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Nano-Biointeractions of Functional Nanomaterials: The Emerging Role of Inter-Organelle Contact Sites, Targeting, and Signaling

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The study of nano-biointeractions, at the forefront of interdisciplinary research, unveils intricate interplays between nanomaterials (NMs) and intracellular organelles, which are pivotal hubs orchestrating diverse cellular processes. Thanks also to the formation of dynamic contacts among their membranes, organelles regulate lipid exchange, calcium signaling, and metabolic pathways. Recently, the potential role of NMs in cellular homeostasis through the regulation of organelle membrane contact sites (MCSs) is emerging, and a complete overview of this issue is still lacking. This perspective aims at elucidating the synergy between functional NMs and organelle contact site research, underscoring the pivotal role of NMs in advancing the comprehension of cell biology mechanisms and fostering therapeutic breakthroughs. This subject represents a crucial aspect of nano-biointeractions, as it can reveal new molecular targets for NMs and potentially revolutionize therapeutic strategies. Nanotechnology may offer unprecedented tools to decipher and manipulate dynamic organelle interfaces with remarkable precision. Engineered nanomaterials may serve as versatile probes and effectors, enabling targeted modulation of organelle contact sites and unraveling the molecular intricacies governing organelle dynamics. Furthermore, nano-biointeraction-driven insights hold promise for therapeutic innovations, offering novel avenues in diseases linked to dysregulated organelle contacts.

1. Introduction

In recent years, the growing interest in nanomaterials (NMs) for nanomedicine has led to a significant rise in promising uses. These applications span from cancer therapy and drug delivery to infection care and bioimaging, highlighting the broad range of NM utilization.[1] The reason for the increasing success of NMs mainly relies on their unique physicochemical properties which depend on factors such as size, surface area, composition, shapes, surface charge, and aggregation state. The possibility of modulating these properties gives NMs significant advantages over their bulk counterpart.[2] To fully harness the potential of NMs in nanomedicine and achieve safer and more effective performance, a comprehensive understanding at the molecular level of the intricate interactions occurring at the interface between nanomaterials and the biological microenvironment (nano-biointeractions) appears essential.[3,4] These interactions define the possible fate of nanomaterials by regulating the blood circulation time, the interaction with the reticuloendothelial system, targeting specific sites, cellular internalization, intracellular localization, and fate.[5] In particular, increasing evidences suggest that the interaction between NMs and biological fluids, influencing the native identity of the nanomaterial, through the formation of the protein corona, is a key factor determining both biological response and targeting efficiency.[6–9] Moreover, NMs are internalized within cells via various and diverse pathways depending on their native physical–chemical properties and their interaction with the extracellular environment (e.g., biological fluids) before reaching the cell membrane. Once taken up by the cells, NMs interact with intracellular organelles by modulating their functions. Recently, the influence of nanoparticles (NPs) on organelle crosstalk and cell signaling through the formation of inter-organelle membrane contact sites (MCSs) is raising increasing interest. The mechanism of action of NMs on/through MCSs is not yet clear, but it represents an important aspect of nano-bio interactions, which could open new scenarios to develop novel therapeutic strategies based on nanomedicine.

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To date, a comprehensive review analyzing the current understanding and areas for further investigation of NMs' effects on organelle functionality is lacking. From the literature, there emerges an ongoing debate regarding the role of NMs in cellular homeostasis through the regulation of contact sites.[10,11] However, this subject represents a crucial aspect of nano-bio interactions, and elucidating the molecular signaling and pathways regulated by MCSs could reveal new molecular targets for NMs, potentially revolutionizing therapeutic strategies.

To advance this emerging domain, in this work we provide a concise overview of the various cellular uptake mechanisms of NMs and examine the influence of biomolecular corona formation on NM biological fate. We further touch upon the intracellular behavior of NMs, emphasizing NM intracellular localization and organelle-targeting. Furthermore, we highlighted the emerging role of inter-organelle membrane contact sites in mediating cellular interactions with internalized NPs and, hence, cell and/or organelle functions. Emphasis is placed on the significance of comprehending molecular-level mechanisms underlying NM-MCS interaction to pave the way for novel therapeutic strategies and disease targeting based on functional nanomaterials.

2. Mechanisms of Uptake and Intracellular Fate of Nanomaterials

2.1. Endocytosis

The cellular uptake mechanisms of NMs are influenced by the physiological state, dispersion fluids, administration routes, and encountered barriers, together with various physical and chemical properties of NMs and their adsorbed biomolecules.[12] NMs can enter the body through inhalation, oral ingestion, dermal and ocular penetration, and injection.[13] Once in contact with the cell membrane, NMs are mostly internalized through endocytosis, involving phagocytosis and pinocytosis for specific NM sizes and surface modifications (**Figure 1**).[14]

Phagocytosis primarily occurs in specialized mammalian cells (such as monocytes, macrophages, and neutrophils) in which large particles (>750 nm in diameter) are internalized following the invagination of the cell membrane and the formation of a phagosome.[15] Nevertheless, phagocytosis can also occur for nanometer-sized particles, as it was observed in previously differentiated THP-1 macrophages, which phagocytosed 5 nm diameter platinum nanoparticles (PtNPs) following their administration.[16] Likely, this event requires a certain degree of NM aggregation when they are close to the cell membrane, as well as the activation state of the phagocyte.

Pinocytosis occurs in most eukaryotic cells through various mechanisms including clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), lipid raft-mediated endocytosis, and macropinocytosis (MP). CME is regulated by clathrin and its adaptor proteins, and it is an important pathway for the internalization of endogenous substances, such as transferrin and low-density lipoprotein. Studies have demonstrated that NPs up to 200 nm diameter enter the cell primarily via CME.[17–19] CvME is coordinated by cup-shaped invaginations of the plasma membrane, named caveolae. It is reported that several NMs are able to enter the cells via CvME, for example, albumin-coated NPs,[20]

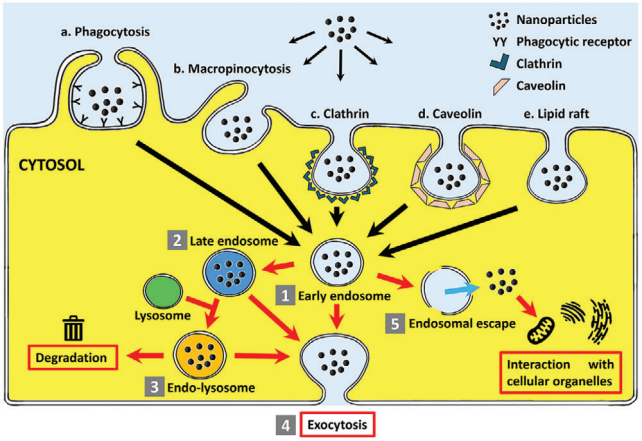

Figure 1. Schematic representation of endocytosis and intracellular fate of NPs. NPs are mainly uptaken via different internalization mechanisms (phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis or lipid raft-mediated endocytosis) and found in early endosomes (1), then into late endosomes (2). The late endosomes may fuse with lysosomes into endolysosomes (3) to proceed toward the degradative pathway. At all the different endosomal stages, NPs could also follow the secretory pathway in which endosomes and/or the endolysosome fuse with the plasma membrane releasing NPs outside the cell (exocytosis) (4). On the contrary, appropriately functionalized NPs can escape from the endosomal compartment (5) to exert their effects in the cytosol.

magnetic NPs containing siRNA of green fluorescent protein,[21] and silica NPs modified with antibodies targeting HER2.[22] Although the specific regulatory mechanisms remain unclear, it is known that large-size NPs cannot be internalized via CvME as the diameter of caveolin-coated vesicles is limited to 30–80 nm.[23]

Lipid rafts are highly compact microregions rich in protein receptors and sphingolipids. Beyond representing a mechanism for the internalization of viral particles,[24] lipid rafts have been shown to be a secondary pathway for the internalization of gold nanorods.[25]

MP is a mechanism of non-selective endocytosis of water-soluble molecules. It depends on actin but is regulated by many other proteins, such as phosphoinositide 3 kinase and Rab family members, and it is sensitive to the pH of the cytoplasm.[23] MP mediates an endocytic pathway of NM uptake by the formation of large and irregular endocytic vesicles derived from the cell membrane, called macropinosomes (0.2–5 µm).[12]

Other pathways of cell uptake involve the direct fusion of the cell membrane mediated by different proteins, mainly SNAREs, Rab proteins, and Sec1/Munc-18 related proteins.[26] This mechanism is utilized by exosomes and membrane-penetrating peptide-modified carriers.[23]

It is noteworthy that, if not appropriately functionalized with molecules that activate a specific endocytic pathway, NMs can use multiple mechanisms of endocytosis[8,27] that contribute to complicating the scenario of NM cellular uptake and final intracellular localization.

In addition to the main internalization routes discussed so far, NMs can enter the cell by passive diffusion via van der Waals forces or steric interactions and the formation of pores.[12] Indeed, it was reported that cationic gold nanoparticles (AuNPs)

were capable of diffusing into the plasma membrane through the formation of hydrophilic pores, thanks to their high surface charge density.[28] However, passive diffusion of NMs through the cell membrane is a significantly rarer event than endocytosis and it is highly dependent on the size, surface charge, and aggregation state of NMs.[29]

2.2. The Relevance of Protein Corona in NM-Cell Interaction

In biological fluids, the NM surface is covered by several adsorbed biomolecules regulating most of the NM interactions with tissues and cells. This adsorption process is described as "biomolecular corona" formation and depends on NM size, surface charge, and composition of the biological milieu, as well as its pH, temperature, and time length of NM-cell contact.[30] The adsorbed biomolecules, mainly soluble proteins, create two distinct layers based on their affinity with the nanomaterial. The proteins with higher affinity quickly decorate the particle forming a stable "hard corona," whereas other proteins can overlap the latter building an outer "soft" layer that can be more easily removed from the NMs by sequential centrifugations.[31] Indeed, multiple molecular layers can be formed over the particle, mainly depending on the bulk nanomaterial and the particle size.[32] General principles to predict the corona formation are difficult to define and the number of specific studies is limited. Most information on biomolecular corona has been obtained investigating AuNPs.[7] Beyond NM size, shape, and charge, more physicochemical features like material concavities, biomolecule/NM size ratio, and transient or long-lasting chemical interactions, seem to play important roles. The recent use of advanced analytical methods and computational tools will certainly lead to a deeper understanding of this phenomenon.

From the biological point of view, the nature of the biomolecular corona is crucial for the NM localization and the following intracellular fate. Biomedical research must consider the derivation of surrounding molecules as a prerequisite for efficient application of nanomaterials, in particular for in vitro experiments and formulations for in vivo release. For example, diverse results can be obtained by releasing the same NM in sera of different species. The distinctive biomolecular coronas created by NM resuspension in fetal bovine serum (FBS), human serum (HS) or human plasma (HP) conditioned medium affect their hydrodynamic diameter, charge, aggregation state, and eventually cellular targeting and uptake.

Corona-forming proteins may encounter cognate receptors expressed onto the cell surface promoting their uptake.[33] On the other hand, specific chemical groups could hamper or delay NM contact with the cell.[34] Hence, the proteins surrounding the NM define its biological identity[35] and this selective adsorption can be used for specific cell targeting[36] increasing the efficiency of drug nanocarriers. An interesting example of the active role of protein corona in cellular uptake is reported using lipoplexes previously incubated with HP.[37] The high affinity of DOTAP/DNA lipoplexes for human vitronectin allowed enhanced cellular uptake in vitronectin-$\alpha v\beta 3$ receptor-positive MDA-MB-435S cells, but not in receptor-negative HEK cells. Recently, different materials or coating polymers have been shown to actively adsorb certain classes of biomolecules allowing their application as delivery carriers for specific diseased tissues.[38] However, the presence of adsorbed proteins is not always sufficient to facilitate particle internalization, even in the presence of their receptors on the cell surface. In fact, nano-sized material can lead to significant structural modification of the adherent proteins due to the above-mentioned physicochemical interactions between the NM surface and the aminoacidic groups, resulting in conformational changes of the corona's biomolecules.[39] To have efficient interaction between the NM-coating proteins and the cell membrane, these proteins should be in the optimal folding to face their potential receptors, namely, being free to rotate to find the proper binding orientation. To overcome this problem while designing novel nanocarriers, covalent or strong-avidity chemical links can be applied between the NM chemical surface and the protein of interest. For example, organic and inorganic NMs were decorated with specific chemokines exploiting covalent bonds between the protein C-terminus and the amino groups on the particle surface[40] or streptavidin–biotin using biotinylated chemokines.[41,42] In both experimental settings, specific localization and increased endocytosis were facilitated by the presence of the desired chemokine receptors on the target cells.

2.3. Intracellular Localization and Organelle-Targeting of Nanomaterials

When an active molecule has passed the external cell membrane by membrane fusion or passive diffusion, it is free in the cytosol and subsequent distribution occurs by diffusion until it encounters another membrane or the cellular target. On the contrary, endocytosed nanoparticles are transported intracellularly through active pathways involving endosomes, lysosomes, the Golgi apparatus, and the endoplasmic reticulum (ER) due to differences in their uptake pathways and characteristics.[23] The endolysosomal pathway is the most common mechanism, with primary endocytic vesicles delivering contents to early endosomes (EEs) which then mature into late endosomes (LEs) and fuse with lysosomes, leading to the degradation of nanoparticles, prevalently.[43] The Golgi apparatus acts as a transit station for intracellular transport, generating new vesicles for transport to endosomes, lysosomes, or the ER. The passage from endosomes to the Golgi apparatus is regulated by various signals (i.e. acid hydrolase receptors, transmembrane enzymes, and SNARE proteins), and the Golgi-ER pathway is considered non-degradative, helping nanoparticles to avoid lysosomal degradation.[44] Since endosomal/lysosomal confinement poses a challenge for NMs in drug delivery, three primary categories of strategies to overcome these barriers have been identified: a) promoting endosomal/lysosomal escape (the proton sponge effect of outstanding pH buffering,[45] osmotic lysis resulting from pH-responsive disassembly of nanoparticles,[46] as well as the swelling effect of pH-responsive nanoparticles,[47] and membrane destabilization induced by pore formation,[48] membrane disruption,[49] membrane fusion,[50] and photochemical internalization[51]); b) directly crossing the cell membrane without entering endosomes or lysosomes (virus fusion protein,[52] cell membrane-coated nanoparticles,[53] cell-penetrating peptides[54]); c) utilizing

different pathways to evade lysosomal degradation (a signal peptide that activates the Golgi/ER retrograde pathway[55]).

However, it is noteworthy that many current nanocarriers lack specific targeting capability, leading to compromised therapeutic effectiveness due to poor tissue accumulation, inefficient cellular internalization, or inaccurate subcellular localization. This issue has raised increasing attention to organelle-targeted nanomedicines, which can deliver drugs to specific intracellular sites, achieving greater therapeutic efficacy. The targeting moiety toward specific organelles allows the nanodrugs to avoid/escape from the endo/lysosomal system and to deliver the drug to the nucleus, mitochondria, lysosomes, endoplasmic reticulum, or Golgi apparatus, specifically. Several strategies for nuclear targeting have been explored,[56] including the use of nuclear-targeting moieties (i.e., nuclear localization signals,[57] trans-activating transcriptional activator protein,[58] the nucleolin aptamer AS1411[59]) as well as nuclear pore complexes dilators (i.e., Dexamethasone[60]) with the aim to functionalize the nanodrugs and facilitate their transport into the nucleus. Mitochondrial delivery has been explored with the help of mitochondrial targeting signals/sequences,[61] mitochondria-penetrating peptides,[62] and triphenylphosphonium.[63] On the other hand, lysosome targeting has been achieved by functionalizing nanoparticles with lysosomal sorting peptides[64] or the morpholine moiety.[65] Targeting the ER using specific retrieval signals like KDEL peptides[66] and other strategies[67] demonstrated the improvement of nanodrug delivery to this organelle. Finally, chondroitin sulfate[68] and six-cysteine peptide[69] have been applied as Golgi apparatus-targeting moieties.

Despite the numerous approaches proposed, further research to optimize these strategies is necessary. For instance, the development of a unique organelle targeting strategy that performs well for different cellular contexts is not straightforward. Achieving an accurate subcellular localization of nanomedicines through organelle targeting requires a detailed understanding of the multifaced physicochemical properties of each organelle, as well as the mechanisms of cellular uptake and intracellular trafficking.[70] Moreover, to get an efficient transport of NMs to specific organelles, innovative strategies to overcome the endo-lysosomal compartment to reach the cytoplasm are still required.[71]

2.4. NM Extracellular Release: Exocytosis and Transcytosis

Another important aspect of NMs engineered for detecting and treating complex diseases is the understanding of their possible exit from the cells. Since exocytosis plays a crucial role in removing nanoparticles with drugs and contrast agents from the body, understanding this pathway is vital for the safe and effective therapeutic application of nanoparticles. The retention of NMs in target cells is directly related to their cytotoxicity and is critical for the efficacy of NM-based drug carriers in nanomedicine.[72] As discussed above, after NMs enter cells, they may reside in, and move to various vesicles and organelles. The recognized exocytosis pathways of NMs mainly include diffusion,[73] rapid recycling pathway,[74] lysosomal pathway,[75] ER/Golgi apparatus pathway,[76] and other pathways.[77] The lysosomal pathway is identified as the most important exocytosis mechanism of NMs, followed by the ER/Golgi pathway.[78] Microtubules are involved in all exocytosis pathways of NMs, aiding in the movement of NMs out of the cell.[79] The pathways chosen for NM excretion are influenced by various factors, including the physicochemical properties of NMs (size, shape, and surface groups), cell type, and cell culture conditions.[79] However, there is no widely recognized conclusion about the parameters regulating intracellular NMs to leave cells via specific pathways.[78] It is worth noting that the formation of a protein corona around NMs complicates the study of exocytosis, as the corona significantly influences the cellular behavior and fate of NMs.[80,81] Moreover, the stability of NMs and the detachment of surface modification groups within cells can impact exocytosis and cytotoxicity.[82] The exocytosis of NMs was found to be influenced by cell type, with cancer cells displaying different exocytosis behaviors compared to normal cells.[83,84] The exocytosis process is also regulated by external factors, such as serum and Ca^{2+} in the culture medium, and the composition of the culture medium affecting the release of intracellular molecules.[85,86] Overall, the exocytosis of NMs is a complex process that can be modified by multiple interrelated factors, making it challenging to determine consistent conclusions on the release of previously internalized NMs from diverse types of cells.[78]

When endocytosis and exocytosis are coupled and occur in polarized cell types, another transport mechanism called transcytosis is identified. Transcytosis is the transport process of cargo within membrane-bounded carriers, between two environments having different compositions.[87] It is involved in the transport of macromolecules such as lipoproteins, antibodies, and albumin in epithelial or endothelial tissues of microvasculature including the blood–brain barrier (BBB)[88] and intestinal barrier. Despite the endothelial barriers represent one of the primary obstacles for the drugs to reach target tissues,[89] various nano-formulations took advantage of transcytosis to make nanomedicines actively extravasate and infiltrate solid tumors.[90,91] Nanomedicines capable of efficient transcytosis must be able to meet some fundamental requirements such as the fast internalization by the endothelial or tumor cells via receptor- or adsorption-mediated endocytosis, proceed through intracellular trafficking via non-degradative pathways (mainly via Golgi apparatus, whose role is to package endogenous materials for exocytosis), should maintain the rapid endocytosis/exocytosis capabilities (to achieve transcytosis across multilayers of tumor cells away from the blood vessels) and the drug release kinetics of the nanomedicine should balance the intracellular drug release and excretion of the nanomedicines.[92]

In this context, by avoiding or reducing the accumulation of NPs into the endolysosomal compartment, through the gene gun method or by the functionalization of NP surface with a membranotropic peptide, gH625, derived from the Herpes simplex virus type 1, it is possible to enhance BBB crossing.[93,94] However, many questions remain about the properties of nanomedicines required to trigger transcytosis, the intracellular pathway, and the selectivity of transcytosis. Future studies are necessary to deeply understand transcytosis and to design effective nanomedicines for such applications. A recent study, for instance, demonstrated that cargos with high avidity to the low-density lipoprotein receptor–related protein 1 (LRP1) bias toward internalization associated with fast degradation, while mid-avidity augments the formation of syndapin-2 tubular carriers promoting a fast shuttling across the BBB.[95]

Table 1. Tethering complexes and hypothesized functional roles of specific membrane contact sites.

Contact sites	Biological activities suggested	Tether proteins involved	Refs.
ER–mitochondria	Ca^{2+} and lipid transfer, mitochondria dynamics, autophagy	IP3R, GRP75, VDAC, TG2, Mfn2, Mfn1, Lam6	[105–108]
ER–endosome	Organelle dynamics, lipid transport, Ca^{2+} signaling	VAP, protrudin, STARD3, ORP1L, RAB7, TPC2, PI3P	[109–113]
ER–lysosome	Ca^{2+} exchange, organelle dynamics	RAB7, ORP1L, VAP	[114]
Mitochondria–lysosome	Ca^{2+} and lipid transfer, organelle dynamics, mitochondrial fission	RAB7	[115]

3. Exploring the Nexus of Cellular Communication with Internalized NMs: Inter-Organelle Membrane Contact Sites

Eukaryotic cells possess a complex system of endomembranes that enable them to confine specific cell functions in separated compartments. The cellular organelles such as the endoplasmic reticulum, the mitochondria, and the lysosomes belong to this system and help the eukaryotic cells to maintain their homeostasis. However, the intracellular organelles are not individual entities in the cytoplasm, but their functions are finely regulated by the MCSs, an interplay mediated by diffusible signals and direct contact among organelle membranes. In the intricate world of cellular biology, MCSs stand as the bustling hubs where organelles engage in intimate communication. These microdomains, characterized by close proximity between different cellular compartments, have emerged as pivotal orchestrators of various cellular processes, ranging from calcium signaling[96] and lipid metabolism[97] to apoptosis,[98] autophagy,[99] and mitochondria dynamics.[100] In the last few years, there has been a growing interest in MCSs, and new tools to visualize and study these interactions have revealed that they are ubiquitous and that organelles can interact to serve various cellular functions.[101] Briefly, MCSs are areas of close proximity between membranes of different organelles mediated by specific proteins (called tethers) allowing the transfer of substances between them (**Table 1**).[102] Recent evidence supports the idea that MCSs allow the exchange of key cellular components between different organelles, regulating the transport of cellular signals and membrane dynamics.[103,104]

At the heart of the contact sites, a complex interplay lies between organelles such as the endoplasmic reticulum (ER), mitochondria, lysosomes, and plasma membrane (**Figure 2**).

For instance, the ER–mitochondria contacts are involved in organelle fission/fusion, facilitating lipid transfer and calcium signaling, which is vital for mitochondrial function and cellular homeostasis.[116] Similarly, the ER–lysosome contacts are crucial for lipid exchange, autophagy, Ca^{2+} dynamics, and organelle motility.[117]

The dynamic behavior of inter-organelle contact sites is one of the most fascinating and intriguing aspects to unveil and characterize. Such proximity regions are strongly affected by cellular cues and environmental stimuli. Several tethering proteins, lipid transfer proteins, and signaling molecules modulate the intricate dynamic interplay among different organelles.[102] Recent experimental evidence suggests that the tethering distances span a range of 10–80 nm.[118] In some mammalian cells, such distances are found to be 19–22 nm,[119] while they are larger in yeast cells, i.e., 17–57 nm.[120] Such distances can reach over 300 nm as observed for Num1, a protein able to anchor the mitochondrial surface to the plasma membrane in yeast cells.[121]

It is worth underlining that the contact time between different organelles is related to the cell type, its function, and regulation.[118] For some MCSs, it lasts less than 1s, while for muscle cells they can persist for the entire cell life.[101]

Nowadays, the MCSs represent a hot research topic, as dysregulation events are primarily involved with the development and progression of various human diseases including neurodegenerative disorders, metabolic syndromes, inflammatory diseases, and cancer.[10,101,122] Therefore, a detailed understanding of the mechanisms at the molecular level underlying these interactions opens up new therapeutic routes to target the diseases at their core.

3.1. Lysosome–Mitochondria Contact Sites

Mitochondria and lysosomes are mainly involved in metabolic and signaling events in cells. Both organelles can act as sensors of the functional status of the cell through different modulation processes. For instance, lysosomes can retain calcium, iron, and cholesterol, whereas mitochondria can vary the calcium uptake and release metabolites and reactive oxygen species (ROS).[123,124] The specific crosstalk between mitochondria and lysosomes finely regulates the physiological cell function and some imbalances are strictly related to neurodegenerative disorder.[125] Interestingly, recent experimental evidence demonstrates that malfunctions in one type of organelle have effects on the other one, highlighting the importance of the mutual interaction between mitochondria and lysosomes.[126,127] Therefore, understanding the mechanisms underlying the coordination between mitochondria and lysosomes is crucial to developing innovative therapeutic strategies. This is particularly important given that, for many of these syndromes, therapeutic intervention focuses primarily on symptom management.[128]

Mitochondria–lysosome contacts allow bidirectional crosstalk between them as well as the regulation of the organelle network.[115] The dynamic formation of mitochondria–lysosome MCSs is proposed to facilitate cholesterol exchange, Ca^{2+} dynamics, and iron metabolism.[129] The tethering is promoted by active GTP-bound lysosomal Rab7, a small GTPase that plays a crucial role in regulating the dynamics and function of mitochondria–lysosome contacts. It localizes onto lysosomal and late endosomal membranes and acts as a master regulator of lysosomal dynamics by binding to Rab7 effector proteins in its active GTP-bound state. Current studies suggest that Rab7 GTP hydrolysis is a key driver of untethering events at mitochondria–lysosome contact and it may be further regulated by additional protein complexes.

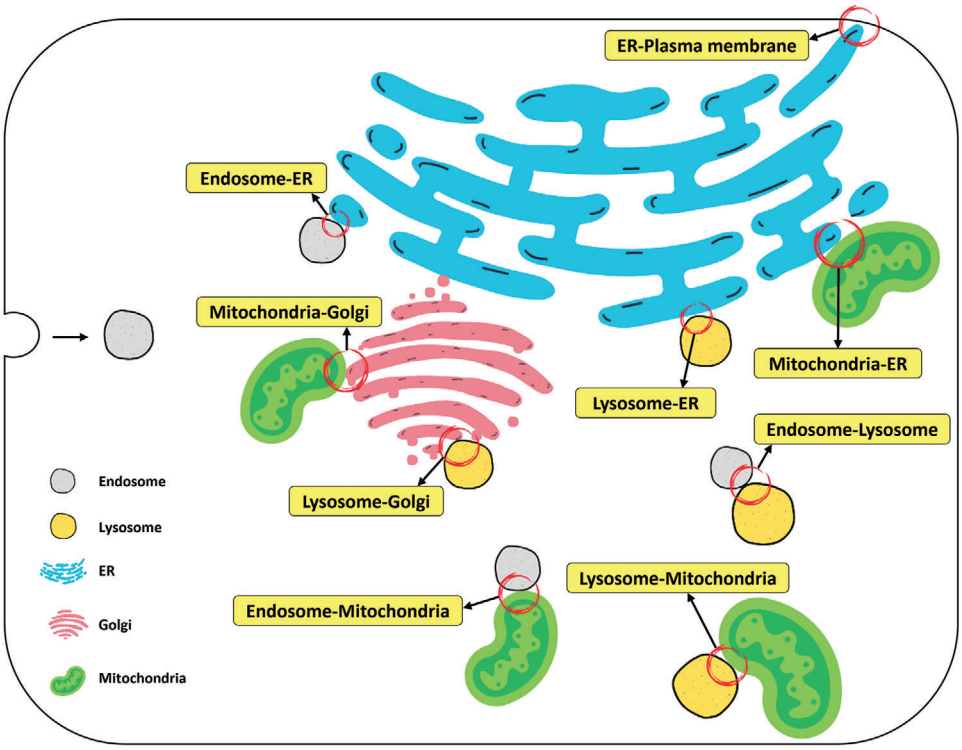

Figure 2. Schematic representation of the main MCSs in mammalian cells.

The transition of Rab7 from an active GTP-bound to an inactive GDP-bound state is mediated by GTPase activating proteins (GAPs). Among them, TBC1D15, interacting with an outer mitochondrial membrane protein Fis1, governs and regulates Rab7 GTP hydrolysis.[130]

Actually, in TBC1D15 GAP-domain or Fis1 mutants, the inhibition of Rab7 GTP hydrolysis leads to inefficient untethering events by prolonging the mitochondria–lysosome contact. Experimental models of human genetic mutations show a correlation between defective mitochondria–lysosome MCSs with neurodegenerative diseases such as Charcot–Marie–Tooth disease, Parkinson's disease, and lysosomal storage disorders.[129]

It has been also demonstrated that the activation or the impairment of the transient receptor potential mucolipin 1 (TRPML1), localized on the lysosome membrane, is strictly related to regulating mitochondrial calcium levels. Therefore, TRPML1 results to be mainly involved in the transport of calcium from lysosomes to mitochondria at the mitochondria–lysosome contact sites. Furthermore, the importance of this channel in the regulation dynamics of Ca^{2+} homeostasis is confirmed by the adverse effects observed in lysosomal storage disorders. In this context, the loss of TRPML1 function is associated with an altered dynamics of contacts between mitochondria and lysosomes and a dysregulation in mitochondrial calcium uptake.[131]

Additionally, at the mitochondria–lysosome contact sites, Transferrin receptor 2 (TfR2), a transmembrane glycoprotein receptor that plays a crucial role in iron homeostasis, is implicated in the transfer of iron from lysosomes to mitochondria. Dysregulation of iron levels within mitochondria can lead to oxidative stress, impaired energy production, decreased mitochondrial size, and heme content in erythroid progenitors contributing to neurodegenerative diseases.[132]

Finally, regarding mitochondria fission processes, recent findings demonstrate the formation of three-way contacts between ER–mitochondria–lysosomes.[114] These data suggest that the ER forms contact with lysosomes at the division site through the interaction of VAMP-associated proteins (VAPs) with the lysosomal lipid transfer protein ORP1L to promote a three-way contact between the ER, lysosome, and the mitochondrion. Furthermore, it was observed that phosphatidylinositol-4-phosphate (PI(4)P), transported by ORP1L from lysosomes to mitochondria, is crucial in the process of mitochondrial fission (**Figure 3**).

Further research is needed to elucidate the precise molecular pathways involved in mitochondria–lysosome crosstalk. Understanding the role of MCSs in this context could provide insights into the molecular basis of neurodegenerative diseases associated with disrupted calcium/iron homeostasis and mitochondrial dysfunction.

3.2. Experimental Methods and Techniques to Characterize MCSs

The detailed characterization of dynamics and ultra-structure of MCSs requires research tools with extremely high temporal and spatial resolutions.[103] Since the first experimental evidence of the existence of physical contacts between ER and mitochondria by electron microscopy in the 1950s,[133,134] several steps forward have been made in the development and design of novel techniques and experimental protocols allowing in-depth study of contact sites from a biochemical, morphological and functional

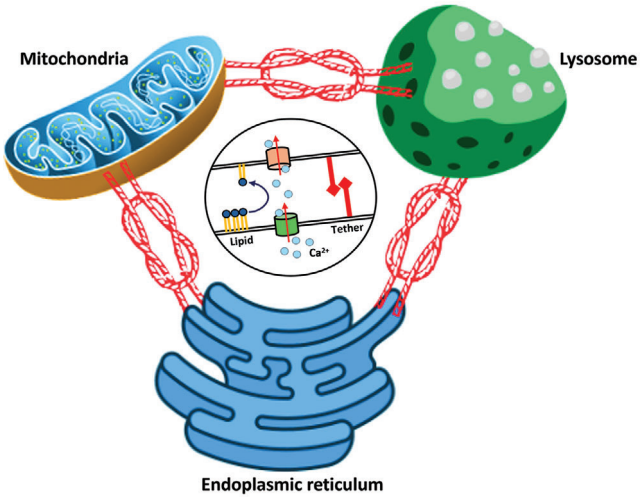

Figure 3. A schematic representation of tripartite MCSs between ER–mitochondrion–lysosome membranes. The inset (black circle) reports a graphic of the molecules involved in the formation of membrane contacts (tethers) and the lipids and Ca^{2+} ions that move among the three organelles.

point of view (**Table 2**). The techniques currently used for the characterization of MCSs can be grouped into three main categories: i) biochemical approaches, ii) microscopy-related techniques, iii) proximity-driven fluorescent probes. Each of these techniques allows us to obtain specific information on a particular aspect of the contact sites, such as the proteins involved, the intracellular localization over time, and the biological function. To fully understand the complex intracellular process of MCSs, the use of different complementary techniques is necessary.

Biochemical methods, i.e., cell fraction, mass spectrometry, proximity-based biotin identification (BioID), and ascorbate peroxidase (APEX) to mention a few examples, are the most employed techniques for the identification of binding complexes and molecular players involved in a specific contact site.[103,135,140,141] In addition, some proximity-driven fluorescent probes, such as proximity ligation assay (PLA), fluorescence resonance energy transfer (FRET), and bioluminescence resonance energy transfer (BRET), are also useful to this scope by combining the use of fluorescence, antibodies and/or molecular biology techniques.[103,107,139,141] Regarding the morphology of the contact sites, microscopy-based techniques, such as electron microscopy, with a high spatial resolution—compared to standard optical microscopy—are highly recommended. To study how contact sites rearrange themselves within the cellular space, all microscopy-related techniques could be useful i.e., transmission electron microscopy (TEM), focused ion beam-scanning electron microscopy (FIB-SEM), and confocal microscopy), but super-resolution microscopy (SRM) methods and some proximity-driven fluorescent probes are more suitable due to the possibility of tracking living cells, making them ideal methods for studying changes in contact sites over time.[103,119,136,137,141] Actually, the variation dynamics of contact sites in the time domain require appropriate experimental approaches that are still under development.[141] Currently, the techniques mentioned above represent a valid tool for investigating the effects of NMs on the dynamics and function of MCSs. From a future perspective, however, NMs themselves could improve the study of MCSs by acting as intracellular probes capable of recognizing specific targets involved in the formation of contact sites between organelles at a molecular level.

4. MCSs and NMs

Some recent studies have highlighted that NMs, once internalized, influence the subcellular compartments by acting on the regulation of MCSs (**Table 3**). These promising results have sparked an ever-increasing interest in the modeling and study of complex regulatory and signaling mechanisms, which to date are still poorly understood.[10,11,142]

Different types of NMs, including crystalline silica nanoparticles, multi-walled carbon nanotubes, silver nanoparticles (AgNPs), and AuNPs, can cause adverse effects by affecting organelles, and their toxic effects are closely linked to their physicochemical properties.[10] Internalized nanoparticles, such as AuNPs and AgNPs, have been found to induce changes in lysosomes affecting their interactions with mitochondria. It has been reported that internalized AuNPs located in lysosomes may lead

Table 2. Methods applied in studying MCSs and main discoveries.

Methods	Techniques	Application field	Main breakthroughs
Biochemical approaches	Cell fraction, Co-immunoprecipitation, mass spectrometry, affinity chromatography, BioID, APEX, Split-APEX	Identification of tethers and molecules involved	Mapping of the proteome at mito-related MCS[135]
Microscopy-related techniques	TEM, SEM, FIB-SEM, ET, cryo-ET, SRM, confocal microscopy, TIRFM	Morphological characterization, Dynamic imaging at super-resolution	Mitochondrial fission regulated by ER-PM MCS,[136] 3D ultrastructure reconstruction of ER-related MCS,[119] Organelles interactome[137]
Proximity-driven fluorescent probes	FRET, BiFC, PLA, ddFP, BRET	Intracellular localization and functional characterization	Mapping of contact sites,[138] Quantification of ER-mito MCS,[139] Identification of Mfn2 as ER-mito MCS tether[107]

Table 3. Suggested biological effects of NMs on specific MCSs.

Nanomaterials	Contact sites	Biological effects	Refs.
AuNP	Lysosome–mitochondria	Mitochondrial fission	[142]
AgNP	ER–mitochondria	Mitochondrial Ca^{2+} alteration, apoptosis, ER stress	[143]
PtNP	Lysosome–mitochondria	Protection from oxidative stress damage	[11]
CBNP	Lysosome–mitochondria	Increased lysosomal membrane permeability and rupture, mitochondrial damage, autophagy inhibition, ROS imbalance, and decreased mitochondrial membrane potential.	[10]

to lysosomal swelling and altered motility. This is achieved by increasing "kiss-and-run" events mediated by dynamin-related protein 1 (DRP1), which enhances mitochondrial fission.[142] On the other hand, the interaction between silver nanoparticles, mitochondria, lysosomes, and ER can lead to complex cellular responses. For instance, only endocytosed AgNPs appear to directly determine the cell fate leading to mitochondrial swelling and vacuolation without direct interaction. Li et al. reported the apoptotic effects of AgNPs on human neuroblastoma cells by the induction of ER stress and perturbation of Ca^{2+} homeostasis. It has also been observed that the increase in MCSs and the alteration of the inositol trisphosphate receptor (IP_3R) function leads to an increase in calcium transfer from the ER to the mitochondria, ultimately triggering the mitochondrial apoptotic pathway.[143] Interestingly, the restoration/upturn of mitochondrial homeostasis and energy metabolism disrupted by AgNPs can be achieved by reacidifying lysosomes through cyclic adenosine monophosphate (cAMP) and rescuing the lysosomal autophagy degradation process.[144] Furthermore, experimental evidence indicates that nanoparticle exposure can cause lysosomal damage and affect lysosome–mitochondria interactions, leading to disruptions in autophagy and mitochondrial homeostasis through mitochondrial ROS.[145] In addition, several other studies report the effects of black carbon nanoparticles in mediating the dysfunction of lysosome–mitochondria interactions through membrane-associated proteins, inducing several organelle perturbations such as increased lysosomal membrane permeability and rupture, mitochondrial damage, autophagy inhibition, ROS imbalance, and decreased mitochondrial membrane potential.[10]

In recent work, the formation of MCSs as mediators of the antioxidant mechanism of action of PtNPs has been suggested.[11] In particular, the protective effect of 5 nm PtNPs was studied on a human hepatic (HepG2) cell line exposed to dichlorodiphenylethylene (DDE), an environmental pollutant, known to cause oxidative stress and mitochondrial impairment. Experimental data indicated that PtNPs decreased cell damage induced by DDE in HepG2 cells very efficiently and to an extent depending on the DDE dose, despite their confinement in the endo-lysosomal compartment. In particular, PtNPs increased the expression of the mitochondrial enzyme SOD2 which is involved in oxidative stress recovery in the cells. PtNPs were also able to hinder the imbalance of mitochondrial dynamics induced by DDE. More precisely, PtNPs maintained mitochondrial morphology constant and regulated mitochondrial dynamics in the presence of DDE. Remarkably, in HepG2 cells treated with PtNPs, alone or in combination with DDE, mitochondria formed MCSs with rough ER and endo-lysosomes containing nanoparticles. After incubation with PtNPs, an increase in mitofusin 2 (Mfn2) protein expression was observed. Mfn2 is a marker protein of mitochondria fusion, and it is involved in the formation of inter-organelle contact sites. TEM analysis revealed a direct interaction between PtNPs containing endo-lysosomes and mitochondria suggesting that the formation of inter-organelle contact sites could actively regulate the mechanisms of action of PtNPs within the cell. These findings demonstrated that inter-organelle crosstalk mediates the protective capability of PtNPs through their intrinsic antioxidant properties and the modulation of mitochondrial functionality.

PtNPs are mainly located in the endo-lysosomal compartment, and so the mechanism of action at the mitochondrial level is not obvious. Inside the cell, the lysosomes are deputy to ensure the optimal physicochemical environment for enzymatic activities along with the degradation and recycling of defective cellular material through autophagy and extracellular material reaching lysosomes by endocytosis or phagocytosis.[146] The stability of the lysosomal membrane is strictly related to the cell survival–death signaling, while the efficient lysosomal enzymatic activity depends on its composition.[147,148] The presence of ROS can compromise the lysosomal integrity due to membrane lipid peroxidation and alkalinization events (i.e., proton leakage) increasing the luminal pH, which leads to many pathologies such as the TRPML1-mediated calcium dyshomeostasis and autophagy defects.[149–151] Cellular homeostasis and the response to stress conditions are subtly mediated by lysosomes–mitochondria contact sites that act as critical communication hubs and their coordination complexes. The mutual interaction engaged at proximity regions contributes to the organelles' function and integrity such as the regulation of mitophagy, lysosomal biogenesis, Ca^{2+} homeostasis, and mitochondrial division.[115,129,152] In this regard, we hypothesize that in the presence of oxidative stress, membrane integrity and pH of lysosomes are preserved by PtNPs due to their intrinsic ROS scavenging activity. In addition to the direct catalytic effects of PtNPs on ROS, secondary mechanisms could contribute to a broad anti-inflammatory action.[153] One intriguing possibility might involve Ca^{2+} release from the lysosomes.[115] In particular, Ca^{2+} is crucial for mitochondrial destabilization leading to the activation of NLRP3 inflammasome and the activation of T-lymphocyte-mediated pathogenic inflammation.[154] In mitochondria–lysosome contact sites, the close localization of calcium channels is important to allow a fast and efficient Ca^{2+}-dependent protein cascade.[131] By reducing the ROS stimulus, PtNPs could contribute to decreasing Ca^{2+} release adding further regulation to the respiratory burst and to the signaling leading to calcium-dependent inflammatory pathways, protecting mitochondria from oxidative stress injury (**Figure 4**).

In addition, another interesting mechanism of mitochondria protection by PtNPs concerns their ability to act indirectly on

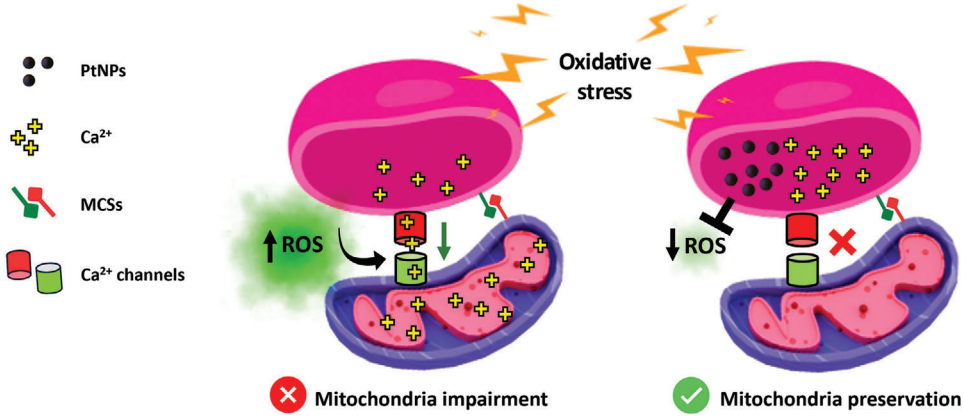

Figure 4. Possible mechanism of action of PtNPs in regulating mitochondrion activity through mito–lyso MCSs. In oxidative stress conditions (left panel), the increment of ROS alters the pH and membrane integrity of the lysosomes leading to an increase in Ca^{2+} efflux from these organelles to the mitochondria, favored by the proximity of the Ca^{2+} channels mediated by mito–lyso MCSs. Calcium dyshomeostasis leads to the impairment of mitochondrial functions. The presence of PtNPs in the lysosomes (right panel) reduces the production of ROS thus contributing to decrease Ca^{2+} release to the mitochondria and protecting them from oxidative stress damage.

ROS levels by stimulating the production of SOD2, an antioxidant enzyme that catalyzes the conversion of O_2^- into H_2O_2 helping to maintain the redox balance.[11] Indeed, defects in SOD2 expression have been associated with liver damage, while overexpression of SOD2 helps protecting mitochondria and the cell from oxidative damage.[155] It has been widely reported in the literature that SOD2 activity is mediated by inflammatory signals and the transcription factor NF-kB pathway plays a key role in upregulating its expression.[156–158] It might be that PtNPs can regulate inflammatory signals that induce increased SOD2 expression (**Figure 5**). As a proof of concept, a recent study reported the statistically significant alteration of 60 genes in THP-1 monocytes exposed to PtNPs through RNA microarray technology, despite PtNPs demonstrated antioxidant activity by reducing ROS levels without significant effect on macrophage viability and immune response.[159] In particular, some of the downregulated genes, such as tlr1, birc3, vstm1, or upregulated such as mtn2, have been correlated with the regulation of inflammatory pathways and ROS by the transcription factor NF-kB signaling pathway.[160–164]

These findings suggest that PtNPs may act via a more complex cascade mechanism and further studies on the regulation of gene expression are needed to elucidate their intriguing properties.

Overall, the understanding of MCSs and their role in facilitating organelle interactions is crucial to disclose the mechanisms of organelle impairment and interactions in the toxicological impact of nanoparticles.

5. Conclusion

Nanomaterials have emerged as versatile tools in the study and manipulation of cellular processes. The key challenge for the development of functional NMs with high therapeutic potential and low adverse effects is the understanding of the biological mechanisms underlying the crosstalk between NMs and cellular organelles and their consequent exploitation to control/modulate organelle functions, including the formation of organelle MCSs. These interfaces, crucial for cellular function, govern various physiological processes, such as lipid metabolism, calcium signaling, and apoptosis. Moreover, it has been recently reported that dysregulation of MCSs is correlated with several diseases. Leveraging on nanotechnology tools, researchers may develop innovative approaches to probe and modulate organelle contact sites with unprecedented precision and specificity. Among the inter-organelle MCSs, lysosome-mediated contacts are of particular interest to understand the fate of NMs. Indeed, cellular internalization of most NMs relies on endocytosis process, ending in endo-lysosome vesicles. Lysosome–mitochondrion MCSs may be one of the key regulatory mechanisms to be targeted in order to exploit some NMs' functional features. NMs like PtNPs below 5 nm in diameter have demonstrated the capability to act in a "positive way" on the formation of MCSs, allowing the protection of cells by oxidative stress conditions/insults by the synergic combination of intrinsic catalytic properties and the modulation of mitochondria activities. NM stability is also very

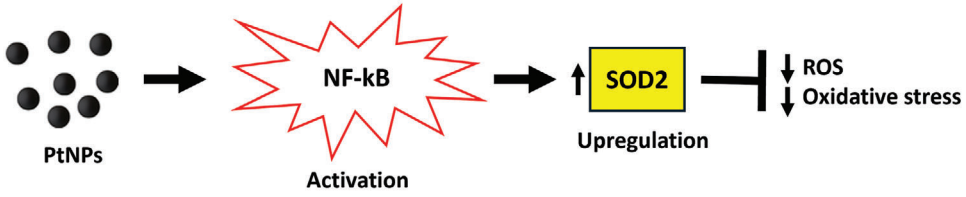

Figure 5. Possible mechanism of action of PtNPs on upregulation of SOD2 enzyme involving the activation of NF-kB signaling pathway.

important to perform studies or develop applications for intracellular vesicles. AgNPs, for example, induce mitochondrial swelling and vacuolation by releasing toxic Ag^+ ions impairing precise investigation of NM interaction with the organelles. Anyway, engineered nanomaterials, ranging from quantum dots to lipid-based nanoparticles, offer unique advantages such as tunable size, surface chemistry, and multifunctionality enabling targeted delivery of probes and effectors to specific organelles. Such nanomaterial-based platforms may facilitate real-time imaging and manipulation of organelle dynamics, shedding light on the intricate molecular mechanisms underlying cellular functions. In principle, different surface chemistries of NMs could allow precise localization in different vesicles, nevertheless, the biomolecular hard corona acquired in the medium represents a potential limitation for some applications.

Nanotechnology-enabled strategies hold promises for therapeutic interventions offering potential avenues for the treatment of diseases associated with aberrant organelle contacts. However, only a few studies are reported to date in this research field representing a limitation. In this direction, it is mandatory to increase the knowledge about the crosstalk between NMs and MCSs. A future challenge will be better clarifying how chemical–physical and/or intrinsic catalytic properties of NMs modulate organelle functions in a controlled manner. The outcomes will allow the development of general conclusions about the behavior of several NM types. A further challenge will be to identify the molecular targets involved in MCS signaling pathways to engineer functional NMs able to specifically influence these intracellular events. Overall, the marriage of nanomaterials and organelle contact site research presents exciting opportunities to deepen our understanding of cellular biology and to develop novel therapeutic strategies.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

functional nanomaterials, inter-organelle contact sites, nano-biointeractions, nanomaterial-mediated cell signaling, organelle targeting

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