Published in final edited form as:

Bioconj Chem. 2018 June 20; 29(6): 1793–1808. doi:10.1021/acs.bioconjchem.8b00026.

Probing Cellular Processes Using Engineered Nanoparticles

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Abstract

Nanoparticles, the building blocks of nanotechnology, have been widely utilized in various biomedical applications, such as detection, diagnosis, imaging, and therapy. However, another emerging, albeit under-represented, area is the employment of nanoparticles as tools to understand cellular processes (e.g., oxidative stress-induced signaling cascades). Such investigations have enormous potential to characterize a disease from a different perspective and unravel some new features that otherwise would have remained a mystery. In this review, we summarize the intrinsic biological properties of unmodified as well surface modified nanoparticles and discuss how such properties could be utilized to interrogate biological processes and provide a perspective for future evolution of this field.

Graphical Abstract

INTRODUCTION

Nanotechnology is a means by which engineered nanoparticles (usually 1 to 100 nm in dimension) are utilized to develop materials, structures, devices, and systems for various purposes.^{1–4} Currently, there are a wide variety of nanomaterials, including metals, metal oxides, and quantum dots, which exhibit unique optical, electrical, and magnetic properties, and have been widely used for a number of biomedical and nanotechnological applications.

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The authors declare no competing financial interest.

Other material types, such as polymers, lipids, small molecules, and organic molecules, can be assembled into carriers incorporated with contrast agents and drugs for applications in molecular and cellular labeling, tracking, detection, drug delivery, medical imaging, and therapy.^{2,4,5} These engineered nanoparticles (ENPs) can enter cells by various intracellular uptake pathways such as phagocytosis, macropinocytosis, clathrin-dependent endocytosis, caveolin-dependent endocytosis, or the crossing of membranes by diffusion (Figure 1).^{6–16} Thus, these ENPs may serve as unique baits to catch and identify new adapter molecules that characterize uptake pathways and broaden our understanding of how malignant cells tweak their intracellular uptake pathways to survive and thrive under nonconductive environments.

Some nanomaterials, particularly nanoparticles of gold, possess intrinsic biological activity. Intrinsic biological activity is dependent on many parameters, such as physical characteristics (size, shape, surface, porosity, agglomeration state, and critical structure) and chemical properties (solubility, chemical compositions, and surface modification).^{17–19} Accumulated evidence shows that the majority of ENPs induces oxidative stress (OS) upon the activation of an array of cell signaling cascades and produces reactive oxygen species (ROS) and cytotoxicity.^{19–21} Several ways by which ENPs can mediate ROS production are (i) they can catalyze oxidation–reduction reactions through their surfaces; (ii) they can interact with cellular components and ROS production machineries, such as mitochondria and the NADPH oxidase system; and (iii) they can decrease cellular antioxidant defense mechanisms by deactivating or decreasing the production of antioxidants (Figure 1).¹⁷ The level of ROS generated within the cell directly influences the cellular response. At low levels, the cell generates an antioxidant defense response, whereas at higher levels, cells induce an inflammatory response and proliferate. However, at very high ROS levels, cells undergo either programmed cell death (apoptosis, autophagy, pyroptosis, or programmed necrosis) or nonprogrammed death (accidental necrosis).^{17,22} Utilizing gold nanoparticles (AuNPs) of various surface charges, our group recently demonstrated that ovarian cancer cells overcome the cytotoxic effects of positively charged gold nanoparticles (⁺AuNPs) by exploiting overexpression of mitochondrial uptake protein 1 (MICU1) as a negative regulator of Ca²⁺ entry to the mitochondria.^{23–25} Previously, we also demonstrated how the protein-corona formation around the surface of functionalized and nonfunctionalized nanoparticles could be exploited to identify new therapeutic targets in ovarian cancer and identified HDGF and SMNDC1 as potential therapeutic targets.^{26,27} Recently, Rotello's groups reported how the protein-corona around ⁺AuNPs could be tailored for selective recognition by macrophages.²⁸ In addition, AuNPs without surface functionalization possess intrinsic therapeutic properties that had been used for the treatment of cancer,^{29,30} rheumatoid arthritis,³¹ ocular disease,^{32,33} and obesity.³⁴ Recently, AuNPs had also been reported to promote osteogenic differentiation of mesenchymal stem cells (MSCs) through activation of the p38-MAPK pathway.^{35,36} Mirsadeghi et al. also probed the inhibitory effects of AuNPs on the fibrillation process of amyloid beta (A β) proteins and observed how the protein-corona around AuNPs affects the fibrillation process.³⁷ Previously, Rotello's groups reported that amino-acid-functionalized AuNPs effectively interacting with the enzyme α -chymotrypsin via surface complementary charges can make supramolecular complexes, resulting in a decrease in its enzymatic activity. The structural diversity of amino acids allowed them to probe the role of many functional groups on regulating enzymatic

function.^{38,39} Recently, our group reported how unique chemical affinities of AuNPs could be exploited to reprogram the tumor microenvironment in pancreatic cancer.⁴⁰ In this review, we will attempt to provide the reported mechanistic cellular responses upon exposure to various ENPs, including metallic, metallic oxide, polymeric, and lipid nanoparticles. Additionally, we will address how inter-rogations utilizing ENPs can help us to better understand these cellular processes and provide opportunities to identify new molecules responsible for poor pathological outcome.

Effects of Engineered Nanoparticles on Cellular Processes.

By using ENPs in biological studies, the vast complexity of different cellular processes can be elucidated and consequently modified. A growing number of nanoparticles are being investigated for their use in intracellular applications, such as labeling, tracking, detection, drug delivery, medical imaging, and therapy.^{1,2,4,5} Based on their compositions, these nanoparticles may be classified into two categories: inorganic and organic. Inorganic nanoparticles are mostly metallic (Au, Ag, Cu, Pt, and Pd), metallic oxides, and sulfides (Ti, Fe, Ce, SiO₂, Zn, Al, Cu⁺, Co, Se, etc.), whereas organic nanoparticles are mainly liposomes, polymeric micelles, dendrimers, polymers, and lipids, as shown in Figure 2. In general, the activities of inorganic ENPs depend on the catalytic property of the core metal/metal oxide, toxicity of the shell components, intrinsic biological properties, and other aspects. On the other hand, organic ENPs elicit their effects on the basis of their compositions and physicochemical properties. Based on the activity, we herein discuss each ENP and provide information on how to probe cellular signaling mechanisms using ENPs.

Metallic Nanoparticles.

Gold Nanoparticles (AuNPs).—AuNPs are biocompatible, easy to synthesize and characterize, and are functionalizable via thiol-metal chemistry.^{4,41} As shown in Figure 2A, AuNPs can act as a self-therapeutic agent or be used to deliver small molecules such as anticancer drugs, proteins, DNA, or RNA.^{29,41} AuNPs bind to heparin-binding growth factors (HB-GFs), such as VEGF165 and bFGF, through the HB domain, leading to the unfolding of their protein structure and inhibition of their function in normal cell lines, including HUVEC and NIH3T3.^{42,43} Since HB-GFs are critically important for angiogenesis and epithelial-mesenchymal transition (EMT), a mechanism that confers metastatic potential to tumor cells, AuNPs may find wide applications as therapeutic agents in angiogenesis-dependent disorders and in preventing tumor growth and metastasis.⁴⁴ Furthermore, AuNPs can enhance apoptosis via p53, bax/bcl-2, and caspase pathways in MCF-7 human breast cancer cells in a dose-dependent manner, indicating potential application of AuNP as a therapeutic molecule in breast cancer.⁴⁵ Moreover, Tsai et al. reported that AuNPs induced apoptosis in K562 cells through ER-stress.⁴⁶ There is also exciting evidence indicating that treatment with AuNPs can alter gene expression levels in normal human lung fibroblast cells (IMR-90) via up- and down-regulation of microRNA-155 and PROS-1 genes, respectively, while observation using TEM suggested no change in the DNA methylation of the PROS-1 gene and induction of chromatin condensation in the nucleus.⁴⁷ A similar study by Li and colleagues⁴⁸ reported that 20 nm AuNPs can trigger OS and autophagy in human lung fibroblasts.⁴⁸ AuNPs treatment also not only upregulated many autophagy related genes (e.g., ATG-7) but also improved lipid

peroxidation levels, as confirmed by the formation of malondialdehyde (MDA) adducts. Furthermore, authors detected formed autophagosome and a notable increase in the inflammatory enzyme gene production of cyclooxygenase-2 (COX-2) and PNK (poly-neucleotide kinase 3'-phosphatase).⁴⁹ The biological effect of 60 nm AuNPs on normal murine macrophage cells was also studied by the Goering group.⁴⁹ Although AuNPs appeared in intracellular vesicles, as evidenced by TEM images, no pro-inflammatory response was induced by the cells. Schaeublin et al.⁵⁰ showed that treatment of keratinocytes (HaCaT) with various charges (positive, negative, or neutral) of 1.5 nm AuNPs distinctly disrupted cell morphology in a dose dependent manner.⁵⁰ In addition, it should be noted that mitochondrial distress was only observed in cells which were exposed to charged AuNPs. Both an enhanced caspase-3 expression and nuclear localization of p53 were found by using the charged AuNPs; however, only an increase in p53 localization in the nucleus and cytoplasm was mediated by the neutral AuNP. Moreover, it was notable that induction of apoptosis was observed by the treatment with charged AuNPs while necrosis was induced by neutral AuNPs. Rotello's group⁵¹ also reported the cytotoxicity of 2 nm cationic AuNPs with various hydrophobicities in HeLa cells using mitochondrial, ROS, and comet assays, which measures DNA damage. The results strongly suggested that hydrophobicity was linearly correlated with the observed acute toxicity and increased DNA damage. Clearly, these AuNPs can generate significant amounts of ROS that oxidatively impair DNA at doses where mitochondrial activity was not affected.⁵¹ Taken together, AuNPs, irrespective of normal or diseased cells, may have the potential to activate and/or down-regulate the cellular signaling cascades which can then be utilized as a probe for intracellular processes.

Silver Nanoparticles (AgNPs).—AgNPs exhibited anti-angiogenic effects, as reported by Eom and colleagues.⁵² They showed that 40 nm AgNPs had anti-angiogenic effects in normal bovine retinal epithelial cells (BREC) *in vitro* as well as an *in vivo* matrigel plug assay. The report stated that the proliferation and migration of VEGF-induced angiogenesis of BRECs could be prevented by the treatment of AgNPs, thus suggesting that AgNPs are in some way related to the activation of the PI3K/Akt signaling pathway.⁵² Therefore, they went on to a conclusion that AgNPs may act as a suppressor of the formation of new blood vessels *in vivo*. This group has also been found the antitumor effects of 50 nm AgNPs *in vitro* and *in vivo*. Moreover, incubation of Dalton's lymphoma ascites (DLA) cell lines with AgNPs executed toxicity in a dose-dependent manner which was related to the activation of caspase-3 and inhibition of cellular proliferation. Furthermore, injection of AgNPs into tumor harboring mice displayed a 65% decrease in ascites production and tumor growth compared to mice treated with sham.⁵³ AgNPs treatment has been displayed a considerable amount of apoptosis in squamous cell carcinoma through TLR2. The outcome of their findings showed that nanoparticle-mediated cell death was significantly decreased via the targeted inhibition of TLR-2.⁵⁴ AgNPs also exerted a strong apoptosis in normal murine dendritic cell lines in a ROS-dependent manner.⁵⁵ AgNPs have been shown the cytotoxicity in lung cancer cells (A549) which was strongly correlated with the ROS levels and early apoptosis-mediated mitochondrial damage. Moreover, the production of ROS also seems to be an arbitrator of genotoxicity. However, such nanocarrier-mediated cytotoxicity can greatly be reduced via the pretreatment of cells with an antioxidant.⁵⁶ In addition, treatment of MDA-MB-231 breast cancer cells with AgNPs has also been shown apoptosis-induced

cytotoxicity in a dose-dependent manner via caspase-3 activation and ROS generation.⁵⁷ Finally, AgNPs induced ER stress-dependent apoptosis.⁵⁸ Such apoptosis-operated cell death may be helpful to consider AgNPs as a potential self-therapeutic agent for human breast cancer therapy. Moreover, increased intracellular Ca^{2+} by AgNPs treatment resulted in the inhibition of the proliferation of IMR-90 and human glioblastoma cells (U251).⁵⁸ Therefore, AgNPs could have the potential to inhibit the signaling cascades and the initiation of the cellular apoptosis pathways.

Platinum Nanoparticles.—This type of nanoparticle is implicated in the improvement of bone loss after estrogen deficiency. PtNPs can damage the receptor activator of nuclear factor- κB (NF- κB) ligand signaling and inhibit osteoclast formation.⁵⁹ Moreover, pectin coated bimetallic nanoparticles of Pt and Au (CP-Au/Pt) also show mitochondrial NADH: ubiquinone oxidoreductase⁶⁰ and free radical scavenging activities,^{61,62} suggesting its implication in OS-induced diseases such as Parkinson's disease.

Metallic Oxide Nanoparticles.—Similar to metallic nanoparticles, metallic oxide nanoparticles (MONPs) can also generate and induce ROS and subsequently activate the signaling cascades, as shown in Figure 1. MONPs can form reactive groups due to the structural defects and transition metals on the particle surface.¹⁹ In addition to their inherent ROS producing properties, MONPs are also indirectly able to generate ROS by triggering cellular mechanisms through damage or activation of mitochondria.^{19,63} Moreover, MONPs can regulate the intracellular ROS content by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.⁶³ The produced ROS can then interact with critical signaling molecules, which modulate cell signaling pathways and subsequently, affect a variety of cellular processes (e.g., proliferation, metabolism, differentiation, and survival).^{17,19}

Titanium Dioxide Nanoparticles.—Treatment with titanium dioxide nanoparticles (TiO_2 -NPs) was associated with the OS-induced ROS and malondialdehyde (MDA) generation with a decrease in the activity of catalase and glutathione (GSH) in A549 cells, resulting in an increase of the expression of p53, p21, and cleaved caspase-3 with the suppression of Bcl-2 at both mRNA and protein levels.⁶⁴ In transformed cells (Bax/Bak deficient cells), TiO_2 -NPs showed lysosomal damage-mediated cell death.⁶⁵ In addition, apoptotic cell death was also found via the upregulation of Bax and Fas.⁶⁶ Moreover, TiO_2 -NPs could increase the intracellular concentrations of Ca^{2+} through the opening of L-type Ca^{2+} channels on normal mast cells and the nonspecific influx of extracellular Ca^{2+} by the permeation of the plasma membrane via an OS-dependent mechanism.⁶⁷ Recently, it has been reported that a sustained elevation of Ca^{2+} was achieved by inducing the release of Ca^{2+} from ER stores, therefore, leading to histamine secretion. Mucin secretion in normal human bronchial chaGoK1 epithelial cells is also induced by TiO_2 -NPs treatment by inducing extracellular Ca^{2+} influx and calcium release from the endoplasmic reticulum.⁶⁸

Iron Oxide Nanoparticles.—Iron oxide nanoparticles (IONPs) are strong inducers of ROS generation. IONPs have shown peroxidase-like activity in the acidic environment of lysosomes,⁶⁹ resulting in the production of ROS and activation of cell-signaling cascades.^{70–72} They have also been shown to induce necrosis and apoptosis in murine macrophages

and increase human microvascular cell permeability.²¹ IONPs can also activate NF- κ B and AP-1, inflammation in human epidermal keratinocytes (HEK) and murine epidermal cells (JB6 P (+)).⁷³ Even bare Fe₃O₄ has shown toxicity in A549 cells in terms of cell death, mitochondrial damage, DNA damage, and acute cytotoxicity.⁷⁴ Furthermore, Raucht et al. demonstrated that superparamagnetic iron oxide nanoparticles (SPIONs) can stimulate EGFR and downregulate ERK and Akt signaling independent of ROS production in colon cancer cells (HCT116 and HKE3) and breast cancer cells (MCF10CA1), while less activity was observed in nonmalignant cells (MCF10A).⁷⁵

Copper Oxide Nanoparticles.—Cytosolic cuprous oxide nanoparticles can be nonspecifically taken up by the mitochondria, therefore, resulting in the damage of the mitochondrial membrane and leading to the subsequent release of pro-apoptotic proteins into the cytosol.⁷⁶ They have also shown cytotoxicity *in vitro* in Hep-2 cells and genotoxicity in human lung epithelial cells, and have caused mitochondrial dysfunction, oxidative DNA damage, and cell death in the A549 cell line.^{77–79}

Cerium Oxide Nanoparticles.—Through the ROS-mediated stimulation of ERK1/2, JNK, and p38/MAPK, cerium oxide nanoparticles trigger apoptosis in hepatoma SMMC-7721 cells. ROS scavengers dramatically reduced activation of kinases and simultaneously decreased apoptotic rate.⁸⁰ Cerium oxide nanoparticles induced lung inflammation and alveolar macrophage apoptosis *in vivo* and apoptosis via caspase-3 activation *in vitro*.⁸¹ Chromatin condensation in BEAS-2B cells was also observed.^{82,83} In addition, they have been shown to activate HO-1 induction via the p38-Nrf-2 signaling pathway *in vitro* in BEAS-2B cells and induce lipid peroxidation and membrane damage *in vitro* in lung cancer cells.⁸⁴

Aluminum Oxide Nanoparticles.—Aluminum oxide (Al₂O₃) nanoparticles are notable inducers of mitochondria-mediated OS and cytotoxicity in human mesenchymal stem cells. Moreover, they have been shown to decrease the expression of antiapoptotic protein Bcl-2.^{85,86} Al-NPs exposure also modulate the gene and protein expressions of MAPK and their activities in the brain.⁸⁷

Cobalt Oxide Nanoparticles.—Cobalt oxide nanoparticles produced OS-induced ROS in human hepatocarcinoma (HepG2) cells that resulted in a significant reduction in GSH and a concomitant increase in OS markers, such as superoxide dismutase, lipid hydroperoxide, and catalase activity. Both the initiation and execution of caspase-3 dependent apoptosis was caused by the alteration of these intracellular enzyme levels.⁸⁸ Furthermore, it was found that chitosan-coated cobalt oxide nanoparticles stimulated TNF α -mediated apoptosis in Jurkat cells.⁸⁹

Selenium Nanoparticles.—There is an exciting report indicating that modification of selenium nanoparticles with folic acid (FA-Se-NPs) helps nanoparticles to the entrance of mitochondrial compartments in MCF-7 human breast cancer cells, resulting in the induction of ROS generation, and finally damaged mitochondria. These cells underwent mitochondria-dependent apoptosis, therefore suggesting that selenium nanoparticles could be a potential targeted therapy for cancer.⁹⁰

Nickel Oxide Nanoparticles.—Nickel oxide nanoparticles activate apoptosis-mediated cell signaling cascades in human epithelial airway cells, A549 cells, MCF10A cells, HepG2 cells, and WI38 cells.^{91,92} Moreover, nickel ferrite nanoparticles were able to induce OS in A549 cells which led to ROS generation and GSH depletion. Additionally, nanoparticle-treated cancer cells displayed an increase in apoptotic mediators Bax, caspase-3, and caspase-9 expression, while survivin and Bcl-2 were downregulated.⁹³

Zinc Oxide Nanoparticles.—Zinc oxide nanoparticles execute apoptosis via both ROS generation and mitochondria-dependent intrinsic pathway activation. Treatment with these nanoparticles triggered the mitochondria-mediated apoptosis pathway via a reduction in the membrane potential of mitochondria and an enhancement in the ratio of Bax/Bcl-2 in human fetal lung fibroblasts, rat retinal ganglion cells, and HepG2 cells. In cancer cells, including human skin melanoma cell line (A375), human alveolar adenocarcinoma cells, human colon carcinoma, and human amnion epithelial (WISH) cells, apoptosis is mediated via p53, survivin, and bax/bcl-2 pathways.^{94–102}

Silica Oxide Nanoparticles.—Several studies showed that cytotoxicity, inflammation, and apoptosis were mediated through nanoparticle-dependent activation of p53, Bax, cell cycle arrest, caspase pathway, and cytokine inflammatory responses in various cells, including human embryonic kidney cells, human hepatic cells, Kupffer cells, human epithelial carcinoma, WI-38, myocardial H9c2 (2–1) cells, peripheral blood mononuclear cells (PBMC), and lung cancer cells. These nanoparticles have also been shown to execute inhibitory effects by interfering with the MAPK/ERK1/2 and Nrf2/ARE signaling pathways.
103–111

Polymeric Nanoparticles.

Polymeric nanoparticles, as shown in Figure 2D, are mainly composed of biodegradable synthetic cationic, anionic, neutral, and/or water insoluble polymers that can either covalently attach or encapsulate therapeutic payloads.¹¹² Examples of these polymers include chitosan, polyethylene amines (PEI), (cationic), poly(2-propylacrylic acid), heparin, poly-(dicarboxylatophenoxyphosphazene) (anionic), polyethylene glycol (PEG), pluronic acid (neutral), and poly(D,L-lactide-co-glycolide) (PLGA) (water insoluble polymers).¹¹³ These polymers have been used extensively as delivery vehicles for various biomedical applications.¹¹² Among these polymers, nanoparticles having a positive charge exhibit major toxic effects. This is mainly due to the activation of several signaling pathways, including apoptosis (type 1 cell death), autophagy (type 2 cell death), necrosis (type 3 cell death), and pyroptosis.¹¹⁴ Interestingly, it was recently reported that incorporation of a low concentration of silver nanoparticles into chitosan nanocarriers induced apoptosis in human colon cancer (HT 29) cells, via an intrinsic apoptotic pathway and increased production of intracellular ROS.¹¹⁵ PEI is a polymer (high in positive charge) which forms an extremely branched network¹¹⁶ and cellular toxicity (e.g., stress-induced toxicities) is mainly dependent on geometry (linear or branched), shape, and size.^{117,118} In addition, it was found that PEI–PLGA copolymers caused OS on cells by producing ROS that damaged DNA with apparently no effect on cell viability.^{119,120} Bexiga et al. also reported that a human brain astrocytoma cell line showed a rapid increase in ROS generation when treated with cationic

polystyrene nanoparticles.¹²¹ Therefore, an emerging area of interest is how scientists can exploit the intrinsic biological effects of these nanomaterials to characterize a disease or to identify molecular machineries responsible for these effects. These identified machines may then be exploited as potential targets to modulate disease outcome. Additionally, identifying proteins or biomolecules around NPs, from disease/normal plasma, subcellular fractionation (cytosol, mitochondria, nucleus), or whole cell lysates from normal and malignant cells may provide a representative signature of the disease, which may be exploited as a diagnostic biomarker and therapeutic intervention. In conclusion, we can say that polymeric nanoparticles involve in mediating cellular signaling mechanisms, especially ROS generation.

Dendrimers.

Dendrimers are well-defined, homogeneous, and monodisperse polymeric structures that are composed of a branch of repeating units around a central core and a multivalent functional group containing outer layer, as shown in Figure 2C. Polyamidoamine (PMAM) dendrimers exhibit apoptosis of KB cells via lysosomal damage as well as ROS and autophagy flux in neuronal cells.^{122–125} However, in human lung cells, these ENPs have been shown to be toxic to the mitochondria. In addition, 60 nm polystyrene nanoparticles can mediate their toxicity through both charge-dependent and lysosomal damage in RAW264.7 cells.¹²⁶

Liposomes.

Liposome are spherical vesicles having a bilayer membrane structure that is made of one or more concentric amphiphilic phospholipids and an aqueous core,¹²⁷ as shown in Figure 2F. They are capable of carrying payloads not only in hydrophilic compartments but also in hydrophobic layers, which protects the payload from the external environment.¹²⁷ There is no direct evidence that bare liposomes can augment any cellular signaling pathways. However, while cationic lipids are used in either liposome or nucleic acid/lipid complexes, toxicity may arise from both high positive charge and large size of complexes crucial for facilitating the cellular uptake.^{128–132} Moreover, both the administered dose of lipoplexes and nitrogen/phosphate (N/P) charge ratio of cationic lipids and nucleic acids are normally associated with such toxicity.¹³³ Recently, there is an interesting piece of evidence showing that thermosensitive liposomes containing functionalized protease substrates and magnetic nanoparticles can be a promising tool to remotely probe tumor specific activity profiles of matrix metalloproteinases.¹³⁴

Protein Corona.

Protein corona offers a new biological identity to ENPs. Corona is formed via adsorption of plasma, serum, or cellular proteins on nanoparticle surface either *in vitro* and *in vivo* or both.¹³⁵ Mainly two types of protein coronas are formed, soft and hard corona.¹³⁶ Initially formed soft corona usually turns into hard corona within a few minutes, where proteins remain strongly adsorbed to the surface of ENPs by various affinity forces (e.g., van der Waals interactions, electrostatic and hydrophobic interactions, and hydrogen bonding).^{135–141} Protein corona around nanoparticles could be modulated by engineering surface properties of the nanoparticles and their sizes, and thus, each type of nanoparticles could have a unique corona signature. This coronic signature might influence nanoparticle

properties *in vivo* including clearance and toxicity. Another unique *ex vivo* application is to exploit the corona formation to identify disease specific signatures from patient samples. This approach is not only help understanding how nanoparticles interact with proteins and provide guiding principles for protein nanoparticle interaction; it may also be used to identify new therapeutic targets, e.g., in cancer, as recently demonstrated. Formation of protein corona could be modulated by surface modifications of the nanoparticle, as for example, PEGylation reduces adsorption of proteins around nanoparticles and thus decrease uptake by reticulo endothelial systems (RES) and increase circulation time. Controlling of blood resident time is essential to deliver nanotherapeutics through passive targeting exploiting the enhanced permeability and retention effect. Evidence demonstrated that apolipoproteins were the most abundant class of proteins in the corona, and that can be reduced by PEG-coating on the surface of either lipid or polymeric nanoparticles and/or metallic nanoparticles. One group found that among different lengths of PEG chain, PEG2 kDa is the best candidate to avoid such serum protein-ENPs interaction.¹⁴² Bertrand et al. showed that the surface PEG density of poly(ethylene glycol)-*b*-poly(lactic co glycolic acid) (PEG-PLGA) nanoparticles plays a pivotal role for their early clearance *in vivo*. This finding identified a PEG density threshold value (approximately 20 poly(ethylene glycol) chains (MW 5 kDa) per 100 nm²) below which blood clearance is rapid, while PEGylation beyond this value does not significantly extend the circulation times, irrespective of various sizes of nanoparticles, such as nanoparticles with diameters of 55, 90, and 140 nm.¹⁴³ Hossen et al. also showed that a combination coating of a shorter PEG2 kDa as a biostabilizer and a longer PEG5 kDa as spacer for ligand attachment on the surface of liposomes demonstrated promising biostability, active targeting efficiency, and accumulation.¹⁴⁴ Walkey et al. also reported that by applying various PEG densities from low (0–0.32 PEG/nm²) to high (>0.96 PEG/nm²) on AuNPs, around 147 serum proteins were identified and then categorized into cluster A, B, C, and D. Among these clusters, cluster A and B proteins were involved in mediating macrophage uptake and combined with complement component C3 that increases clearance of pathogens, apoptotic cells, and particles via binding with macrophage complement receptors.¹⁴⁵ In addition, in a similar approach, we also identified new targets such as SMNDC1, PPA1, PI15, and HDGF and in cellular lysate proteins using AuNPs.^{23,26,27} Thus, ENPs can be utilized as a probing tool to identify the fate associated signaling proteins for rational design of delivery systems and discovering new targets for cancer therapeutics.

Nanoendocytosis.

Nanoendocytosis is a common process by which ENPs can enter cells using various proteins associated pathways, including phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis,^{6–16} as shown in Figure 1. Phagocytes (e.g., macrophages, neutrophils, and monocytes) utilize the phagocytosis process to take up relatively large ENPs (typically a few micrometers) via the complexation with opsonic proteins, e.g., immunoglobulin (IgM and IgG), complement component (C3, C4, and C5), and blood serum proteins. These opsonized ENPs bind to cell surface, internalize via the formation of phagosomes, which subsequently fuse with lysosomes and are destroyed by acidification or enzymolysis in the lysosomes.¹⁴⁶ Macropinocytosis is an actin-driven endocytosis which involves the internalization of a large

quantity of extracellular fluids and ENPs via plasma membrane ruffling. The membrane ruffling forms a large vesicle called a macropinosome. Membrane ruffling is associated with the activation of signaling cascade such as the receptor tyrosine kinases which play an imperative role in the uptake process.^{147,148} In clathrin-dependent endocytosis, ENPs binding with receptors activate the formation of clathrin-coated pits on the cytosolic side of the plasma membrane, which move inside the cell upon the function of GTPase activity of dynamin and actin.¹⁴⁹ Caveolin-dependent endocytosis is also involved in the formation of caveolin coats (enrich in caveolin protein) on the cytosolic side of the plasma membrane upon interaction with receptors. The entry of ENPs through this pathway is proceeded with complex biochemical signaling cascades that still remain poorly understood. However, ENPs following this route could bypass lysosomes, thereby considered as a beneficial pathway for the improvement of therapeutic efficacy.^{150–152} Bhattacharyya et al. demonstrated that nanoconjugation modulates antibody mediated receptor endocytosis and endocytosis pathway can be switched by nanodesign. They demonstrated that AuNPs partially coated with anti-EGFR antibody cetuximab enters the cells through macropinocytosis pathway whereas nanoconjugates with complete coverage on AuNPs endocytosed via dynamin 2 dependent caveolar uptake. Such switching of endocytosis pathway by nanodesign is interesting and may be exploited to identify new adapter molecules for macropinocytosis and caveolar uptake pathway by identifying protein coronas around partially covered and completely covered nanoparticles.^{172,173}

Effect of ENPs Size and Composition on Signaling Mechanisms.

The effect of physicochemical properties, particularly size and surface charge of ENPs, plays a pivotal role in the activation of signaling cascades. In general, owing to a high surface area, smaller ENPs are more biologically active and induce adverse chemical reactions, such as ROS generation, compared to larger ENPs.¹⁵³ It was reported that 1.4 nm AuNPs caused rapid cell death by necrosis, while larger, 15 nm AuNPs showed low toxicity irrespective of cell types.¹⁵⁴ Sykes et al. reported that tumor accumulation of actively targeted 60 nm AuNPs was 5-fold faster and 2-fold higher than that of passively targeted 60 nm AuNPs, while using other sizes of AuNPs, including 15, 30, and 100 nm, showed no difference in tumor accumulation.¹⁵⁵ Arvizo et al. reported that among various sizes of AuNPs including 5, 10, 20, 50, and 100 nm, 20 nm AuNPs showed highest antiproliferative activity against cancer cells whereas normal cells remained unaffected.^{29,156} Park et al. reported that 4 nm AgNPs induced excessive levels of ROS production and interleukin-8 secretion than 20 and 70 nm AgNPs.¹⁵⁷ Others showed size dependent toxicity when treated with 50 and 200 nm SiO₂¹⁵⁸ and 45 and 90 nm polymer nanoparticles.¹⁵⁹ Thus, the size of ENPs plays a critical role in mediating intrinsic biological activities. However, such an effect is dependent not only on size but also on surface properties such as surface charge, hydrophobicity, hydrophilicity, and so forth. In general, cationic ENPs exhibit more toxic effects than neutral or anionic ones, probably because of their high affinity toward the negatively charged plasma membrane and induction of various signaling cascades.¹⁵³ Arvizo et al. demonstrated that positively charged AuNPs depolarized the plasma membrane potential of both cancer and noncancer cells that induced influx of extracellular Ca²⁺ leading to Ca²⁺-overload. Interestingly, normal bronchial epithelial cells (BECs) and airway smooth muscle cells did not survive upon such treatment, while there was no effect on cancer cells.

Investigating how cancer cells survived such high Ca^{2+} -overload led to the identification of MICU1 as a glycolytic switch that helped cancer survive not only Ca^{2+} -overload but cytotoxic effects of chemotherapeutics such as cisplatin.^{23,164} In addition, both cationic and anionic ENPs (e.g., liposomes) have a short blood circulation time that increases complement activation and macrophage uptake. They also exhibit poor tumor penetration properties. In comparison, neutral ENPs are relatively stable, exhibiting longer blood circulation time and high in tumor penetration properties.^{160–164} Collectively, these results suggest that ENP size and composition play a vital role in generating cellular response. Thus, identification of biomolecules associated with different types of nanoparticles may provide molecular insight in their mechanisms of actions as well as identifying new molecules to interrogate the disease.

Exploring Engineered Nanoparticles as a Probe for Cellular Processes.

Recently we demonstrated how a basic understanding of cell–nanomaterial interactions led to the identification of MICU1 as a glycolytic switch in ovarian cancer, which is responsible for tumor growth and therapy resistance. $^{+}\text{AuNPs}$ depolarize the plasma membrane of normal and cancer cells, thereby allowing the entry of extracellular Ca^{2+} into the cytosol and inducing ER stress. Ovarian cancer cells exploit the overexpression of MICU1, that functions as a gatekeeper, to prevent Ca^{2+} entry into the mitochondria and thereby shunts the metabolic requirement toward glycolysis.¹⁶⁴ Therefore, it is conceivable that isolation and identification of the protein corona around $^{+}\text{AuNPs}$ by mass spectrometry may lead to the discovery of new regulators of glycolysis in ovarian cancer.^{23–25} Moreover, amino-acid-functionalized AuNPs can be a potential probe to elucidate the role of many functional groups in restraining enzymatic function.³⁸ It is obvious that such modulation of enzyme activity can help us to capture cellular processes, including signal transduction, replication of DNA, and metabolism.^{38,39} We have identified a number of potential targets in ovarian cancer including HDGF, PPA1, SMNDC1, and PI15 by modulating protein corona around either surface-functionalized or naked AuNPs. Other groups have found the protein corona-dependent fate of these nanoparticles via the recognition of macrophages.^{26–28} Unfortunately, mass spectroscopy is currently the only way to allow for a quantitative analysis of the protein corona composition. However, this method is too complex to allow the extraction of quantitative parameters of individual protein species. Mounting evidence indicates that AuNP–fluorescent polymer “chemical nose” sensors may have the precise potential to more simply detect, identify, and quantify target proteins.^{165,166} In this novel methodology, AuNPs quench the polymer fluorescence. The interaction between the nanoparticle and the polymer is disturbed by the presence of proteins, resulting in the production of distinct fluorescent response patterns of each protein species. This nanomaterial-based protein detection method could offer an interesting solution to the complexities associated with other established protein detection assays, such as mass spectrometry. Moreover, DNA-functionalized AuNPs could be an option for navigating complex environments in cells.¹⁶⁷ The system is composed of small satellites around a core nanoparticle, where the changes in conformation are caused via a toe-hold displacement mechanism in response to DNA, resulting in the alteration of the optical properties and biological interactions of this nanosystem. Some natural biomolecules (e.g., proteins) change their function by altering their 3D conformation regarding biological signals.¹⁶⁷

Such ability of a nanosystem helps us to provide a lot of conformations to stimulate functions or remain dormant until needed. Another example is the use of functionalized magnetic nanoparticles (fMNPs) as a nanoscale template to examine the formation and function of molecular complexes within living cells. fMNPs bind with signaling proteins that are responsible for the local perturbation and manipulation of signaling cascades within the cytosol. Investigators applied this strategy to unravel the role of the Rho-GTPase family in the subcellular context in terms of morphological changes and remodeling of the actin cytoskeleton.¹⁶⁸

As the field continues to evolve, a better and more thorough understanding of ENP functions and cellular effectors will develop a stronger and more efficient probing tool. Utilizing such a tool will help guide future endeavors of discovering and optimizing therapeutic targets and/or pathways and ultimately lead to the development of next-generation therapies.

CONCLUSION

ENPs, in general, are inducers of OS-mediated ROS generation and subsequent activation of cell signaling pathways, including apoptosis (caspase, p53, and Bax/Bcl-2), DNA damage, inflammation, proliferation, and other processes (Table 1 and Table 2). These effects are mainly dependent on the physicochemical properties of the nanoparticles such as surface charge. Similarly, core of the nanomaterials such as whether metallic or metal oxide also play pivotal role. Other mechanisms including inhibition of growth factor mediated signaling and reversal of epithelial to mesenchymal phenotype are also operative depending on the types of nanoparticles. However, a generalized conclusion regarding the mechanisms of action for any particular type of material is difficult as that would also depend on the types of cells, tissue origins of cells, and disease types that are used for the study. Therefore, understanding molecular signatures (as for specific proteins) may define the cellular response, and that in turn may characterize a disease. This is an area with tremendous potential to uncover new molecular machineries to define a disease and identify new therapeutic targets.

PERSPECTIVE

The idea of nanotechnology was conceived in a visionary lecture by Dr. Feynman, “There is plenty of room at the bottom”, where he envisioned the possibility of maneuvering materials atom by atom. It was this concept that led to the development of nanoparticle synthesis by a “bottoms up” approach; start with the smallest unit and build up until the desired size is achieved.¹⁶⁹ Scientists have now mastered the bottoms-up approach of nanoparticle synthesis to precisely tune size, shape, and surface functionalization for various nanotechnological and biomedical applications.^{23,26,27,135,143,165,166,170} Another evolving but under-represented area is probing cellular processes using engineered nanomaterials: utilizing well-characterized nanomaterials as tools to unravel the intricacies of the cellular processes and identify new molecular machineries responsible for poor outcome, as, for example, in cancer.^{134,171} The necessity of identifying new molecular targets is documented by paucity in improving overall survival in a number of cancers including pancreatic cancer and ovarian cancer. We along with others have taken strides toward that goal.

23,26,27,135,143,165,166,170 The protein corona provides a new biological identity to nanoparticles. The corona is formed via adsorption of plasma, serum, or cellular proteins on the nanoparticle surface either *in vitro*, *in vivo*, or both.¹³⁵ This corona signature might influence nanoparticle properties *in vivo* and provide information regarding clearance and toxicity. Another unique *ex vivo* application is to exploit the corona formation to identify disease specific signatures from patient samples. This approach is not only helpful in understanding how nanoparticles interact with proteins, but also useful for the identification of new therapeutic targets, e.g., in cancer.

Exploiting the self-therapeutic property of AuNPs, we have identified new targets such as SMNDC1, PPA1, and PI15 by characterizing the protein corona around it. These newly identified targets may be exploited to interrogate ovarian cancer to improve patient outcomes. Trying to understand the interaction of surface engineered AuNPs with cells, we have identified HDGF and MICU1 as new therapeutic targets in ovarian cancer (Figure 3). Similarly, understanding how nanoparticles enter the cells (nanoendocytosis) may lead to the discovery of new molecular machineries involved in the endocytosis of materials, including nutrient uptake, and thus provide opportunities to modulate those processes to inhibit tumor growth.^{172,173} Therefore, exploiting nanomaterials as tools to understand cellular processes is a promising area that may significantly impact health and diseases and would continue for decades to come.

ACKNOWLEDGMENTS

In carrying out this work, we used 2R01CA136494, CA213278, and HL120585 grants.

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Figure 1.

Engineered nanoparticle entry, intracellular appearance, ROS generation, and ROS-mediated signaling cascades. Engineered nanoparticles (ENPs) can enter animal cells by phagocytosis, pinocytosis, macropinocytosis, clathrin/caveolin-independent endocytosis, or diffusion and appear in the cytosol. Subsequently, ENPs can generate and induce the production of reactive oxygen species (ROS) in the mitochondria via intrinsic (formation of reactive groups) and cell-mediated (triggering cellular mechanisms for the damage or activation of mitochondria) pathways. Activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase by ENPs can also regulate the intracellular ROS content. The produced ROS can interact with critical signaling molecules, which modulate cell signaling pathways and subsequently affect a variety of cellular processes (e.g., proliferation, metabolism, differentiation, and survival).

Figure 2.
Schematic illustration of common nanomaterials used in various biomedical applications.

Figure 3.

Discovery of unknown-target signaling protein. ENPs either naked (a) or surface modified (b) interact with signaling cascades initiating proteins in cytosol and form protein-ENP complex. The resulting trap proteins can be identified using nanoLC-MS/MS spectroscopy and discovered targets via bioinformatics.

Table 1.

Summary of Signaling Processes Mediated by Metallic Nanoparticles

nanoparticles	compositions	size (nm)	surface charge (mV)	cell name and type	main signaling mechanisms	reasons for action	refs
Metallic	Gold Nanoparticles (AuNPs)	5, 10, and 20	Negative	HUVEC and NIH3T3 normal cell lines	Bind and unfold heparin growth factors, thereby inhibiting their functions	Metallic core size	42, 43
		Unknown	Unknown	MCF-7 breast cancer	Enhanced apoptosis via p53, bax/bcl-2, and caspase pathways	Unknown	45
		1–3, 5–6, and 15–20	Positive	K562 leukemia	Induces apoptosis through ER-stress	Unknown	46
		20	Negative	IMR-90 lung fibroblasts	Upregulates microRNA-155, downregulates PROS-1, and induces chromatin condensation	unknown	47
		20	Negative	IMR-90 lung fibroblasts	Triggers oxidative stress and autophagy; enhances lipid peroxidation levels and upregulates autophagy-related genes, stimulates autophagosomes formation, and increases inflammatory enzyme gene production	Adsorbed protein	48
	Silver Nanoparticles (AgNPs)	1.5	Neutral/ Positive/ Negative	HaCaT keratinocytes	Disrupts cell morphology in dose dependent manner; increases caspase-3 and nuclear localization of p53; induces mitochondrial stress and apoptosis	Surface Charge	50
		2	Positive	Hela	Increases ROS production and damages DNA	Surface charge and Core size	51
		50	Unknown	Bovine Retinal Endothelial cells (PEDF)	Halts angiogenesis via inhibiting cell proliferation and migration, perhaps by targeting the PI3K/Akt signaling pathway	Intrinsic biological properties	52
		50	Unknown	Dalton's Lymphoma Ascites	Activates caspase-3 and inhibits cellular proliferation;	Shell component	53
		56	Negative	Human juvenile costal chondrocytes and PDL cells	Activates TLR-2 to induce apoptosis in a dose dependent manner	Dose	54
Metallic	Silver Nanoparticles (AgNPs)	2.3	Unknown	Murine dendrites	Induces ROS-mediated apoptosis	Core component	55
		121	Negative	A549 lung cancer	Induces ROS-mediated apoptosis	Core component	56
		20	Unknown	MDA-MB-231 breast cancer	Induces ROS, ER-stress, and consequential apoptosis	Concentration	57
		Unknown	Unknown	IMR-90 and U251 glioblastoma	Inhibits cell proliferation by increasing intracellular Ca ²⁺	Dose effect (Metallic composition)	58
		>100	Negative	Osteoclasts	Damages the receptor activator of NF- κ B ligand signaling and inhibits osteoclast formation	Unknown	59
	Platinum Nanoparticles (PtNPs)	2, 4.7	Unknown	<i>in vitro</i> activity measurements without cells	Antioxidant like activities via scavenging free radicals	Unknown	60–62
	Hybrid Nanoparticles (AuNPs+PtNPs)						

Table 2.
Summary of Signaling Cascades Mediated by Metallic Oxides and Other Nanoparticles

nanoparticles	compositions	size (nm)	surface charge (mV)	cell name and type	main signaling mechanisms	reasons for action	refs.
Metallic Oxides	Titanium Dioxide (TiO ₂ -NP)	434	Slightly	A549 Lung Cancer	Induces ROS and malondialdehyde generation, decreases GSH and catalase activity; increases expression of p53, p21, and cleaved caspase-3 and suppresses Bcl-2 expression	Adsorbed proteins	64
		25	Unknown	Bax/Bak deficient cells	Induces lysosomal damage-mediate cell death	Unknown	65
		70, 130, 300	Unknown	Chang (normal human liver), MCF10A (normal breast epithelial) and WI38 (normal lung fibroblast) cell lines	Upregulates Bax and Fas	Size	66
		83	Unknown	Mast cells	Increases intracellular concentrations of Ca ²⁺ by opening L-type channels and allowing nonspecific Ca ²⁺ influx	Concentration	67
		<75	Unknown	Bronchial chaGo-K1 epithelial	Induces extracellular Ca ²⁺ influx and Ca ²⁺ release from ER stores thereby leading to Mucin secretion	Unknown	68
	Iron Oxide (IONP)	<10, 15–30	Negative	Human glioma cells U251, microvascular endothelial cells (HMVECs), murine macrophages, and progenitor cells	Induces ROS generation; has shown peroxidase-like activity in lysosomes	Catalytic properties of metallic oxides	69–71
		14	Negative	Murine 3T3 fibroblasts and C17.2 neural	Increase endothelial cell permeability ROS producing leading to cell injury and death	Core	72
		15, 20, 50	Unknown	Normal human epidermal keratinocytes (HEK) and murine epidermal cells (JB6 P?)	Affects actin cytoskeleton architecture, formation of focal adhesion complexes and cell proliferation	Size	73
		9 and 15	Negative	HCT116 colon cancer and MCF10CA1 breast cancer	Activates EGFR and downstream ERK and Akt signaling independent of ROS production.	Core size	75
	Copper Oxide	40–110	Unknown	Hel.a cells, 293T cells, MEF cells and melanoma cell line YUMAC	Damages mitochondria membrane due to nonspecific uptake of NPs resulting in the release of pro-apoptotic proteins into the cytosol.	Unknown	76
		66	Negative	Human lung epithelial cells (A549)	Genotoxic	Dose (component)	77
		42 and 3000	Unknown	Hep-2 hepatocarcinoma human	Cytotoxic	Size	78

nanoparticles	compositions	size (nm)	surface charge (mV)	cell name and type	main signaling mechanisms	reasons for action	refs.
		30	Unknown	A549 lung cancer	Induces mitochondria dysfunction, oxidative DNA damage and cell death	Dose	79
	Cerium Oxide	20–30	Unknown	SMMC-7721 hepatoma	Triggers apoptosis via ROS-mediated activation of ERK 1/2, JNK, and p38/MAPK	Unknown	80
		Unknown	Unknown	Alveolar macrophages	Induces apoptosis through caspase-3 activation	Unknown	81
		30	Unknown	BEAS-2B	Induces chromatin condensation and p38/Nrf2-mediated HO-1 activation	Dose	82, 83
		15, 30 and 45	Unknown	Lung cancer cells	Induces lipid peroxidation and membrane damage	Size	84
	Aluminum Oxide (Al ₂ O ₃)	160	Unknown	Mesenchymal stem cells	Promotes mitochondria-mediated oxidative stress and cytotoxicity; decreases expression of antiapoptotic Bcl-2	Dose	85, 86
		100–400	Unknown	Brain cells	Augments MAPK expression and activity	Unknown	87
	Cobalt Oxide	265	Negative	HepG2 hepatocarcinoma	Promotes oxidative stress-induced ROS and caspase-3 dependent apoptosis	Concentration	88
		62	Unknown	Jurkat and normal lymphocytes	Stimulated TNF α -mediated apoptosis in Jurkat whereas no effect on normal cell at a dose of 100 μ g/mL	Dose	89
	Folic Acid-Mediated Selenium (FA-Se-NP)	70	Negative	MCF-7 breast cancer	Enters mitochondria thereby generating ROS and inducing mitochondria-dependent apoptosis	Composites	90
	Nickel Oxide	151 and 97	Unknown	Epithelial airway cells, MCF10A and A549	Induces apoptotic-mediated cell signaling cascades	Dose	91, 92
		311 1nd 350	Negative	Human liver cells (HepG2)	Increases apoptosis mediators (Bax, caspase-3/9) and decreases antiapoptosis mediators (Bcl-2)	Absorbed proteins	93
	Zinc Oxide	328, 431, 196, and 60	Negative	Human fetal lung fibroblasts, Rat retinal ganglion cells, and Hep-2G hepatocarcinoma	Decreases mitochondrial membrane potential, increases Bax/Bcl-2 ratio, thereby inducing mitochondria-dependent apoptosis	Unknown	94–98
		263, 267, 127–131, and 57	Negative	A375 melanoma, human alveolar adenocarcinoma, human colon carcinoma, and human colon carcinoma, and human amnion epithelial (WISH) cells	Induces apoptosis via p53, survivin, and Bax/Bcl-2 pathways	Shell components	99–102
Polymeric Nanoparticles	Chitosan-AgNPs	173	Unknown	Human colon cancer cells (HT 29)	Increases ROS and induces mitochondria-mediated apoptosis	Inorganic core	115
	Polyethylene Amines-Poly(D,L-lactide-co-glycolide) (PEI-PLGA)	110	Positive	Human umbilical vein endothelial cells (HUEVC)	Triggers oxidative stress in cells by inducing ROS production; causes DNA damage but does not impact cell viability	Core particle charge	119

nanoparticles	compositions	size (nm)	surface charge (mV)	cell name and type	main signaling mechanisms	reasons for action	refs.
Dendrimers	Amino-modified Polystyrene	Unknown	Positive	human brain astrocytoma cells	Cell death through an apoptotic mechanism mediated by damage to the mitochondria	Charge	121
	Polystyrene nano-sphere	63–297	Positive	Macrophage (RAW 264.7), epithelial (BEAS-2B), hepatocyte (HEPA-1), endothelial (HMEC), and pheochromocytoma (PC-12) cell lines	Mediates toxicity via charge-dependent and lysosomal damage	Charge	126
	Polyaminoamine (PMAM)	Unknown	Positive	KB cells (a subline of the cervical carcinoma HeLa cells)	Apoptosis mediated cell death via lysosome-induced pathway	Functional amino groups	122
Liposomes	Cationic Liposomes	5–6	Positive	Neuronal cells	Triggered ROS and autophagy	Composites	123
		7–11	Slightly negative	SK-Mel-28 cells, human melanoma cells (ATCC, HTB-72), HaCaT cells, immortalized human nontumorigenic keratinocytes, Caco-2 cells, and human colonic adenocarcinoma cells	Slightly toxic	Composites	124
		Nanometer sizes	Positive	Various cells either normal or abnormal	Cytotoxic	Size and charge of Core and cationic lipid components	128–133