FLOW CYTOMETRY IN NANOTOXICOLOGY: A BRIEF OVERVIEW

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Abstract

The paper deals with the role of flow cytometry in assessing the biocompatibility and safety profiles of nanomaterials. Flow cytometry is a powerful tool to characterize the impact of various exogenous factors on different cell populations due to its ability to register optical and fluorescence characteristics of cells analyzing multiple parameters simultaneously. An overview of flow cytometry application for evaluating the redox state of cells, viability and cell death modes (apoptosis, necrosis, necroptosis, pyroptosis, autophagy), and pro-inflammatory effects of nanoparticles is provided. Flow cytometry offers rapid, informative, quite cost-effective and multi-angled analysis of safety profiles of nanomaterials taking into account the key mechanisms of their toxic action. Recent advances in flow cytometry technologies and the availability of commercial automated cell counters make flow cytometry a convenient research tool for in vitro nanotoxicology. However, the field requires the development of standardized flow cytometry protocols for nanotoxicity testing.

Keywords: *nanomaterials, nanoparticles, cytotoxicity, cell death, reactive oxygen species*.

Introduction

Nanomedicine is a rapidly growing field of medicine, which implies the application of nanotechnologies for medical purposes. In general, nanomaterials are defined as materials that have at least one dimension ranging from 1 to 100 nm [1]. Nano-sized materials possess unique physicochemical characteristics compared to the large-sized substances of the same composition due to quantum effects, higher surface area, which increases the surface-to-mass ratio, and higher reactivity [2]. These size-dependent effects of nanostructured materials make them promising agents in medicine. Over the recent years, a plethora of applications have been suggested for nanomaterials. In particular, nanomaterials are used as diagnostic and therapeutic agents [3-7], antibacterial agents [8, 9], drug delivery tools [10, 11], photodynamic and photothermal agents for the treatment of neoplasms [12], contrast agents for magnetic resonance imaging [13], gene delivery agents [14], wound healing nanodrugs [15, 16], etc. However, the field faces significant obstacles and challenges that have to be overcome to successfully translate the results of experimental research into clinical practice. The major issues that limit the progress of nanomedicine are targeted delivery, poor biocompatibility and safety of nanomaterials, pollution of environment with nanostructured materials, lack of cost-effectiveness and full-scale industrial production, and imperfect governmental regulations [17, 18].

Toxicity remains one of the major concerns and severe challenges to nanomedicine. It has been reported that toxicity of engineered nanomaterials is dependent on multiple factors, including composition, size, which affects the surface area, shape, surface chemistry and charge, dose, protein corona, exposure routes, environmental factors, etc. [19, 20]. Hazardous effects of nanomaterials are mediated via multiple mechanisms. However, it has been revealed that reactive oxygen species (ROS) generation and oxidative stress are key factors of their toxicity [20-22]. It is important to note that ROS generation is usually proportional to the surface-to-volume ratio, which is associated with a higher reactivity of nanostructured materials [23]. In turn, excessive ROS formation causes oxidative damage to phospholipids, promoting lipid peroxidation, DNA

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molecules, resulting in genotoxic and carcinogenic effects of nanomaterials, and proteins. Nanomaterials-induced ROS overgeneration can be indirect and mediated via NADPH oxidase-dependent or mitochondrial mechanisms [24, 25]. In addition to direct ROS-mediated damage to macromolecules, nanomaterials-induced oxidative stress triggers apoptosis, necrosis, necroptosis, autophagy, pyroptosis, mutations, inflammation, fibrosis, and cancer [24, 26].

ROS overproduction mediated by nanomaterials can trigger mitogen-activated protein kinase (MAPK) and the c-Jun-N-terminal kinase (JNK) signaling, initiating apoptosis [27]. Moreover, there is accumulating evidence that nanoparticles can enhance apoptosis not only via intrinsic, but also extrinsic pathways, in particular, through FAS-mediated mechanisms [28]. Both pathways result in activation of caspases.

Oxidative stress-mediated pathway has been stated to be a key mechanism of nanoparticles-induced necrosis and necroptosis [29]. The latter is referred to as a regulated form of necrosis. Both necrosis and necroptosis lead to similar morphological changes, rupture of cell membranes and release of strongly pro-inflammatory damage-associated molecular patterns (DAMPs) [30].

Nanomaterials have been demonstrated to induce autophagy [31], which is a cellular degradation process crucial for the maintenance of homeostasis in response to nutritional and metabolic dysregulation [32]. Changes in the redox status induced by nanostructured materials inhibit the PI3K/Akt/mTOR signaling pathway, which results in activation of autophagy [33]. The feature of nanoparticles to affect authophagy makes them a promising anticancer therapeutic agents, given the role of autophagic cell death in cancer.

Another cell death mode regulated by nanomaterials is pyroptosis, which is a strongly pro-inflammatory form of caspase-1-dependent cell death of mainly macrophages associated with pore-mediated leakage of pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 through the cell membrane with the subsequent influx of ions and cell lysis [34, 35]. Nanomaterials have been shown to induce pyroptosis [36, 37]. Increasing evidence demonstrates that NLRP3 inflammasome, which plays a key role in pyroptosis, responds to changes in the redox status, in particular, nanoparticles-mediated ROS overgeneration [37, 38], which implies the importance of ROS-mediated mechanisms in nanomaterials-induced pyroptosis activation.

In addition to pro-oxidant action and induction of various cell death modes, nanoparticles are characterized by immunotoxicity [39, 40]. Nanoparticles-triggered ROS-mediated activation of signaling pathways and transcriptional factors, including NF-κB (nuclear factor κB) and activator protein (AP)-1, upregulates cytokines such as TNF-α (tumor necrosis factor-α), IL-2, IL-6, and IL-8 [26]. It is worth mentioning that the pro-inflammatory cytokines enhance ROS generation in cells, which causes secondary oxidative stress and exacerbation of toxic effects [41]. In addition, IL-1β and IL-18 can be secreted by cells via ROS-associated NLRP3 inflammasome pathway activation [38].

Furthermore, ROS-mediated pathways are involved in the development of nanoparticles-induced fibrosis. TGF-β (transforming growth factor-β) is known to be a key driver of fibrosis, which can act via canonical (Smad-associated) and non-canonical (non-Smad-associated) pathways. TGF-β signaling activates fibroblasts, epithelial-mesenchymal transition, production of extracellular matrix (ECM) components, downregulating of ECM-degrading metalloproteinases and upregulation of tissue inhibitors of metalloproteinases (TIMPs) [42]. TGF-β is known to be upregulated in oxidative stress [43], which provides evidence that nanoscale materials can induce fibrosis via ROS/TGF-β pathways. The ability of nanoparticles to induce fibrosis via oxidative stress/TGF-β signaling pathway has been proven experimentally [44, 45].

The mechanisms of oxidative stress-mediated nanotoxicity are summarized in Fig. 1.

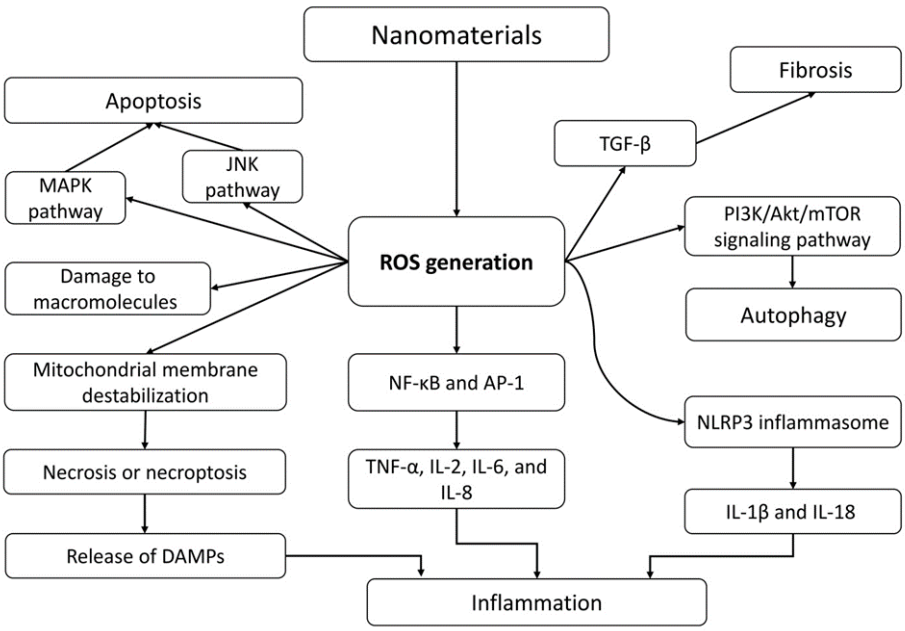

Figure 1. ROS-mediated pathways of nanomaterials-induced toxicity.

All the parameters outlined above can be assessed by flow cytometry. Flow cytometry is a sophisticated technology, which is used to separate and characterize populations of cells suspended in a fluid based on their morphology, size, granularity and fluorescent parameters using fluorescent dyes and labeled antibodies [46]. Flow cytometry is widely used in immunophenotyping, analyzing the expression of both surface and intracellular antigens, ROS generation, cytokines, the content of intracellular ions, and various cell death forms [47, 48]. In addition, flow cytometry can be used to detect proteins underwent post-translational modifications, including phosphorylation, which is crucial for analyzing cellular signaling [49]. Flow cytometry has been widely used to test the toxicity of various xenobiotics in vitro [50-52].

In this paper, we want to highlight the flow cytometry-based approaches to detect major toxicity factors of nanomaterials, including oxidative stress, apoptosis, necrosis, necroptosis, pyroprosis, autophagy and inflammation.

The major flow cytometric assays used for testing nanotoxicity are available in Table 1.

Cell redox homeostasis and flow cytometry

Flow cytometry is a common tool to assess ROS generation in cellular populations. It has been reported that several ROS-sensitive probes can be used for this purpose [53]. The most common oxidative stress-detecting probes are: 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), DHE (dihydroethidium), and CellROX green.

In particular, H2DCFDA is a ROS sensor, which is catabolized into H2DCF (dichlorodihydrofluorescein) by esterases inside the cells. In turn, H2DCF is converted to a highly fluorescent DCF (dichlorofluorescein) whose fluorescence is registered by flow cytometry. One of the advantages of H2DCFDA staining is the fact that this dye is sensitive to multiple ROS, such as H2O2, hydroxyl radicals, peroxy radicals, and reactive nitrogen species (RNS), such as ·NO and ONOO- [54]. H2DCFDA staining is used to assess ROS generation in cells exposed to nanoscale materials [55-61]. It is important to note that H2DCFDA is less sensitive to superoxide ion compared to DHE [62]. When DHE enters a cell, it interacts with superoxide ion to produce fluorescent ethidium and 2-hydroxyethidium [63]. Redox status of cells has been reported to be assessed by DHE staining with the registration of fluorescence by flow cytometry [59, 61, 64, 65]. CellROS green dye is used to distinguish oxidatively stressed viable cells from the non-stressed ones. It is used primarily to detect hydroxyl radical. The use of this dye for evaluating the impact of nanomaterials on the redox status of cells has been reported [66, 67]. Our analysis suggests that H2DCFDA staining is more commonly used due to the fact that it is less specific

Table 1

Flow cytometry-based approaches used to assess nanotoxicity

Mechanisms of nanotoxicity	Techniques used	Reports on the use in nanotoxicology
Oxidative stress induction	H2DCFDA staining	Onishchenko et al., 2021 Tkachenko et al., 2020 Kermanizadeh et al., 2018 Zhang et al., 2018 Gu et al., 2016 Han et al., 2014 Zhao et al., 2013
	DHE staining	Sadhu et al., 2018 Gu et al., 2016 Lehman et al., 2016 Zhao et al., 2013
	CellROX staining	Quan et al., 2020 Sabido et al., 2020
Apoptosis	Annexin V/7AAD staining (both apoptosis and necrosis)	Azizi et al., 2017 Wu et al., 2017 Kumar et al., 2015
	Annexin V/PI staining (both apoptosis and necrosis)	Vuković et al., 2020 Yang et al., 2019 Kai et al., 2011 Lu et al., 2011
	Cleaved caspase-3 staining	Plackal Adimuriyil George et al., 2018 Ma et al., 2015 Kai et al., 2011
	Mitochondrial transmembrane potential ($\Delta\psi_m$) detection	Plackal Adimuriyil George et al., 2018 Zhao et al., 2018 Kai et al., 2011
Necroptosis	Combination of PI staining with other methods	Niu et al., 2019 Sonkusre & Cameotra, 2017
Pyroptosis	FLICA caspase 1 assay	No data available
Autophagy	MDC staining	Liu et al., 2020
	LysoTracker dyes	Liu et al., 2020 Wang et al., 2018
Inflammation	Changes in leukocyte subpopulations	Michelini et al., 2021 Hazan-Halevy et al., 2019 Gamucci et al., 2014 Hardy et al., 2013 Kourtis et al., 2013 Hanley et al., 2009
	Changes in intracellular cytokine production	Brzóska et al., 2018 Bancos et al., 2015 Strehl et al., 2015

and covers more ROS types. Thus, CellROS and DHE can be used as additional dyes in combination with H2DCFDA to figure out the role of particular ROS types in nanomaterials-induced oxidative stress.

Cell death modes and flow cytometry

Flow cytometry is routinely used to detect apoptosis of cells. Several types of staining have been proposed, which focus on different hallmarks of this suicidal cell death mode. The commonly applied cytometric assays to analyze apoptosis are a combined staining with annexin V and 7-aminoactinomycin D (7AAD) or propidium iodide (PI), detection of the content of intracellular active caspases and the mitochondrial transmembrane potential (Δψm) [68].

The cytofluorimetric staining of cells with annexin V and 7AAD or PI is based on the ability of annexin V to bind phosphatidylserine (PS) located on the surface of cells and the capacity of 7AAD or PI to interact with DNA and become fluorescent upon binding. The former is used to detect PS externalization, which is a hallmark of apoptosis, while the latter indicates the loss of membrane integrity, which occurs in late apoptosis or necrosis. Thus, this staining can be used to discriminate viable, early apoptotic, late apoptotic/necrotic and dead necrotic cells [69]. Both techniques are convenient for analyzing nanoparticles-induced apoptosis [70-76].

Caspases are intracellular proteases that are involved in orchestration of apoptosis. They are widely used as markers of apoptosis, especially active caspase-3 produced both in intrinsic and extrinsic apoptotic pathways, including for flow cytometry [77]. Identification of cleaved caspase-3 in cells treated with nanostructured materials is the most common and informative approach to detect caspases by flow cytometry [76, 78, 79].

In normally functioning mitochondria, the mitochondrial transmembrane potential (Δψm) is created by constant proton pumping from matrix to intermembrane space by electron transport chain complexes I, III and IV and is used to generate ATP by oxidative phosphorylation [80]. The depolarized mitochondrial membrane is a sign of apoptosis [81], which is used as a marker for assessing the influence of nanomaterials on apoptosis by flow cytometry using primarily a mitochondrial transmembrane potential-sensitive JC-1 probe [76, 78, 82]. According to our estimates, other methods to detect apoptosis by flow cytometry such as analysis of cytochrome c release or DNA fragmentation are less frequently applied.

The major technique to detect necrosis is 7-AAD (or PI) staining, which indicates the loss of cell membrane permeability to impermeable fluorescent probes. Usually it is combined with annexin V staining, since there are no specific markers for necrosis, in contrast to necroptosis, a programmed lytic cell death. Canonically, necroptosis is mediated by RIPK1 (receptor interacting protein kinase 1)–RIPK3 (receptor interacting protein kinase 3)–MLKL (pseudokinase mixed lineage kinase domain-like protein) axis [83]. In particular, TNFα signaling recruits RIPK1 and RIPK3 involved in MLKL phosphorylation. MLKL compromises the cell membrane integrity forming pores, which results in lytic cell death [84].

Flow cytometry can be used to detect necroptosis in several ways, including with the help of a combination of imaging flow cytometry and annexin V/PI staining, labeled antibodies to RIPK1 and caspase-3 plus cell viability dye staining, and using fluorescently labeled antibodies to phospho-MLKL [85, 86]. Data on the impact of nanomaterials on necroptosis are scarce. In particular, selenium nanoparticles were reported to induce it in a ROS-dependent manner [87]. In addition, necroptosis-inducing features of nanomaterials can be detected by a combination of flow cytometry with other methods, e.g., western blotting [88].

Pyroptosis, a pro-inflammatory caspase-1-mediated cell death mode, is detected by flow cytometry using mainly fluorescent-labeled inhibitors of caspases (FLICA) caspase-1 assays [89]. However, this approach is not widely used in nanotoxicology researches due to the prevalence of immunobloting-, confocal microscopy- or ELISA-based detection of pyroptosis-associated proteins.

Several flow cytometric assays have been developed to assess autophagy. They include determination of the microtubule associated protein LC3B and the use of LysoTracker dyes or monodansylcadaverine (MDC) staining [90, 91]. There is accumulating evidence that nanomaterials can modulate the autophagic process in cells [92, 93]. However, confocal microscopy

is a preferential method for autophagy-detecting assays.

Inflammation markers and flow cytometry

Flow cytometry is widely used to assess inflammation markers [94, 95]. Flow cytometry can be applied for evaluating nanomaterials-mediated changes in leukocyte subsets and intracellular cytokine production. Expectedly, both approaches have been reported to be used for testing nanotoxicity [96-104], since flow cytometry is a generally recognized approach to assess inflammation-associated cells and intracellular cytokine expression.

However, due to the heterogeneity of nanomaterials there are no standard guidelines for testing nanotoxicity. In addition, novel screening methods to assess biological effects of nanoparticles are required [105]. Recent advances in flow cytometry, including the application of more lasers and development of novel fluorochromes, multiplexed analyses and the availability of new commercial dyes and florescent-labeled antibodies increase the scope of opportunities for flow cytometry in nanotoxicity testing. Thus, flow cytometry has become an essential tool in nanotoxicology and since the field is expanding this instrument seems promising.

Declarations

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The author has no ethical conflicts to disclosure.

Consent for publication

The author gives her consent to publication

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