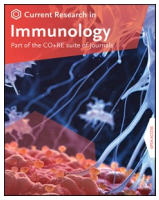
Current Research in Immunology 4 (2023) 100073

Contents lists available at ScienceDirect

Current Research in Immunology

journal homepage: www.sciencedirect.com/journal/current-research-in-immunology



Nanoparticles cellular uptake, trafficking, activation, toxicity and *in vitro* evaluation

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A B S T R A C T

Nanoparticles (NPs) physicochemical properties, such as size, shape, surface chemistry, charge, etc., play a critical role in biological systems interactions, which include NPs' cellular uptake, trafficking, activation, and toxicity. Although nano-bio interactions are multifaceted and complex, their assessment is essential for future therapeutic and diagnostic use since being carriers that deliver specific molecules (i.e., active pharmaceutical ingredients and imaging agents) in intracellular sites. The journey of NPs begins by reaching the plasma membrane and entering the cell mainly through endocytosis. After vesicles pinch off the cell membrane, the intracellular trafficking is mediated by a network of cellular endosomes which direct NPs to the different cellular components. Otherwise, NPs or their contents are released into the cytoplasm. In both cases, NPs can pass undetected or be recognized by the cell leading to a pro or anti-inflammatory response. Indeed, the cell response mostly depends on cell type and NPs physicochemical properties. The principal mechanism by which NPs activate the cell response is RONS production. Other mechanism includes signaling pathways modulation related to metabolic and enzymatic reactions, cell transduction, and immune modulation. Hence, the underlying mechanisms of cellular and subcellular interactions *in vitro* should be performed to provide insights into NPs' effect. This information helps us to improve their synthesis and design to maximize the clinical benefits while minimizing side effects. Most *in vitro* tests to evaluate NPs' effect in cells were developed focusing on cell dysfunctions, cytotoxicity, genotoxicity, immunogenicity, and cell death.

1. Introduction

Researchers have focused on the interaction of nanoparticles (NPs) with biological systems as it provides insights into how NPs modify downstream cell signaling pathways. NPs drive a desired biological response or enhance cellular uptake and trafficking to deliver therapeutic and diagnostic payloads into the cell. NPs-cell interaction *in vitro* is essential to provide not only preliminary results of how NPs affect cells but also to understand the underlying mechanisms behind it. In this graphical review, we described four distinct aspects of NPs-cell interactions: NPs cellular uptake, NPs intracellular trafficking, cell response to NPs, and the *in vitro* analysis used to evaluate cell response (see Figs. 1–4).

2. Cellular uptake of nanoparticles

Cellular uptake of NPs involves highly regulated mechanisms that are classified into endocytosis-based uptake pathways and direct cellular entry of NPs.

Endocytosis is a multi-step process that includes: particular ligands binding to the cell surface receptors forming a ligand-receptor complex, nucleation of cytosolic proteins involved to form a coated pit; plasma membrane invagination; scission of invagination to form an intracellular vesicle; and uncoating and recovery of the endocytotic proteins from the vesicle (Sabourian et al., 2020), (Donahue et al., 2019).

Endocytosis includes: (a) Clathrin-dependent is initiated by binding NPs (or their ligands) to cell membrane receptors (e.g., transferrin receptors, low-density lipoprotein receptors, epidermal growth factor receptors, and β2 adrenergic receptors). The cargo within early endosomes formed by this endocytic pathway will eventually reach lysosomes. (b) Caveolin-dependent can bypass lysosomes, protecting the contents from hydrolytic enzymes and lysosomal degradation. (c) Clathrin- and caveolin-independent takes place in cells devoid of both receptors. This pathway includes Fast endophilin, CLIC/GEEC, Arf6, and RhoA-mediated endocytosis. All the ways mentioned above end up in an early endosome. (d) Phagocytosis is exercised by professional phagocytes (i.e., macrophages, monocytes, dendritic cells, neutrophils, and B lymphocytes). Clearance of NPs is mediated by opsonization and adsorption of immunoglobulins (i.e., antibodies), complement proteins, and other serum proteins (e.g., laminin and fibronectin) onto NPs'

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https://doi.org/10.1016/j.crimmu.2023.100073

Received 18 April 2023; Received in revised form 12 October 2023; Accepted 15 October 2023

Available online 9 November 2023

surface. These complexes are recognized by Fc, mannose/fructose, scavenger, and complement receptors, which initiate a signaling cascade, actin assembly, cell surface extensions, engulfing, and internalization (Behzadi et al., 2017). (e) Macropinocytosis is a non-specific cellular uptake mechanism characterized by the engulfment of extracellular fluids and solutes through actin-stabilized plasma membrane extensions. All endocytic uptake pathways are highly regulated and mediated by different types of lipids and transport proteins, which end up confined within intracellular vesicles, such as endosomes, phagosomes, or macropinosomes. (Donahue et al., 2019), (Foroozandeh et al., 2018), (Oh, 2014).

Direct cellular entry means that NPs can cross the cell plasma membrane by biochemical or physical means and includes: (i) Direct translocation in which NPs disrupt the cell plasma membrane and enter the cell, avoiding endosomal entrapment and energy-dependent transport. (ii) Lipid fusion, in which lipid bilayer-coated NPs fuse with the cell membrane, and the cargo is delivered directly to the cytoplasm. (iii) Electroporation is the pore formation by the electrical pulses through which NPs can be internalized. (iv) Microinjection is injecting NPs into the cytoplasm using specialized microinjectors (Donahue et al., 2019).

3. Intracellular trafficking of nanoparticles

After successful cellular entry, NPs overcome intracellular barriers to reach cellular compartments and organelles. Motor proteins and cytoskeletal structures transport NPs throughout the cell in complex trafficking patterns to various intracellular destinations. After cellular internalization, NPs are confined within a membrane-lined vesicle called an early endosome, which can undergo an aging process. Early endosomes (pH 6.5) fuse with vesicles and ferry the cargo to the desired cellular destination (i.e., cytosol, nucleus, mitochondria, Golgi apparatus, and endoplasmic reticulum). Nevertheless, part of the cargo (proteins and lipids) is recycled back to the plasma membrane in a recycling endosome (Behzadi et al., 2017). The other part is sorted into intraluminal vesicles (ILVs) giving rise to multivesicular endosomes (MVB), referred to as late endosomes (pH 5.5), by a maturation and differentiation process. Then, the cargo will be directed to one of the following possible fates: (i) specific cell organelles, (ii) integration with lysosomes (~pH 5) to form endolysosomal vesicles (pH 4.5) whose hydrolytic enzymes (proteases, lipases, phosphatases, nucleases, etc.) degrade trapped NPs, (iii) secretion in exosomes, or (iv) recycling to the plasma membrane via back-fusion (Behzadi et al., 2017), (Foroozandeh et al., 2018), (Sohrabi et al., 2021). Although the endosomal network is the most common intracellular trafficking pathway of NPs metabolization, some can escape and be released into the cytoplasm via membrane fusion, membrane destabilization, particle swelling, and osmotic rupture to reach the cytosol (Behzadi et al., 2017).

4. Cell immune response to nanoparticles

The induction of cytotoxicity by NPs is closely related not only by their physicochemical properties but also by the entry pathway,

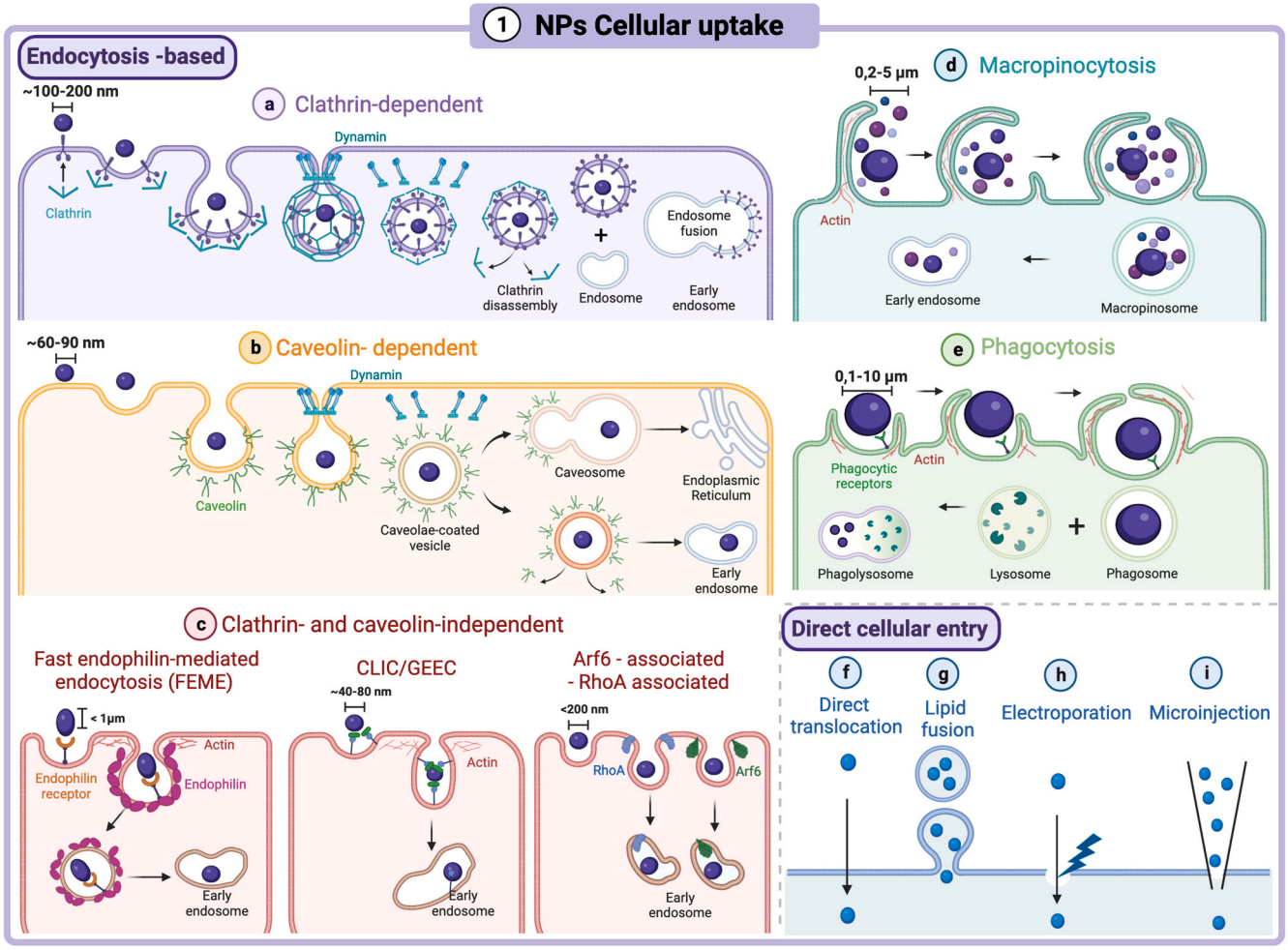

Fig. 1. Schematic representation showing the mechanisms of nanoparticle cellular internalization divided into endocytosis–based mechanism (left), which includes (a) clathrin-dependent; (b) caveolin-dependent; (c) clathrin- and caveolin-independent; (d) phagocytosis; and (e) micropinocytosis; and direct cellular entry (right), which includes (f) direct translocation, (g) lipid fusion, (h) electroporation, and (i) microinjection.

intracellular localization, cell type, and cell microenvironment (Wang et al., 2021). Briefly, NPs can pass undetected or be recognized by the immune system. Once detected, they can be tolerated or induce defensive (inflammatory) or anti-inflammatory (healing) responses. In the first scenario, undetected NPs reach their target when they escape cellular receptors or when they are not opsonized by complement molecules or antibodies. To avoid cell recognition, polymers, such as albumin, polyethylene glycol (PEG), retinol, CD47, and erythrocyte membrane fragments, camouflage the surface of the NPs. The second scenario is when NPs are detected and tolerated, which means NPs are silently removed without inducing inflammation. Small sizes, hydrophilicity, and negatively charged surfaces often result in tolerable NPs. The third scenario, the most common, is when NPs are detected and not tolerated, which affect cellular pathways function that lead to cell dysfunctions. Some factors promote a non-tolerable response, such as non-biocompatible size or shape, homo or hetero aggregation excess, chemical transformations, corrosion, and ion or soluble compounds released by NPs (Ernst et al., 2021).

The main toxicity mechanism of NPs is reactive oxygen species (ROS) and nitrogen species (RNS) production (Ray et al., 2021), (Mohammadinejad et al., 2019). ROS and RNS lead to oxidative and nitrosative stress that induces DNA, lipid, and protein damage. Other mechanisms include but are not limited to, impaired calcium homeostasis, perturbed mitochondrial activity, loss of cell membrane integrity, protein interactions disruption, unfolded proteins, ER stress, and genotoxicity (Mohammadinejad et al., 2019), (Kumar et al., 2017).

Moreover, NPs modulate signal transduction pathways (metabolic and enzymatic reactions) since activate pattern recognition receptors (PRRs) which include Toll-like receptors (TLRs), Rig-like receptors (RLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), mannose-binding ligands (MBLs), ficolins and DNA/RNA sensors. PRRs recognize pathogen-associated molecular patterns (PAMPs) of NPs, or endogenous stress signals, termed danger associated molecular patterns (DAMPs) caused by NPs (Perciani et al., 2021), (Kotsias et al., 2019). It leads to cytokines cascade production and costimulatory molecules expression (Kumar et al., 2017), (Natarajan et al., 2020).

NPs can also modulate immune response via cell recruitment and cell activation. First, the complement system is a non-specific defense that favors NPs opsination. Then, NPs are recognized and eliminated by phagocytes (e.g. macrophages, neutrophils, and dendritic cells) and leukocytes (e.g. natural killer cells, mast cells, eosinophils, and basophils) (Liu et al., 2022). Once detected, NPs activate not only innate but also adaptive immune responses through antigen presentation. Intracellular antigens are degraded by the proteasome and are loaded onto MHC class I molecules in the ER to be presented on the cell surface to $CD8^{+}$ T cytotoxic cells. Alternately, extracellular antigens are internalized and fused with autophagosomes where they are degraded and loaded onto MHC class II molecules to be presented to $CD4^{+}$ T helper cells (Th) (Kotsias et al., 2019), (Liu et al., 2022). Th1 cells create a pro-inflammatory cell-mediated immunity. Th2 cells are responsible for an anti-inflammatory response and promote antibody production by B lymphocytes to induce humoral immunity. Th17 cells are involved in

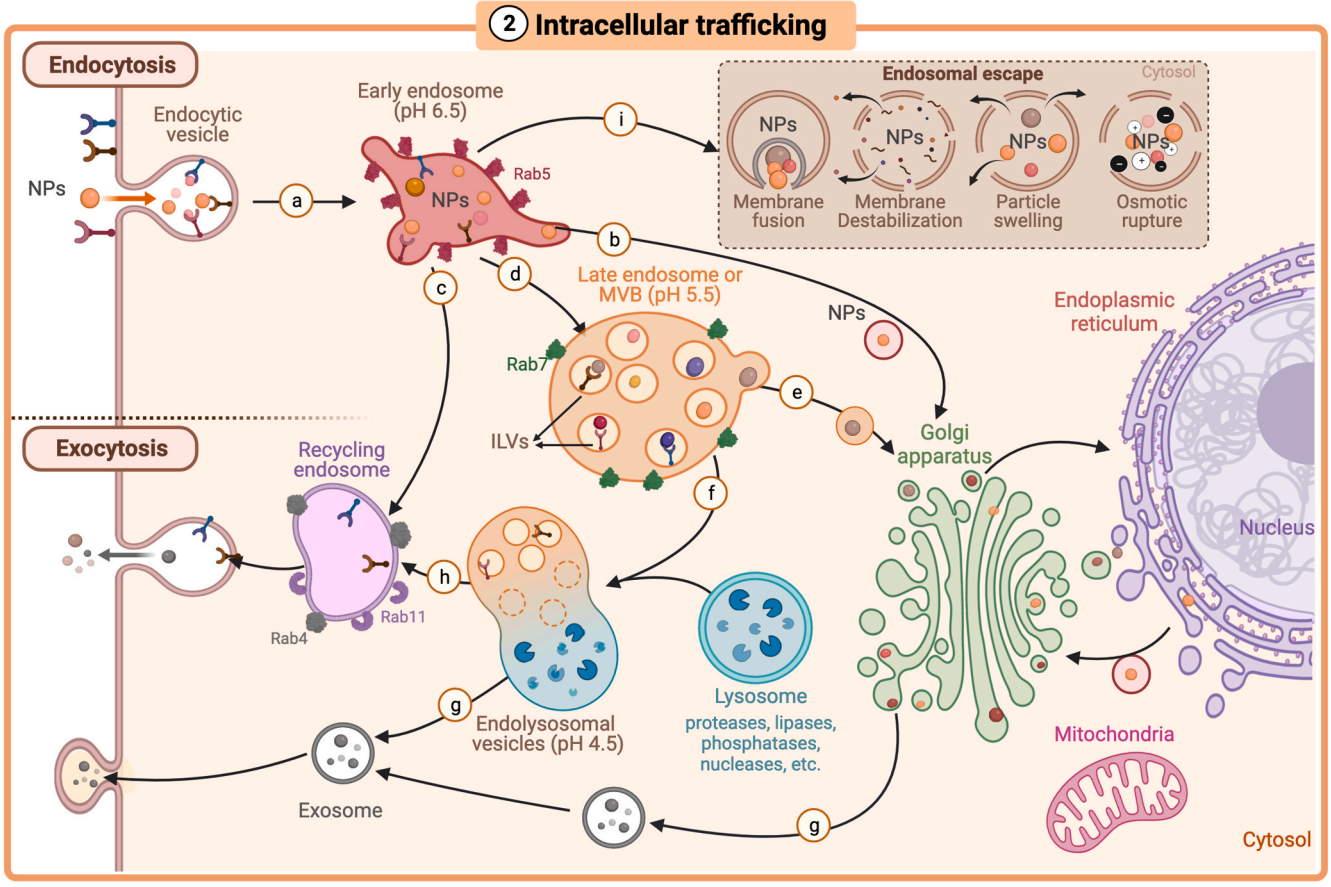

Fig. 2. Schematic representation showing nanoparticle intracellular trafficking mediated by a network of cellular endosomes. (a) After cellular internalization, nanoparticles are confined in early endosomes. (b) They fuse with vesicles and ferry the cargo to the desired cellular destination (i.e. cytosol, nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum). (c) Part of the cargo is recycled back to the plasma membrane in a recycling endosome. (d) The other part is sorted into intraluminal vesicles (ILVs) giving rise to the late endosome. Then, the cargo will be directed to one of the following possible fates: (e) specific cell organelles, (f) form endolysosomal vesicles whose hydrolytic enzymes degrade trapped NPs, (g) secretion in exosomes, or (h) recycling to the plasma. (i) Some NPs can escape from the endosome and be released into the cytoplasm via membrane fusion, membrane destabilization, particle swelling and osmotic rupture to reach the cytosol.

autoimmune response and cell-mediated inflammation, whereas Treg cells are in charge of immuno-regulation (Liu et al., 2022).

NPs also lead to cell death through non-programmed (necrosis), programmed-apoptotic (apoptosis and anoikis), and programmed non-apoptotic cell death via vacuole formation (autophagy, entosis, methuosis, and paraptosis), mitochondria damage (mitoptosis and parthanathos), immune activation (pyroptosis and NETosis) and other (ferroptosis, necroptosis) (Mohammadinejad et al., 2019), (Yan et al., 2020), (Wang et al., 2022).

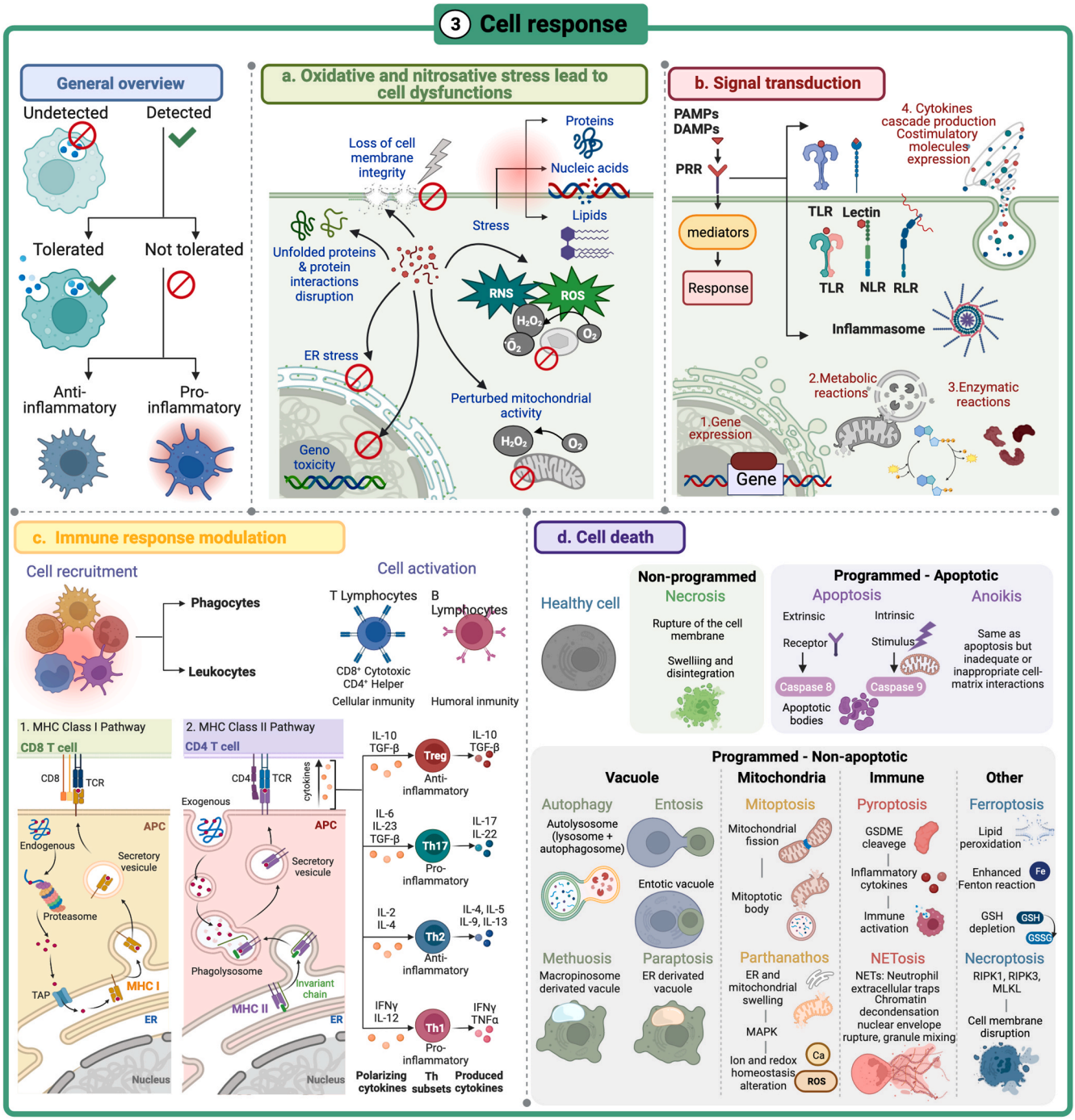

Fig. 3. It describes the cell response after nanoparticle exposure. In the big picture (left), NPs can pass undetected or be recognized by the cell leading to a pro or anti-inflammatory response. Once detected, NPs can modulate immune response via cell recruitment, cytokines cascade production, costimulatory molecule expression, and cell activation. At the intracellular level, NPs can cause: (a) Oxidative and nitrosative stress, which leads to cell dysfunctions and genotoxicity. (b) Signal transduction that leads to metabolic and enzymatic reactions, gene expression, and cytokine cascade production. (c) Immunomodulation through cell recruitment and cell activation via MHC-I and II antigen processing and presentation pathway. 1) Intracellular antigens are degraded by the proteasome and are loaded onto MHC class I molecules to be presented to $CD8^+$ T cytotoxic cells. 2) Extracellular antigens are internalized in a vesicle and fused with autophagosomes, where they are degraded and loaded onto MHC class II molecules to be presented to $CD4^+$ T helper cells. (d) Cell death mechanisms include non-programmed (necrosis), programmed-apoptotic (apoptosis and anoikis), and programmed nonapoptotic cell death via vacuole formation (autophagy, entosis, methuosis, and paraptosis), mitochondria damage (mitoptosis and parthanathos), immune activation (pyroptosis and NETosis) and other (ferroptosis, necroptosis).

5. *In vitro* analysis

In vitro tests are suited for high-throughput screening because they provide insights into the initial effects on target cells, the primary mechanisms of toxicity, and the scope for design improvements at relatively lower cost and in reduced time. In addition, all NPs' physicochemical properties are crucial design criteria that researchers need to consider and analyze *in vitro* when engineering NPs for future applications such as Tissue engineering, two-dimensional (2D) and three-dimensional (3D) scaffolds since deliberately designed NPs may change upon exposure to a biological environment. (Wang et al., 2019).

The first step for *in vitro* NPs-cell interaction is the evaluation of viability and cytotoxicity by tetrazolium-based assays (MTT/MTS/XTT) and monosodium salts (Alamar blue, CCK-8 and WST-1) assays (Kumar et al., 2017), (Chen et al., 2018). A second approach is the evaluation of cell metabolism and oxidative and nitrosative stress. The following molecules are often tested: ROS, superoxide dismutase (SOD), glutathione enzymes (transferase GST, reductase GSH, and peroxidase GPx), thioredoxin (peroxidase and reductase), lipid peroxidation (LPO), Vitamin E, catalase (CAT), nitric oxide (NO), Dichlorodihydrofluorescein (DCF), Dihydroethidium (DHE), Dichlorodihydrofluorescein Diacetate (DCFDA), and NADPH (Chen et al., 2018)– (Singh, 2019).

Another subject of analysis is cell death (Mohammadinejad et al., 2019). For example, autophagy is performed by Dansylcadaverine. Apoptosis is assessed by Caspase-3 and caspase-9 activity, Annexin V and Propidium Iodide (PI), and Acridine orange and Ethidium bromide (AO/EtBr) staining. Necrosis is carried out by dye uptakes such as Neutral Red and Trypan Blue, which also evaluate lysosome homeostasis and membrane permeability, respectively (Kumar et al., 2017), (Arora et al., 2012), (Bozzuto et al., 2021). Genotoxicity is evaluated using comet assay, chromosomal aberration assay, micronucleus (MN), TUNEL assays, and 8-Hydroxy-2′-deoxyguanosine (8-OHdG) quantification (Kumar et al., 2017). Other assays evaluate specific cellular sites like the membrane integrity by lactate dehydrogenase (LDH) or Calcein acetoxymethyl (calcein AM)/ethidium homodimer, and mitochondrial activity by mitochondrial membrane potential (Wm) or MitoSOX assay. Cellular morphology by microscopy also could provide insights into toxicity since NPs may interact with cytoskeletal proteins and change cell organization(Wang et al., 2019).

Many other tests evaluate pro or anti-inflammatory response by the expression of molecules, interferons, interleukins, growth factors, and genes related to cell metabolism. The most common tests evaluate oxidative and nitrosative stress measuring superoxide, peroxide, and oxidoreductase molecules. A second approach is measure of inflammation molecules such as interleukin (IL)-1β, IL-6, IL-8, IL-18 tumor necrosis factor-alpha (TNF-α), Nuclear Factor-kβ (NF-κB), cyclooxygenase-2 (COX-2) (Ong y and Nyam, 2022). They can also evaluate apoptosis and cell cycle moelcules such as heme oxygenase-1 (HO-1), thioredoxin reductase 1 (TXNRD1), C-X-C motif ligand 2 (Cxcl2), monocyte chemoattractant protein-1 (MCP-1/CCL2), and more (Natarajan et al., 2020), (Horie et al., 2012). In addition, there are test that evaluate cell receptors activated by NPs, such as ficolins, MBLs, TLRs, RLRs, NLRs, and CLRs and downstream cascade to characterize NPs entry pathway (Perciani et al., 2021).

All the cell markers and tests above-mentioned could be determinate via microscopy (inverted, confocal, fluorescence), q-RT-PCR, ELISA, flow cytometry, Western Blot, or immunolabeling (Chen et al., 2018), (Horie et al., 2012). One big approach is Multi-OMICS which include genomics, transcriptomics, proteomics, metabolomics, etc., in conjunction with bioinformatics analysis.

Finally, bio-mechanical characterizations could be performed by microscopy such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), Atomic Force Microscopy (AFM), mass spectroscopy, UV–Visible and fluorescent spectroscopy, nanoparticle tracking analysis, microfluidic techniques and others (Behzadi et al., 2017), (Sohrabi et al., 2021), (Arora et al., 2012). Furthermore, this approach has proven beneficial for estimating the relative distribution of NPs inside specific compartments and to following intracellular trafficking.

6. Conclusions

In vitro study is critical for researchers to understand and explore NPs' cellular interactions, cellular uptake, intracellular trafficking pathways, cell activation, and corresponding toxicity to ensure targeted delivery systems. The immunological and molecular test developed for

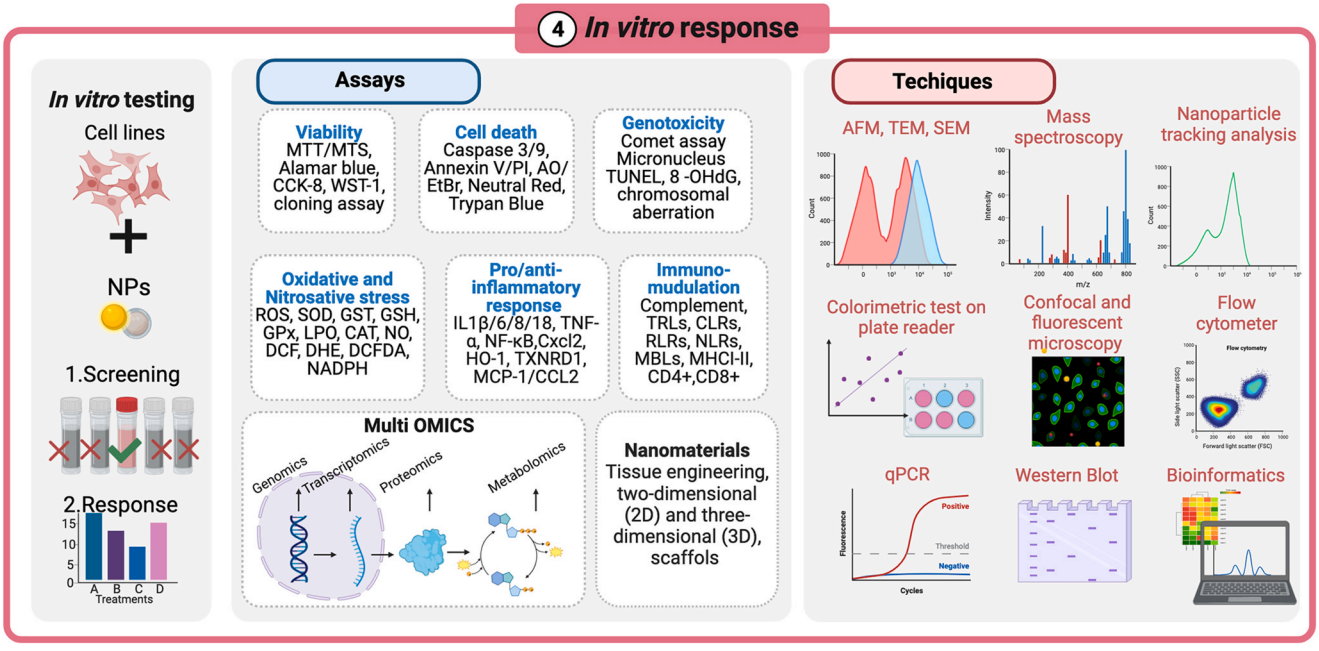

Fig. 4. Schematic representation of *in vitro* test after nanoparticle exposure. *In vitro* tests are suited for high-throughput screening and provide insights into the initial response of target cells (left). Techniques available to determine viability, cell death, genotoxicity, oxidative and nitrosative stress, pro or anti-inflammatory response, and immunomodulation. In addition, the figure shows equipment and platforms available for *in vitro* study (right).

in vitro applications provides preliminary information about cell dysfunctions, oxidative and nitrosative stress, cytotoxicity, genotoxicity, cell death, immunogenicity, immunomodulation, signal transduction, and others. The aim of the evaluation *in vitro* is crucial to achieve a balance between efficacy and toxicity not only to enhance NPs properties but also to avoid triggering undesirable immunological responses.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marbel Torres reports was provided by Universidad Fuerzas Armadas ESPE. Marbel Torres reports a relationship with Professor that includes: employment. I have no conflict of interest.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crimmu.2023.100073.

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