxidation ultimately increases intracellular levels of acetyl coenzyme A, which is capable of inhibiting glucose metabolizing enzymes. New therapeutic targets for diabetes may involve mitochondrial targets such as prevention of lipid peroxidation by scavenging ROS, gene therapy to express specific mtDNA genes for normal function and increase glucose metabolism within the mitochondria.

4.0 Therapeutic Barriers

Key cellular barriers for mitochondrial drug delivery include the plasma membrane, outer mitochondrial membrane, and inner mitochondrial barrier (Figure 1 and Table 2). In addition, depending on the drug structure, there are metabolic barriers that can degrade the drug prior to reaching the mitochondria.

4.1 Cellular Membrane

After extravasation of the drug from the circulation into the extracellular space, the first hurdle the drug must overcome is the cellular membrane to enter the cytoplasmic space (Figure 1A). Drug molecules of relatively small molecular weight (< 500 mw) and lipophilicity (log P > 5) are more likely to passively diffuse across the cellular membrane. For this reason, many therapeutics on the market are lipophilic. However, this may cause dissolution and administration issues due to poor solubility. For example, Doxil® is a liposomal formulation of doxorubicin to improve the bioavailability and solubility of the drug. Other mechanisms of cellular entry include receptor mediated endocytosis and transporter mediated.

Receptor mediated mechanisms of cellular entry may be used if there is a targeting ligand such as folate to bind folate receptor, which is over expressed on malignant cells (Gabizon et al., 2010). Other approaches may include targeting cell-surface gangliosides, Her2 receptor, EGF-receptor, and transferrin-receptor. A major advantage of targeting ligands is higher cellular uptake compared to non-functionalized therapeutics. For instance, Gabizon et al. have shown that folate targeted liposomal doxorubicin is more efficacious than plain liposomal doxorubicin in reducing tumor volume and increasing survival in multiple different tumor mouse models (Gabizon et al., 2010).

Common mechanisms of cellular entry by nanoparticles are phagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis (Figure 1A). Phagocytosis is most associated with virus and bacterial cell uptake by macrophage or other immune cells as a method for protecting the host from infection or to fight infection. Therefore, particle size for uptake by this pathway is generally on the order of bacterial cell or viral size (approximately 750 nm). Nanoparticles will most likely enter cells by receptor mediated pathways including clathrin-mediated endocytosis and caveolae-mediated endocytosis. Clathrin-mediated endocytosis is facilitated by the protein clathrin, which deforms the cellular membrane to help form a vesicular structure (the early endosome). Once the particle has bound the receptor and the vesicular pit has been formed, clathrin coats the pit to help pinch off the vesicle from the cell membrane. The most common form of receptor endocytosis is clathrin independent, caveolae-mediated endocytosis. Caveloae is a lipid raft protein that is associated with cholesterol rich portions of the cell membrane and

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is found in many clathrin independent endosomes budded from the cell membrane. Macropinocytosis is a third form of receptor mediated endocytosis whereby the receptor recognizes its target and encapsulates its target plus the surrounding fluid of which is then fused with the cellular membrane. This method is less commonly observed for particles, but can be induced by specific targeting ligands such as CD91 (Ogden et al., 2001). Further, the mechanism of cell entry is dependent on particle size: micron sized particles (0.75 μm) undergo phacotysosis; particles between a micron and 500 nm undergo caveolae-mediated endocytosis; particles of a few hundred nm (less than 500 nm) undergo clathrin-mediated endocytosis.

The above mechanisms of particle uptake result in formation of early endosomes, late endosomes and eventually, fusion with lysosomes. Endosomes are relatively large vesicles (1 μm in diameter) that have similar characteristics to the cell membrane including the receptor the particle bound for cell entry. These endosomes mature into late endosomes, which are mutli-vesicular bodies (meaning containing multiple vesicles inside a larger vesicle structure). These late endosomes then fuse with the membrane of the lysosome organelle to degrade the contents of the endosome. This occurs by proton pumps that pump protons into the lysosome to increase the acidity of the pH and promote acid mediated degradation. For those particles that do enter cells via the endosomal pathway, they must escape the endosome before fusion with the lysosome to prevent degradation. This has been achieved by integrating proton sponges into nanoparticles. For example, Bulmus et al. have created a pH responsive copolymer consisting of a poly disulfide acrylate group attached to a methyacrylic acid and butyl acrylate copolymer (Bulmus et al., 2003). The disulfide group of poly disulfide acrylate is capable of accepting protons to prevent acid mediated degradation of the particle. All particles that enter the endosomal pathway must exit before fusion with the lysosome in order to exert its therapeutic effects.

4.2 Cytosolic Barriers

The diffusion of molecules through the cytosol is not insignificant and the hurdles molecules must face to travel from the plasma membrane to the mitochondrial organelle must be addressed. The cytosol is a dynamic environment packed with macromolecular species and small molecules with minimal free space available. Specific components of the cytosol include: water, which makes up approximately 70% of the total volume of the cell (Luby-Phelps, 2000); ions such as potassium, sodium, chloride, magnesium, calcium, etc; and macromolecules including proteins, which make up approximately 20% of the cell volume (Ellis, 2001). The high concentration of macromolecules up to 400 mg per milliliter (Ellis and Minton, 2003) is referred to as molecular crowding, which makes the cytosol a highly viscous structure compared to water. At 20°C, water has a viscosity of 1.002 cP and at 30°C, the viscosity of water reduces to 0.7978 cP. The viscosity of the cytosol near the plasma membrane in Madin-Darby canine kidney cells (MDCK) was estimated to be 1.0 ± 0.2 cP at 37°C (Bicknese et al., 1993). The molecular crowding of the cytosol forms a significant diffusion barrier for molecules. For instance, the diffusion of fluorescein isothiocyanate (FITC) through the cytosol of Swiss 3T3 fibroblasts was only 28% as rapid as that in free solution (Luby-Phelps et al., 1987). In addition to the reduced diffusion of molecules in the cytoplasm, collisional interactions and binding to intracellular components are also barriers for molecules targeted to the mitochondria (Garner and Burg, 1994; Seksek et al., 1997).

4.3 Mitochondrial Membranes

Therapeutic agents targeted to the mitochondrial matrix must cross two barriers, the outer and inner mitochondrial membrane. The outer mitochondrial membrane has no membrane potential and is a relatively weak barrier compared to the inner membrane. The most abundant protein in the outer mitochondrial matrix are the voltage-dependent anion channels

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(VDAC, also known as porin), which can transport gold nanoparticles of 3 nm, but not 6 nm gold nanoparticles (Salnikov et al., 2007). VDACs non-specifically transport molecules less than 5000 mw into the transmembrane space between the outer and inner mitochondrial membranes. These channels provide several functions for the mitochondria including participation in the mitochondrial permeability transition pore complex with adenine nucleotide translocase and cyclophilin D to form contact sites between the outer and inner mitochondrial membranes (Crompton et al., 1998). In addition, VDAC integrates with other transporters in the outer mitochondrial membrane including peripheral-type benzodiazepine receptor (PBR) (Papadopoulos et al., 2006). PBR primarily translocates cholesterol and benzodiazepine derivatives into the mitochondria for metabolism and is found primarily in locations of the outer mitochondrial membrane that interact with the inner mitochondrial membrane. The outer membrane also contains mitochondrial protein transporters for proteins made by the ribosomal protein synthesis machinery with mitochondrial function. Proteins that are translocated to the mitochondria have unique terminal sequences that are generally positively charged and contain alpha helical secondary structure (Jensen and Dunn, 2002). The alpha helix is recognized by transporters in the outer mitochondrial membrane such as the transporter outer membrane (TOM) complex.

If the therapeutic target is within the mitochondrial matrix, the drug will also have to cross the inner membrane. The inner membrane is rich in cardiolipin, a unique two tailed diphosphatidylglycerol lipid, which contributes to the function of mitochondria including apoptosis. The inner membrane contains the electron transport chain, which generates a strong negative membrane potential of approximately −180 mV (Kamo et al., 1979) due to the proton gradient from the matrix toward the intermembrane space. This membrane potential is unique to mitochondria and it can potentially be used to target therapeutics to this organelle. The mitochondrial accumulation of cationic lipophilic drugs is generally regarded as much higher than other molecules including hydrophilic, neutral, and anionic molecules. Horobin et al. (Horobin et al., 2007) and Durazo et al. (Durazo et al., 2011) have further characterized the physiochemical properties of various molecules that exhibit mitochondrial entry. Using over 100 mitochondriotropics identified from literature, Horobin et al. reported that selective mitochondrial accumulation involved electric potential, ion-trapping, and complex formation with cardiolipin. Molecules with low or moderate lipophilicity exhibited non-specific mitochondrial accumulation. Further, they reported that only a third of the so called mitochondriotropics were cationic, lipophilic molecules. Also, the authors noted that drugs that are designated as mitochondriotropics were not exclusively localized to mitochondria but had access to other organelles as well. Durazo et al. quantified the isolated rat brain mitochondrial accumulation of 20 anionic, neutral, and cationic compounds with varying lipophilicities at physiological pH and developed the following equation to explain the uptake based on molecular properties: Log % Uptake = 0.333 Log D + 0.157 Charge − 0.887 Log PSA + 2.032 ($R^2 = 0.738$), where Log D and PSA represent drug lipophilicity and polar surface area. Thus, drug lipophilicity, charge, as well as polar surface area influence mitochondrial uptake. Further, the uptake of the most lipophilic, cationic molecules was inhibited upon membrane depolarization by valinomycin.

Other drug molecules with low affinity for mitochondria may be assisted by transporter proteins for their mitochondrial entry. For instance, anionic solute transporters exist within the inner membrane for transporting substrates for glycolysis and the citric acid cycles as well as solute organic carriers. Transporter channels are also present within the inner membrane that selectively transports proteins with N-terminal basic and hydrophobic amino acids (Horwich et al., 1985). The transporter inner membrane (TIM) 23 complex consists of multiple translocation proteins including Tim23p, Tim17p, Tim44p, and mtHsp70p (Jensen and Dunn, 2002). Tim23p is a voltage-activated cationic selective pore of 20 Å and is capable of translocating two polypeptides simultaneously. Interestingly, the Tim23p protein

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has its N-terminus protruding through the outer mitochondrial membrane and hence, is present on the surface of the mitochondria. It is therefore hypothesized that the TIM23 complex works in conjunction with TOM translocons in order to transport proteins across the outer and inner mitochondrial membranes. Mitochondrial heat shock protein 70p facilitates translocation of the polypeptide through the TIM23 translocon in an ATP dependent manner. Other translocons exist in the inner mitochondrial membrane including TIM22. TIM22 has sequence homology with TIM23 complex proteins Tim23p and Tim17p. However, the pore size is slightly smaller than that of Tim23p and is variable between 11 and 18 Å. Different proteins are translocated through the TIM22 and TIM23 complexes where cationic polypeptides are translocated through the TIM23 complex; specificity for the TIM22 complex is currently unknown.

Targeted mitochondrial delivery may be achieved by integrating mitochondrial translocation ligands and/or positively charged ligands into the therapeutic design. In addition, mitochondrial oligonucleotides, have been shown to successfully target functionalized titanium nanoparticles to mitochondria (discussed in section 5.3.2) (Paunesku et al., 2007).

5.0 Nano-Therapeutics

Nanotechnology provides several advantages in the design of therapeutics to overcome the barriers and limitations discussed in the previous section. Polymeric and metallic nanoparticles and liposomes of sizes ranging from 5–260 nm have been shown to either enter mitochondria or exert effects in mitochondria (Tables 3 and 4). Lipidic nanosystems mainly constituted liposomes. Another potential option for lipidic nanoparticles is solid lipid nanoparticles (Muller et al., 2000), which were not investigated to date for mitochondrial targeting. Polymeric and metallic nanoparticles and liposomes offer unique advantages and disadvantages (Table 5), as elaborated below.

5.1 Liposomes

Liposomes have the greatest translational potential for mitochondrial delivery since these delivery systems are generally biocompatible and non-toxic. Further, research in the field of liposomal drug delivery may be one of the oldest in drug delivery systems and most successful in developing FDA approved therapeutics. Doxil ®, doxorubicin stealth liposomes, FDA approved in 1995, was the first liposomal formulation marketed and since then, several other liposomal formulations have entered the market: AmBiosome ® (amphotericin), DaunoXome® (daunorobicin) and DepoCyte® (cytrabine). Thus, liposomes are pharmaceutically relevant and therefore, the most investigated class of nanosystems for mitochondrial targeted therapeutics.

The advancement of mitochondrial targeted liposomes is largely attributed to the research group of Dr. Volkmar Weissig (Boddapati et al., 2008; Boddapati et al., 2010; Boddapati et al., 2005; D'Souza et al., 2005; Elbayoumi and Weissig, 2009; Patel et al., 2010). In the development of an anti-tumor drug, Boddapati et al. fabricated stearyl triphenyl phosphonium (STPP) functionalized liposomes for ceramide to specifically target the mitochondria (Boddapati et al., 2008). The authors chose STPP as their targeting molecule for mitochondria since it exhibits both cationic and lipophilic properties. Ceramide is a known anti-cancer agent that targets cytochrome c release, ROS production, and apoptosis. Liposomes with a diameter of 55 nm were fabricated by dissolving dioleoylphosphatidyl choline, cholesterol, and STPP (83.5:15:1.5 mol % and total lipid of 25 mg/mL) in 5 mM HEPES (pH 7.4) followed by probe sonication. In vitro and in vivo assays were used to characterize the efficacy of functionalized liposomes compared to non-targeted liposomes. Using an in vitro confocal fluorescence assay and MitoFluor Green dye from Molecular Probes/Invitrogen, they observed that STPP functionalized liposomes localized within the

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mitochondria of 4T1 breast cancer cells. Further, these liposomes induced more apoptosis in 4T1 breast cancer cells, compared to non-targeted liposomes, based on a characteristic DNA ladder that is indicative of apoptosis. The in vivo tumor model was developed in BALB/c mice by subcutaneous injection of 4T1 breast cancer cells. Compared to no treatment and those treated with non-targeted liposomes, wherein animals died at day 12, the STPP functionalized liposomes group survived until day 18. Further, the rate of tumor growth for animals receiving functionalized liposomes was only 15 mm^3/day compared to animals that received non-targeted liposomes or no treatment, wherein the tumor growth rate was greater than 40 mm^3/day. The authors indicated that their dose was 6 times less than the typical dose and still more effective (6 mg/kg vs. 36 mg/kg). Since the drug was targeted to the mitochondria, the drug efficacy was much higher. Thus, conjugation of a cationic, lipophilic ligand to liposomes is an advantageous approach to target drugs to the mitochondria.

Similarly, STPP functionalized liposomes also enhanced the efficacy of sclareol (a colon cancer and leukemia drug with a mitochondrial target) compared to plain drug (Patel et al., 2010). STPP functionalized liposomes of 105 nm loaded with sclareol exhibited 160% increase in apoptotic events in COLO205 cells compared to only 30% increase for non-targeted liposomes loaded with sclareol. Further, the STPP functionalized liposomes loaded with sclareol exhibited 200% increase in caspase-8 activity and 300% increase in caspase-9 activity compared to non-targeted liposomes loaded with sclareol, which only had 75% increase in caspase-8 activity and 125% increase in caspase-9 activity. Hence, the functionalized liposomes reduced the amount of drug required for an effective response (EC_{50}) compared to non-functionalized liposomes. DQAsomes prepared from dequalinium (a quaternary ammonium salt, that self-assembles into liposome-like cationic vesicles) encapsulating paclitaxel also exhibited enhanced efficacy (apoptosis activity) over plain drug in COLO25 cells (D’Souza et al., 2008).

Targeted liposomes can also deliver therapeutics to the outer and inner mitochondrial membranes by fusing with the outer surface of the outer mitochondrial membrane (Yamada et al., 2008). Liposomes were fabricated with dioleoylphosphatidylethanolamine (DOPE): lipid x: octaariginine (R8) at 9:2:0.5 molar ratio, where lipid x was sphingomyelin or phosphatidic acid. The size of these liposomes ranged from approximately 230 to 260 nm. DOPE:SM:R8 and DOPE:PA:R8 liposomes were capable of fusing the outer mitochondrial membrane as determined by transmission electron microscopy. Liposomes functionalized with R8 and encapsulating green fluorescent protein (GFP) delivered GFP to the outer and inner mitochondrial membrane in isolated liver mitochondria as determined by western blotting of fractionated outer and inner mitochondrial membranes. In contrast, liposomes without R8 functionalization did not deliver GFP to either the outer or inner mitochondrial membrane. These results were confirmed in HeLa cells, which further demonstrated that R8 functionalized liposomes deliver GFP to the mitcohondria specifically. The authors validated the ability of these liposomes to deliver cargo by demonstrating delivery of gold nanoparticles to the outer and inner mitochondrial membrane using R8 functionalized liposomes encapsulating gold nanoparticles. This study provides concrete evidence that fusogenic liposomes of about 250 nm can deliver its contents to the mitochondria.

The above studies demonstrate that mitochondrial targeted liposomes enhance mitochondrial delivery of therapeutics and efficacy of mitochondrial therapeutics (Figure 2). The delivery mechanism of non-fusogenic liposomes is unknown and these liposomes may bind the surface of the mitochondria to deliver drug in close proximity to their target. However, cytotoxicity of targeted liposomes has yet to be investigated. The design of liposomes has thus far been for cytotoxic drugs for the treatment of cancers and hence, the potential use of liposomes for other therapeutic applications is unknown.

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5.2 Polymer Nanoparticles

Fabrication of polymeric nanoparticles encapsulating anti-oxidant drugs is becoming increasingly more popular due to the biodegradable nature and biocompatibility of specific polymers. Polymeric nanoparticles also offer an advantage over other materials because they can be easily chemically modified and conjugated to targeting ligands and/or drugs.

A recent study fabricated PLGA nanoparticles loaded with superoxide dismutase (SOD), which were more efficacious in preventing H_2O_2 induced neuronal cell death compared to SOD alone and SOD conjugated to poly-ethylene glycol (Reddy et al., 2008). PLGA is non-toxic and biocompatible and hence, is commonly used for drug delivery applications (Shive and Anderson, 1997; Walter and Merkle, 2002). These nanoparticles were fabricated using a w/o/w double emulsion whereby SOD in a bovine serum albumin containing aqueous solvent was added to PLGA in chloroform. The primary emulsion was prepared under probe sonication of 55W for 2 min and then added to 2 % polyvinyl alcohol and sonicated again to form the secondary emulsion. The double emulsion was stirred overnight to evaporate chloroform and the particles were lyophilized. The resulting 290 nm particles exhibited an SOD encapsulation efficiency of 75 % and over 80 % of the encapsulated SOD was released in the active form. Human neuronal cells were treated with 50 μM of H_2O_2 and monitored for cell survival. Incubation of cells with 100 IU of SOD loaded nanoparticles for 6 h prevented cell death from occurring; however, longer incubation times (12 h and 24 h) had no neuroprotective effect and cell survival was the same as the untreated group. The authors' speculate that the H_2O_2 produced by SOD as a result of ROS reduction, depletes intracellular antioxidants such as catalase, which neutralize H_2O_2. This study demonstrates that polymeric nanoparticles loaded with anti-oxidant species are capable of protecting cells from oxidative stress induced cell death.

5.3 Metal Nanoparticles

Nanoparticles made from metal elements including gold, platinum and titanium dioxide have unique properties including anti-oxidant capabilities, size < 10 nm, and ease of attachment of targeting ligands for mitochondrial targeting. However, the safety and efficacy of using metal nanoparticles is debatable among scientists. Wherever appropriate, studies that report toxicity of metal nanoparticles are included.

5.3.1 Gold Nanoparticles—Gold nanoparticles have been fabricated with various different sizes from <10 nm to about 100 nanometers (Link and El-Sayed, 1999). Gold nanoparticles have surface Plasmon resonance in which the electrons in the gold surface create a magnetic field upon laser irradiation. Altering the surface of the gold nanoparticle with proteins or DNA for example, will change the surface Plasmon resonance properties of the gold nanoparticle. This unique property of gold nanoparticles has become the key to developing novel activated drug delivery systems, diagnostic systems (El-Sayed et al., 2005), sensitive biosensors for detection of protein-ligand binding reactions (BIACORE instrument), biosensing assays (since at least 1984 (Flanagan and Pantell, 1984)), microelectromechanical systems (Voskerician et al., 2003) as well as others. Current literature has very limited information on the application of gold nanoparticles as mitochondrial delivery devices, but some studies allude to the potential application of these devices as novel mitochondrial targeting systems.

In particular, the ease of conjugating biomaterials to the surface of the nanoparticle through a terminal amine or thiol group makes gold nanoparticles an ideal system for fabricating functionalized nanoparticles (Braun et al., 2005). In the design of a diagnostic system for detection of cancer at early stages, gold nanoparticles were easily functionalized with anti-epidermal growth factor receptor (EGFR) antibody by mixing the gold nanoparticle solution

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in HEPES buffer with the antibody at a 1:1 volume ratio at room temperature for 20 min (Sokolov et al., 2003). Separation of unconjugated antibody from gold nanoparticles was obtained by centrifuging at 5000 rpm for 2 h. Conjugation of gold nanoparticles with thiol-containing substrates is also relatively trivial. Thomas et al. conjugated gold nanoparticles with thiol-modified polyethylenimine by mixing gold nanoparticles with the thiol-modified polyethylenimine at molar ratios of 1:3 or 3:1 and stirring for 10 min (Thomas and Klibanov, 2003). Then, 7.93 mM of $NaBH_4$ was dissolved in 4 mL of water and added dropwise to the solution over 40 sec and the reaction was stirred for another 24 h. The resulting gold nanoparticle conjugate was dialyzed against a 12 kDa membrane to remove unconjugated thiol-modified polyethylenimine.

In addition, the synthesis of gold nanoparticles is also easily obtainable. Fabrication of gold nanoparticles is optimized for each purpose and the method of synthesis varies between research groups, but generally a reducing agent is mixed with gold hydrochlorate ($HAuCl_4$) to form nanoparticles. One method is to add 4 mL of 1% sodium citrate to 100 mL of 0.01% boiling gold hydrochlorate and stir until there is a deep red color (Zhang et al., 2003). The method used by Turkevich and Frens (the process was first described in 1951 (Turkevich et al., 1951)) is generally used to fabricate gold nanoparticles, which consists of reducing $HAuCl_4$ with citrate at 100°C (Kimling et al., 2006). Typically, 95 mL of gold solution is brought to a boil and 5 mL of preheated citrate solution is added; whereby the concentration of citrate can be altered to tune the particle size. Others have used sodium borohydride as a reducing agent in the fabrication of gold nanoparticles to make much smaller nanoparticles (<10 nm) (Prevo et al., 2008). Altering the stirring speed, reducing reagents, stabilizing reagents, etc. can be used to optimize the method of synthesis for particle size and stability.

A variety of studies have identified mitochondrial damaging effects in the presence of gold nanoparticles. One study investigated the ability of gold nanoparticles to disrupt the integrity of the outer mitochondrial membrane and cause the release of cytochrome c from the mitochondrial electron transport chain, which is known to cause cell death (Salnikov et al., 2007). The study found that gold nanoparticles of 6 nm were not able to permeate the outer mitochondrial membrane, but 3 nm gold nanoparticles could permeate the outer mitochondrial membrane of heart mitochondria. The mitochondrial entry of 3 nm gold nanoparticles was VDAC-dependent since their entry was inhibited by 15–50 μg/mL König’s polyanion and 100–300 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonate, both VDAC inhibitors. Therefore, the delivery of gold nanoparticles to the mitochondria is mediated by the size of the nanoparticle whereby particles smaller than or equal to 3 nm are capable of crossing the outer mitochondria membrane. This mechanism of entry of gold nanoparticles may be of therapeutic value for induction of cellular apoptosis. However, other therapeutic mechanisms may require that gold nanoparticles avoid mitochondrial entry by this mechanism.

Cellular toxicity of gold nanoparticles is also mediated by nanoparticle surface charge and nanoparticle concentration (Goodman et al., 2004). Cationic gold nanoparticles less than 10 nm (McIntosh et al., 2001) in diameter have an LC_{50} (concentration that causes lethality in 50% of the population) of 1 μM in two human cell lines (cos-1 and red blood cells) and 3 μM in a bacterial cell line (E. *coli*). Anionic gold nanoparticles have an LC_{50} of 7 μM in cos-1 cells, 72 μM in red blood cells, and 28 μM in E. *coli*. Further, toxicity was characterized by lysis activity in anionic 1-stearoyl-2-oleoylphosphatidylcholine (SOPC)/ stearoyl-oleoyl-phosphatidylserine (SOPS) (0.5 mL 25 mg/mL/0.15 mL 10 mg/mL, respectively) liposomes and neutral SOPC (0.5 ml 25mg/ml) liposomes. Cationic gold nanoparticles lysed 10-fold more anionic liposomes (about 20%) than anionic gold nanoparticles (about 2%) in 5 min. Lysis of neutral liposomes (about 15%) was nearly the same as for anionic liposomes (about 20%) for cationic gold nanoparticles at 5 min;

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however, anionic gold nanoparticles lysed more neutral liposomes (about 5 %) than anionic liposomes (about 2%) at 5 min. Lysis of anionic liposomes directly correlated with concentration of cationic gold nanoparticles. Gold nanoparticles at 2, 9, 18, 88, 175, 264, 350, 439 and 875 nM had about 12%, 22%, 25%, 30%, 35%, 37%, 40%, 45% and 50% lysis of anionic liposomes at 5 min, respectively. Concentrations of cationic gold nanoparticles greater than 175 nM had an exponential increase in induced lysis with increased time. In addition to the diameter of the gold nanoparticles, the charge of the nanoparticle and concentration contributes to cellular toxicity.

Although gold nanoparticles can exhibit toxicity, they can also exhibit therapeutic effects. One study in particular showed that chitosan functionalized gold nanoparticles of 6 to 16 nm in size are 80 times more efficient at eliminating ROS in an $H_2O_2/FeSO_4$ system than ascorbic acid (Esumi et al., 2003). The antioxidant activity was independent of gold nanoparticle size, but increased with an increase in chitosan concentration. These results suggest that chitosan may be responsible for the antioxidant activity when complexed with gold. However, chitosan has no known antioxidant effects based on the present literature and may exhibit cytotoxicity (Qi et al., 2005).

The same group has also shown that gold nanoparticles functionalized with polyamidoamine (PAMAM) dendrimers are also capable of behaving as antioxidants by reducing ROS to water and oxygen (Esumi et al., 2004). PAMAM dendrimer functionalized gold nanoparticles were fabricated by reducing the $HAuCl_4$-dendrimer mixture with $NaBH_4$ and stirring for 30 min. Using a spin trapping technique, dendrimer-gold nanocomposites of approximately 3.6 nm with terminal carboxyl groups were able to remove hydroxyl groups at a rate constant of $3.3x10^{13}$/M·s. PAMAM dendrimer alone was not capable of eliminating hydroxyls and hence, the gold nanoparticle may be contributing to the antioxidant effects of dendrimer functionalized gold nanoparticles. The rate constant for ascorbic acid (a natural antioxidant) was only 3.9×10^{11}/M·s and PAMAM functionalized gold nanoparticles with terminal carboxyl groups had a rate constant of 85 times faster. The dendrimer functionalized gold nanoparticles may be more promising than the chitosan-gold nanoparticles due to the smaller size of the dendrimer functionalized gold nanoparticles and slightly higher efficiency at eliminating ROS. The gold nanoparticles can be functionalized to provide for mitochondrial targeting of the dendrimer functionalized gold nanoparticles.

A recent study published in Nano Letters demonstrated that gold nanorods functionalized with CTAB (cetytrimethylamonium bromide, a cationic lipophilic molecule) preferentially induced cell death in cancer cells, but not in normal cells and were able to accumulate in the mitochondria of cancer cells, but not in the mitochondria of normal cells (Wang et al., 2011). CTAB is well known to induce toxicity in both animal and cell models and may be partially responsible for cell death induced by CTAB functionalized gold nanorods (Isomaa et al., 1976). Gold nanorods were fabricated using a common reduction method: CTAB and $HAuCl_4$ in water were mixed followed by $NaBH_4$ addition and 2–5 h of stirring. Nanorods were formed by particle growing with CTAB, $HAuCl_4$, $AgNO_3$, H_2SO_4 and Ascorbic acid. Final particles were made by centrifuging and redispersing with PBS and were of approximately 55 nm in length and 13 nm wide. The authors demonstrated that normal cells eliminated and removed gold nanoparticles through lysosomal clean up. In cancer cells, gold nanoparticles were able to escape the lysosome due to differences in lysosomal protein content between cancerous and normal cells. These effects were determined to be a property of CTAB whereby addition of CTAB alone to cancer cells significantly reduced the integrity of the lysosomal membrane and CTAB had no effect on membrane integrity of normal cells. After 6 h incubation of cancer cells with the gold nanorods, the gold nanorods entered the cytoplasm and localized within mitochondria. The authors hypothesize that the extremely negative membrane potential across the inner mitochondrial membrane is responsible for the

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preferential uptake of cationic CTAB functionalized gold nanorods. Cancer cell death was induced by mitochondrial membrane potential disruption as determined by a JC-1 dye assay and formation of ROS by flow cytometry. JC-1 dye was excited at 488 nm and emission at 530 nm and 580 nm was used to detect the monomer or aggregate form of the dye whereby the aggregate emits at 580 nm in the presence of a strong membrane potential and the monomer emits at 530 nm in the presence of a weak membrane potential. After incubation of cancer cells with gold nanorods for 24 h, JC-1 dye emitted in the green, indicating a reduction in the membrane potential. ROS production was detected using the DCFH-DA probe (Molecular Probes, USA) and flow cytometry analysis. The DCFH-DA dye was excited at 488 nm and the emission at 525 nm was used for detection of ROS. Cancer cells had significantly higher levels of ROS than non-cancerous cells after 24 h treatment with gold nanorods. These results allude to a mechanism of gold nanoparticle mitochondrial entry other than the outer mitochondrial membrane since these particles are much larger than 3 nm. Further, these studies demonstrate that surface modification of gold nanoparticles enhances mitochondrial delivery and gold nanoparticles in the absence of drug elicit anti-cancer therapeutic effect. Gold nanoparticles have the potential to act as both a drug carrier and an antioxidant, which makes them an attractive nanoparticle for mitochondrial delivery.

5.3.2 Titanium Dioxide Nanoparticles—Titanium dioxide particles are found in several consumer products such as sunscreens, paints, and vitamins and recently have entered the nanomedicine field. One research group has investigated the use of these nanoparticles as targeted nanotherapies to enter the mitochondria for gene regulation control (Paunesku et al., 2007). These nanoparticles exhibit their mitochondrial specific effects due to the presence of a mitochondrial-specific oligonucleotide on the surface of the nanoparticle. TiO_2 nanoparticles were fabricated by addition of titanium (IV) tetrachloride dropwise to cooled water before dissolving the colloidal solution in concentrated sulfuric acid (H_2SO_4) (Paunesku et al., 2003). The pH was adjusted to 3.5 with NaOH and oxygen was removed under N_2 or Argon gas. Next, the nanoparticles were coated with 100 μL of glycidil isopropyl ether to prevent nonspecific interaction of oligonucleotide with hydroxyl groups on the TiO_2 surface. While vigorously mixing, 0.2 M LiOH was rapidly added to the solution to adjust the pH to 9.5 and finally, the particles were dialyzed against 10 mM NaH_2PO_4 until pH 6.5 was obtained. The resulting nanoparticles were 3–5 nm and were functionalized with a mitochondrial oligonucleotide (NADH dehydrogenase subunit 2 gene) to demonstrate mitochondrial specificity. Before the particles were conjugated with oligonucleotide, the reactive group, dopamine, was tethered to the surface of the nanoparticle. Dopamine-end labeled oligonucleotides were tethered to the colloidal solution at $8 > pH > 2.5$ resulting in spontaneous adsorption of dopamine to the surface due to the favorable free energy (−7.6 Kcal/mol). The stability of the dopamine complex is much more stable than the glycidil isopropyl ether coating on the TiO_2 nanoparticles, which results in a stable complex in the presence of sunlight and thousands of 10 mJ laser pulses. Conjugation of the dopamine labeled oligonucleotide to the nanoparticle was visualized by the formation of the intense red color due to the charge transfer between dopamine and TiO_2.

Oligonucleotide (NADH dehydrogenase subunit 2 gene) functionalized TiO_2 nanoparticles were then applied to MCF-7/WS8 breast cancer cells and the cells were subjected to electroporation as a transfection tool. After transfection, the cells were allowed to attach to cell culture dishes for 2 h and then the cells were washed to remove any nanoparticles that did not enter the cell. Using TEM images, these nanoparticles were present within only the mitochondria of the cell. The authors also confirmed mitochondrial targeting in a rat pheochromocytoma cell (PC12) line and found nanoparticles within mitochondria that were isolated from cells. These results demonstrate that nanoparticles coated with oligonucleotides specific to the mitochondrial DNA are capable of mitochondrial

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intracellular targeting. However, the mechanism by which the oligonucleotide conjugated nanoparticle is shuttled or trafficked to the mitochondria is unknown.

Interestingly, non-functionalized titanium dioxide particles of 5 nm (anatase) and 23 nm (anatase and rutile) also target the mitochondria with minimal nuclear delivery (Suzuki et al., 2007). Using confocal microscopy, the location of titanium dioxide particles (5 and 23 nm) overlapped with the mitochondrial stain (Mitotracker Red CMXros) indicating their subcellular location after treating CHO-K1 cells. Larger titanium dioxide particles (5000 nm) crossed the plasma membrane, but were unable to enter mitochondria. Thus, mitochondrial delivery appears to be mediated by nanoparticle size and possibly, nanoparticle materials since titanium dioxide can enter mitochondria without functionalization.

However, titanium dioxide particles are capable of inducing mitochondrial dysfunction and toxicity. Titanium dioxide particles of Degussa P25 mixture (70 % anatase/30 % rutile) are reported to be 30 nm, but rapidly aggregate to greater than 500 nm when exposed to DMEM and HBSS medium and reach a steady state size of greater than 830 nm (Long et al., 2006). Exposure of these titanium dioxide particles to BV2 microglia cells caused significant toxicity events to occur including hyperpolarization of the mitochondrial membrane (as indicated by the MitoTracker Dye), rapid increase in H_2O_2 generation, and significant increases in ROS production from the electron transport chain of the mtiochondria. Within 20 min, the mitochondria potential difference was depolarized in the presence of particles ranging from 830 nm to 2400 nm as detected by the increase in the MitoTracker Red ratio of emission at 579 nm to 612 nm. The increase in H_2O_2 generation was detected using the OxyBURST and ImageiT dyes and the increase in emission ratio of 508 nm/525 nm and 495 nm/529 nm, respectively. An increase in ROS generation was detected using the dye MitoSOX and the emission ratio of 510 nm/580 nm. These results are important to consider when designing nanoparticles for mitochondrial drug delivery. Depending on particle stability and aggregation potential, these nanoparticles may exhibit different behaviors in different medium. Therefore, early stages of nanoparticle development should include toxicity tests in appropriate conditions (buffer, pH, temperature) to ensure higher rates of success in in vivo models.

5.3.3 Platinum Nanoparticles—Platinum nanoparticles also exhibit unique antioxidant properties as described by several research groups (Aiuchi et al., 2004); however, limited studies have confirmed platinum nanoparticles as safe materials (Elder et al., 2007) in cell free and cell based assays. Platinum nanoparticles of approximately 20–30 nm and of varying shapes (nanoflowers, spheres, and multipods) did not produce ROS in a cell free system nor inflammatory responses (interleukin-6, and tumor necrosis factor α) in a human umbilical vein endothelial cell (HUVEC) system up to 50 μg of platinum nanoparticles. These results confirm that platinum nanoparticles of various shapes do not exhibit cytotoxicity.

Platinum nanoparticles have been recognized for their antioxidant properties and potential mitochondria therapeutic effects. Hikosaka et al. demonstrated that platinum particles functionalized with pectin were capable of oxidizing NADH to NAD^+ (Hikosaka et al., 2008). Pectin functionalized platinum particles were prepared by citrate reduction of platinum-pectin (H_2PtCl_6): addition of 4 mL of 16.6 mM H_2PtCl_6 to 43.8 mL of water and then refluxing at 100 °C. Then, 8.6 mL of 77.2 mM trisodium citrate dehydrate was added to the mixture and the solution was refluxed for 30 min. Once the reaction cooled, 10 mL of 3.96 mg/mL pectin was added and the mixture was stirred for another 1 h. In 12 h, 85% of NADH was oxidized to NAD when 100 μM NADH was incubated with 50 μM pectin functionalized platinum particles at room temperature. The oxidizing properties of these

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platinum particles may be used to normalize the redox potential by regenerating NAD+ species for glycolysis and other cellular pathways to function properly. The same research group has also demonstrated that platinum nanoparticles (with a size of 5 nm) are capable of quenching superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) (Kajita et al., 2007). The authors hypothesize that since pectin functionalized platinum nanoparticles oxidize NADH to NAD^+ and reduce ubiquinone (CoQ) to ubiquinol ($CoQH_2$), platinum nanoparticles may mimic the functions of complex I. This is an exciting result because if pectin functionalized platinum nanoparticles can replace the functions of complex I, these nanoparticles have the potential to be a unique therapeutic or diseases with complex I deficiencies such as Alzheimer's disease.

Polyacrylic acid (PAA) protected platinum nanoparticles of approximately 2 nm are also capable of acting as antioxidants invitro (Watanabe et al., 2009). In the presence of 25 mL of ethanol, 2 mL of 16.6 mM PtCl6 and 0.39 g of PAA (125 : 1 molar ratio of PAA : Pt) were stirred and refluxed at 90–95°C for 120 min. The reaction mixture was placed in an evaporator to remove solvent and then resdispersed in water. Using a spin trapping method, the ability of PAA coated platinum nanoparticles to scavenge the superoxide anion radicals was determined. The platinum nanoparticles scavenged the radicals in a dose dependent manner and the IC_{50} was 58 μM. PAA alone was not capable of scavenging the radicals and hence, platinum was identified as the source of the antioxidant activity.

Recent in vivo studies also confirm the potential antioxidant activity of platinum nanoparticles. In a pulmonary inflammation model, platinum nanoparticles demonstrated antioxidant properties in vitro as well as in vivo. PAA stabilized platinum nanoparticles were synthesized using sodium citrate as a reducing agent. Particles of approximately 2 nm in diameter at 200 μM were capable of quenching H_2O_2 and O_2^- by 46.7% and 35.5%, respectively, in a cell free assay. The in vitro cell assay was conducted in alveolar epithelial cells (A549) and the platinum nanoparticles were capable of dose dependently preventing cell death of the cells in the presence of 1% cigarette smoke extract (toxic dose). DBA/2 mice were exposed to cigarette smoke from one cigarette every 1 h for 5 times a day. Intranasal administration of the PAA coated platinum nanoparticles once daily for 3 days, increased the antioxidant activity in the lung by 57 % compared to the control mice (no cigarette smoke) and mice given saline injection exposed to cigarette smoke had an 83 % decrease in antioxidant activity. Further, the neutrophil count in mice exposed to cigarette smoke and administered PAA coated platinum nanoparticles had similar levels to control mice (no cigarette smoke). PAA coated platinum nanoparticles are effective ROS scavengers and may be more effective than current antioxidant treatments.

5.3.4 Bimetallic Nanoparticles—Bimetallic nanoparticles are also capable of acting as antioxidant species and may behave as both the delivery vehicle and therapeutic agent. Dual properties of these nanoparticles are likely to simplify manufacturing processes for pharmaceutical development as well as pharmacokinetic profiles by eliminating release kinetics. For instance, gold and platinum bimetallic nanoparticles of approximately 5 nm quenched H_2O_2 with an IC_{50} of approximately 2, 4 and 5 μM with an Au:Pt ratio of 0:100, 25:75 and 50:50 and superoxide anion radicals were quenched with an IC_{50} of approximately 70, 100 and 150 μM for particles of Au:Pt ratio 0:100, 25:75 and 50:50, respectively (Kajita et al., 2007). Bimetallic particles of Au and Pt were fabricated using a simple method of mixing $H_2PtCl_6 \cdot 6H_2O$ with $HAuCl_4 \cdot 4H_2O$ and refluxing before adding trisodium citrate dehydrate. The authors concluded that as the platinum molar percent decreased, the quenching of both H_2O_2 and superoxide anion radicals reduced, indicating platinum has a much stronger quenching activity of ROS than gold. Conjugation of targeting ligands (peptides, proteins or nucleic acids) to the gold surface of the bimetallic nanoparticle

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may be an effective approach to target the mitochondria. However, the cellular toxicity of these bimetallic nanoparticles has yet to be tested.

6.0 Summary

The mitochondrion is a novel therapeutic target involved in the pathology of many degenerative and metabolic diseases. Mitochondria targeted therapeutics are currently under investigation to alleviate oxidative stress in various diseases. Metal nanoparticles, which can be designed at a size <10 nm are most intriguing due to their small size and inherent anti-oxidant properties. In addition, metals such as gold can be easily functionalized with targeting ligands due to thiol reactivity. However, the toxicity of these metal nanoparticles is largely unknown even though metals such as gold are regarded as non-toxic. Polymeric particles and liposomes, the most pharmaceutically relevant delivery systems, have also shown success in exhibiting mitochondrial therapeutic effects. Liposomes are by far the most investigated pharmaceutical nanosystems, which showed preferential mitochondrial delivery, biocompatibility, and enhanced efficacy. However, the size of liposomes and polymeric nanoparticles is generally restricted to about 100 nm or larger.

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Abbreviations

DNA	deoxyribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
NADH	nicotinamide adenine dinucleotide
FDA	Food and Drug Administration
PLGA	poly(lactide-co-glycolide)
ATP	adenosine triphosphate
FAD	flavin adenine dinucleotide
ROS	reactive oxygen species
ADP	adenosine diphosphate
ATP	adenosine triphosphate
RNA	ribonucleic acid
HNE	4-hydroxynonenal
VDAC	voltage-dependent anion channels
PBR	peripheral-type benzodiazepine receptor
TOM	transporter outer membrane
TIM	transporter inner membrane
OMM	outer mitochondrial membrane
IMM	inner mitochondrial membrane
STPP	stearyl triphenyl phosphonium
R8	octaariginine

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DOPE	dioleoylphosphatidylethanolamine
GFP	green fluorescent protein
SOD	superoxide dismutase
EGFR	epidermal growth factor receptor
$HAuCl_4$	gold hydrochlorate
SOPC	1-stearoyl-2-oleoylphosphatidylcholine
SOPS	stearoyl-oleoyl-phosphatidylserine
PAMAM	polyamidoamine
CTAB	cetytrimethylamonium bromide
PAA	polyacrylic acid

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Highlights

- Mitochondrial defects underlie the pathology of Alzheimer's disease and diabetes.
- Plasma membrane, cytosol, and mitochondrial membrane are key barriers for mitochondrial delivery of nanosystems.
- Some nanosystems including metal nanoparticles exhibit mitochondrial entry.
- Mechanisms of mitochondrial entry include protein transporters and channels.
- Functionalization of nanosystems enhances mitochondrial delivery and/or efficacy.

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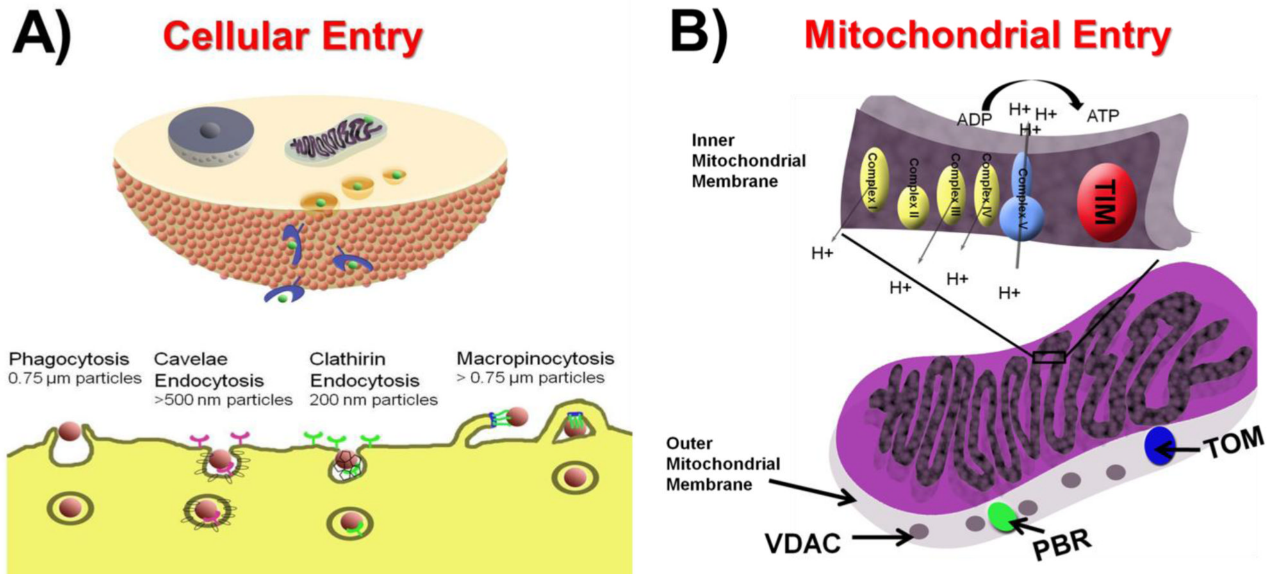

Abbreviations: transporter outer membrane (TOM), peripheral-type benzodiazepine receptor (PBR), voltage- gated dependent anion channel (VDAC), transporter inner membrane (TIM)

Figure 1.

Mechanisms of nanoparticle cellular entry (A) and potential mitochondrial transports for nanoparticle entry (B) including transporter outer membrane (TOM), peripheral-type benzodiazepine receptor (PBR), voltage gated-dependent channel (VDAC), and transporter inner membrane (TIM). Mitochondrial proteins of the electron transport chain: complex I is NADH dehydrogenase, complex III is cytochrome bc_1, complex II is succinate dehydrogenase, complex IV is cytochrome c oxidase, and complex V is ATP synthase are displayed in panel B.

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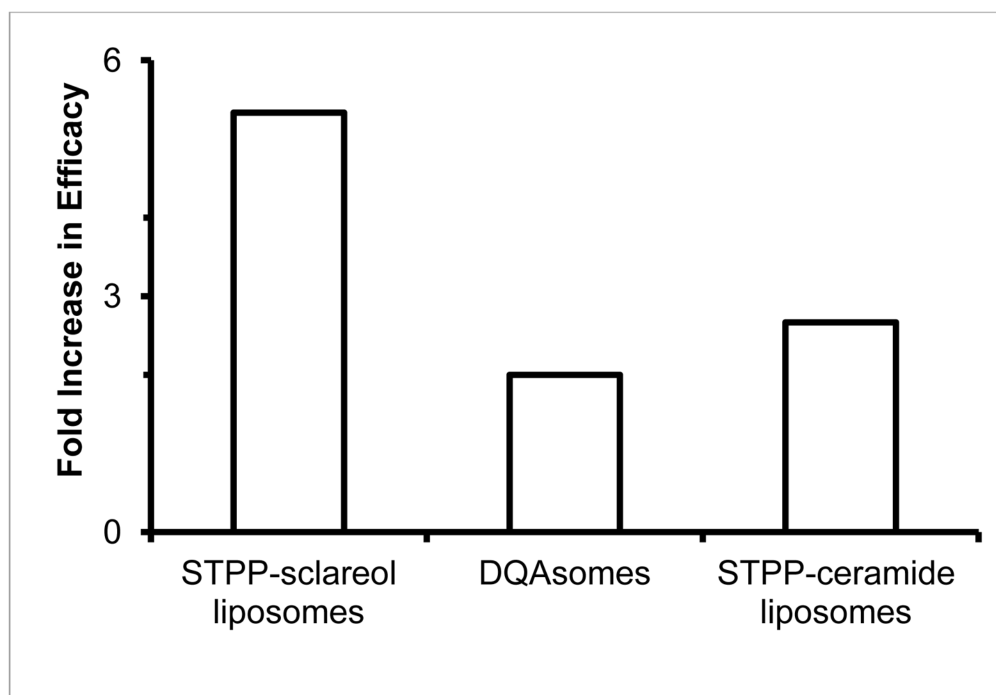

Figure 2.

Superior efficacy of functionalized nanosystems in mitochondria. In various studies, enhanced efficacy was demonstrated for functionalized nanosystems compared to non-functionalized systems. Percent increase in therapeutic efficacy for each mitochondrial targeted nanosystem compared to non-targeted nanosystem is shown. The descriptor before hyphen in the X-axis labels refers to the functionalization. The efficacy parameter for STPP-ceramide liposomes was tumor volume/day in balb/c mice with 4T1 breast tumors (Boddapati et al., 2008); the efficacy parameter for STPP-sclareol liposomes (Patel et al., 2010) and DQAsomes loaded with paclitaxel (D'Souza et al., 2008) was apoptosis in COLO25 cells.

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Table 1

Mitochondrial defects in Alzheimer's disease and Diabetes and potential therapeutic strategies.

Disease	Mechanism of Mitochondrial Dysfunction	Potential Therapeutic Strategy
Alzheimer's	• Aβ aggregates bind cyclophilin D to reduce the membrane potential and increase oxidative stress • Deficiency in cytochrome c oxidase (complex IV) • mtDNA mutations, which can reduce ETC protein activity	• Competitive inhibition to prevent binding of Aβ aggregates to cyclophilin D • Anti-oxidants to prevent oxidative stress • Gene therapy to reverse mtDNA mutation effects • Enzyme replacement (e.g. complex IV)
Diabetes	• Reduced mitochondrial function and content increases intracellular glucose levels and reduces blood insulin levels • mtDNA mutations reduce mitochondrial respiration function and inhibit insulin secretion from β cells • Mitochondrial dysfunction or uncoupling of ETC induces lipid oxidation, which increases levels of acetyl CoA and inhibits mitochondrial metabolism of glucose	• Anti-oxidants to prevent oxidative stress • Gene therapy to reverse mtDNA mutation effects • Inactivate/inhibit intracellular acetyl CoA from interacting with glucose metabolizing enzymes in mitochondria • Activate glucose metabolizing enzymes in mitochondria

Abbreviations: electron transport chain (ETC), mtDNA (mitochondrial DNA)

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Table 2

Biological barriers for mitochondrial delivery and potential approaches to overcome drug delivery challenges.

Biological Barriers	Properties	Approaches to Overcome Barriers
Plasma Membrane	• −70 mV potential difference • Lipophilic membrane • Molecules > 500 mw have difficulty with diffusion	• Positively charged and lipophilic molecules • Small compounds (< 500 mw) • Receptor ligands
Cytosolic Barriers	• High viscosity • Reduced diffusion • Collisional interactions • Binding to intracellular components	• Low molecular weight solutes with low non-productive binding to intracellular components
Outer Mitochondrial Membrane	• Weak, lipophilic barrier • Transporter outer membrane (TOM) for protein translocation • Voltage- gated dependent anion channel (VDAC) for gold nanoparticles • Peripheral-type benzodiazepine receptor (PBR) for cholesterol and benzodiazepine uptake	• Particles <5000 mw • TOM ligands • Benzodiazepine/cholesterol-derived ligands
Inner Mitochondrial Membrane	• −180 mV potential difference • Strong, lipophilic barrier • Molecules > 500 mw have difficulty with diffusion • Transporter inner membrane (TIM) 22 and 23 for protein translocation • Rich in cardiolipin	• Small molecules (< 500 mw) • Positively charged and lipophilic molecules • TIM ligands (e.g., N-terminal 10–80 basic and hydrophobic amino acids)

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Table 3

Properties of nanosystems designed for targeted mitochondrial delivery.

Nanosystem	Size (nm)	Disease Targeted	Therapeutic Approach	Mechanism of Action	References
STPP Functionalized Liposomes	55	Cancers	Apoptosis	Enhanced ceramide mitochondrial delivery	(Boddapati et al., 2008)
STPP Functionalized Liposomes	105	Cancers	Apoptosis	Enhanced sclareol mitochondrial delivery	(Patel et al., 2010)
PLGA Nanoparticles	290	Diseases with oxidative stress	Anti-oxidant	Enhanced superoxide dismutase mitochondrial delivery	(Reddy et al., 2008)
Chitosan Functionalized Au Nanoparticles	6–16	Diseases with oxidative stress	Anti-oxidant	80 times more efficient than ascorbic acid at reducing ROS	(Esumi et al., 2003)
PAMAM Functionalized Au Nanoparticles	3.6	Diseases with oxidative stress	Anti-oxidant	85 times more efficient than ascorbic acid at reducing ROS	(Esumi et al., 2004)
CTAB Functionalized Au Nanorods	55 × 13	Cancers	Apoptosis	Induction of oxidative stress and disruption of mitochondrial membrane potential	(Wang et al., 2011)
Oligonucleotide Functionalized TiO_2 Nanoparticles	3–5	Mitochondrial DNA diseases	Gene and/or drug delivery carrier	Functionalized with mitochondrial gene	(Paunesku et al., 2007)
Pectin Functionalized Pt Nanoparticles	5	Diseases with NADH dehydrogenase deficiency	Anti-oxidant	Platinum accepts and donates electrons similar to complex I of the ETC	(Hikosaka et al., 2008)
Au/Pt Bimetallic Nanoparticles	5	Diseases with oxidative stress	Anti-oxidant	Platinum is capable of reducing H_2O_2	(Kajita et al., 2007)

Abbreviations: stearyl triphenyl phosphonium (STPP), poly-co-glycolic acid (PLGA), polyamidoamine (PAMAM), cetytrimethylamonium bromide (CTAB)

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Table 4

Reported mitochondrial uptake of nanoparticles of varying size.

Mitochondrial Entry/Effect	Size (nm)	Nanoparticle Description	Reference
Yes	260	DOPE:SM:R8 and DOPE:PA:R8 liposomes	(Yamada et al., 2008)
Yes	55	STPP-ceramide liposomes	(Boddapati et al., 2008)
Yes	3	Gold nanoparticles	(Salnikov et al., 2007)
Yes	55×13	CTAB-gold nanoparticles	(Isomaa et al., 1976)
Yes	5	Oligonucleotide-TiO_2 nanoparticles	(Paunesku et al., 2007)
Yes	5	TiO_2 nanoparticles	(Suzuki et al., 2007)
Yes	23	TiO_2 nanoparticles	(Suzuki et al., 2007)
No	5000	TiO_2 nanoparticles	(Suzuki et al., 2007)

Abbreviations: Dioleoylphosphatidylethanolamine (DOPE), sphingomyelin (SM), octaarginine (R8), phosphatidic acid (PA), stearyl triphenyl phosphonium (STPP), cetytrimethylamonium bromide (CTAB)

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Table 5

Advantages and disadvantages of nanosystems targeting mitochondria.

Nanosystem	Advantages	Disadvantages	References
Liposomes	• Mitochondrial specificity • Enhanced ceramide and sclareol delivery	• Cellular toxicity is unknown • Size limitations (>100 nm)	(Boddapati et al., 2008; Patel et al., 2010)
PLGA Nanoparticles	• Biodegradable • Biocompatible	• Size limitations (>100 nm)	(Reddy et al., 2008)
Gold Nanoparticles	• Mitochondrial specificity • Easily functionalized • Anti-oxidant properties with chitosan	• Gold may exhibit toxicity • In vitro efficacy is unknown	(Esumi et al., 2004; Esumi et al., 2003)
Gold Nanorods	• Mitochondrial specificity • Small size (< 100 nm) • Easily functionalized • Cancer specificity with CTAB	• Gold may exhibit toxicity	(Wang et al., 2011)
TiO_2 Nanoparticles	• Mitochondrial specificity • Anti-oxidant properties	• Cellular toxicity is unknown • In vitro efficacy is unknown	(Paunesku et al., 2007)
Au/Pt Bimetallic Nanoparticles	• Inherent anti-oxidant properties due to Pt	• Gold may exhibit toxicity • In vitro efficacy is unknown	(Kajita et al., 2007)

Abbreviations: poly-co-glycolic acid (PLGA), cetytrimethylamonium bromide (CTAB)