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An Overview on Nanotoxicity and Nanomedicine Research: Principles, Progress and Implications on Cancer Therapy

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The toxic paradigms of chemotherapeutic drugs and nanoparticles are tightly linked. Whereas uncontrolled exposure of living systems to therapeutics/nanomaterials leads to toxicity, selective induction of cytotoxicity in cancer cells against cancer. The increasing understanding of nanotoxicity paradigms has recently resulted in important benchmarks for the safe design of nanomaterial-based drug delivery systems aiming to fight cancer. In this context, this review aims to compile and present recent advances, outcomes and interconnections between nanomaterial-based drug delivery and nanotoxicity disciplines in order to provide a comprehensive guidance for future research. First, the basic concepts and mechanisms of nanomaterial-based drug delivery and nanotoxicity are introduced. Second, we present a detailed classification of drug delivery strategies and nanotoxicity paradigms, supported by the most recent research studies with special focus on the interconnects between nanotoxicity and drug delivery research, which are highlighted in order to explore future opportunities for developing advanced therapeutic approaches. Finally, this review is concluded with future prospects on the use of nanoparticles for manipulating the behavior of cells and animals.

1. Introduction

Cancer is one of the world's most devastating diseases with high resilience to conventional treatments.[1] Since P. Ehrlich postulated his visionary concept of "magic bullet" based on the use of targeted medicines to efficaciously attack pathogens without affecting healthy tissues, various chemotherapeutic drugs (e.g. synthetic chemicals, proteins, peptides, etc.) have been used to treat cancer together with other approaches, including surgery and radiotherapy.[2] However, these strategies may not always succeed mainly due to the unfavorable drug pharmacokinetics, strong side effects of drugs, tumor metastasis, and the development of multi-drug resistance.[3] The unprecedented discoveries of cancer targets and cancer genome mapping have dramatically stimulated the development of new chemicals, inhibitors, therapeutic genes and bioactive peptides / antibodies for targeted cancer therapy.[4,5] Unfortunately, these novel drugs still face biological barriers when delivered systemically into the body, which greatly reduce the targeting efficiency and potentially increase side-effects. At the beginning of the new millennium, we have witnessed the intensive multidisciplinary research of nanotechnology across almost all disciplines. In particular, nanotechnology when confronted with cancer biology has triggered new opportunities for improving targeted cancer therapy.

Nanomaterials are commonly defined as those materials with very small components and/or structural features (such as particles and fibers) with at least one dimension in the range of 1–100 nm.[6,7] Nanomaterials can be metals, metal oxides, ceramics, polymers, or composite materials and present novel properties when compared to conventional materials due to their nanoscale features. These materials have enabled promising new opportunities in oncology for treatment of cancer by nanomaterial-based drug delivery strategies, in which anti-cancer drugs are loaded directly onto nanomaterials and transported to the specific tumor tissues for cancer killing.[8] Since nanomaterials as drug carriers are programmed to target cancer cells actively, the drug delivery efficiency should be significantly improved when compared to passive targeting tumor by free diffusion of drug molecules. Although nanomaterial-based drug delivery strategies provide superior advantages over traditional chemotherapy, the potential cytotoxicity associated with nanomaterials still raises significant concerns.[9] Nanoparticles have distinct toxicity patterns as compared with their larger counterparts. The reduced size of materials at nano-scale increases the number of surface molecules and their surface area exponentially, leading to complex biophysicochemical interactions at the bio-nano interfaces when exposed to physiological environments.[10,11] Understanding these interactions and their consequence is of fundamental importance for the identification of potential paradigms of nanotoxicity. It should be note that drug delivery and nanotoxicity have strong correlations. To induce toxicity to cancer cells in a selective manner by nanomaterial-based drug delivery strategies eliminates tumors, whereas unfavourable toxicity of nanomaterials, therapeutic drugs, and the

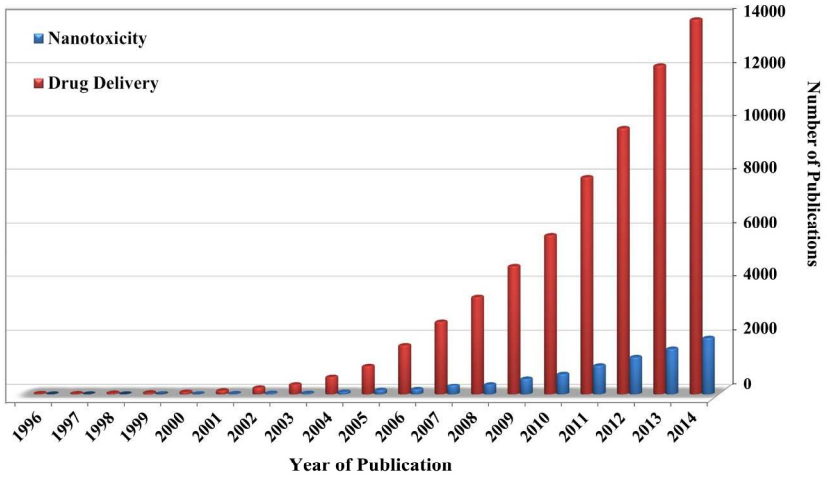

Figure 1 The number of published papers on drug delivery and nanotoxicity from 1996 to 2014 according to the statistics on Google Scholar. Note that the key word "drug delivery" here only refers to the notions of nanomaterials-based drug delivery.

combinations of both, often termed as nanomedicines, causes side-effects and dysfunctions. More importantly, since nanoparticles, therapeutic drugs and nanomedicines share similar biological fates / responses in the body, understanding the interconnections between nanotoxicity and drug delivery profoundly broadens our visions and possibilities to improve cancer therapy. Statistically, the number of publications relating to the scope of nanomaterial-based drug delivery and nanotoxicity increased sharply since 2000. (**Figure 1**) In 2014, over 14200 and 2120 research studies were published on these two areas, respectively, which were approximately two hundred times larger than the number of studies reported in 2000. That means, more than 44 papers per day have been published in these two areas during 2014. To address the significance of research in drug delivery and nanotoxicity, this review aims to provide a comprehensive view about the fundamental principles, concepts as well as the most recent research progress in these two areas. The scope and structure of this review is shown in **Figure 2**.

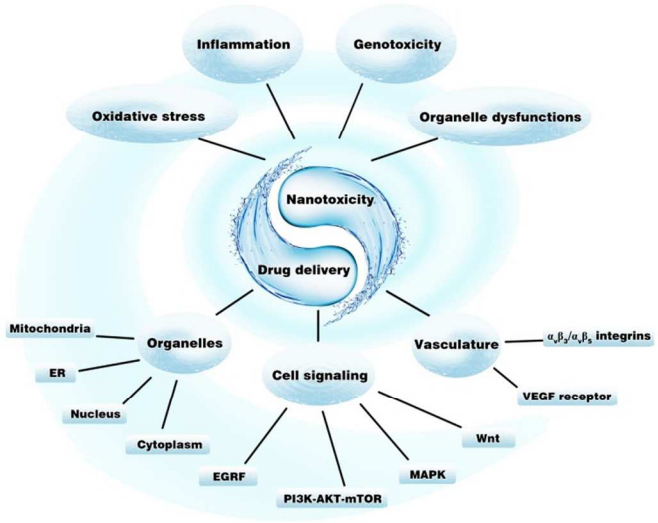

Figure 2 Scheme of the correlations between nanotoxicity and nanomaterial-based drug delivery researches. The studies of nanomaterial-based drug delivery and nanotoxicity are closely interconnected, which is presented as a TaiChi shown in the middle of scheme. Nanotoxicity researches provide direct benchmarks for the safe-design of novel nanomaterial-based drug delivery system; while selective inducing toxicity in cancer cells by nanomaterial-based drug delivery approaches cure cancers. The major nanotoxicity paradigms and specific site-targeting strategies will be discussed in this review.

2. Site-targeted Drug Delivery: Engineered Drug Targeting Approaches by Nanomaterials

Conventional drug therapies suffer from many drawbacks and intrinsic limitations such as low drug solubility of hydrophobic drugs, poor biodistribution, lack of selectivity and unfavourable pharmacokinetics.[7,12,13] The importance of controlling drug pharmacokinetics has been realized since 1950, but challenges still exist for delivering cytotoxic anti-cancer drugs with minimum side-effects.[14] For example, one of the most widely used anthracyclines, doxorubicin, has a rapid plasma clearance and short half-life (5-10 mins in first phase, and 29 h in terminal phase) when administered intravenously.[15] In addition, doxorubicin, like other anthracyclines, potentially induces cardiotoxicity, limiting the therapeutic dosage that can be administered in clinical applications.[16] Comorbid conditions, such as impaired liver or renal functions, can further limit the dose tolerance of these cytotoxic drugs.[14] Biocompatible and bioactive drugs, including proteins, nucleic acids, enzymes and genes, face other problems as they degrade prematurely by metabolism when administered through oral or intravenous routes.[17] Unfortunately, the problems of dosing and off-site targeting of cytotoxic drugs are just some of the issues that patients suffering from cancer must face. Prolonged exposure to drugs may make cancer cells to develop cross-resistance to several structurally unrelated chemotherapeutic agents, a phenomenon known as multi-drug resistance, which significantly diminishes the therapeutic outcomes.[18,19] Therefore, effective treatment of cancer requires a full understanding of cancer biology in order to adjust the features of medicines.

Nanomaterial-based site-targeted drug delivery systems are promising approaches to overcome the inherent limitations of conventional drugs. These systems present several attractive properties, including reduced off-target toxicities, enhanced efficiency of drug delivery by enhanced permeability and retention (EPR) effect and site-targeting strategies. Other advantages such as improved drug-circulation times, controlled drug-release kinetics, and superior dose scheduling for

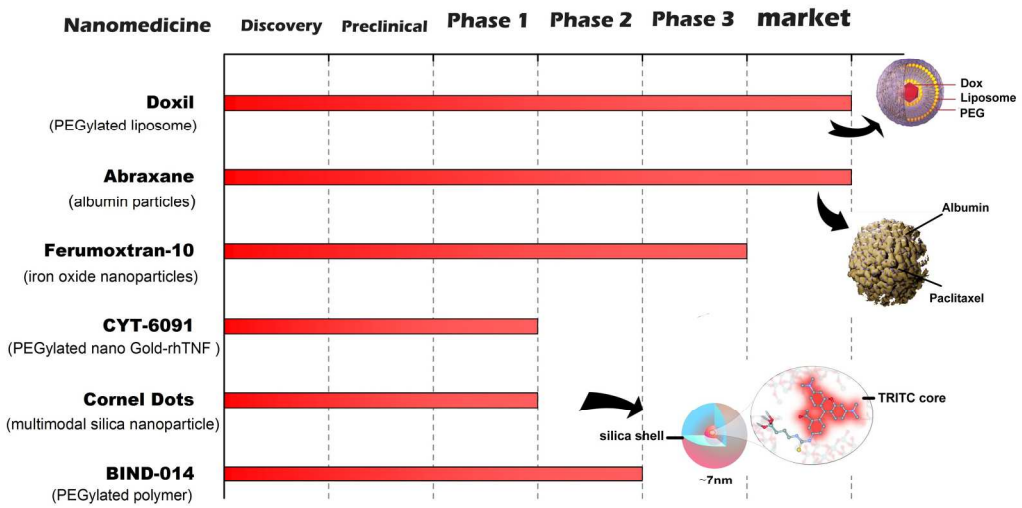

Figure 3 Selected nanoparticle-based programs in clinical development.

improved patient compliance also highlight the potential of nanomedicines in clinical applications.[20] It is noteworthy that micro-sized drug formulations, such as iron and aluminium salt-based drug crystals were established almost a century ago.[21,22] However, the widespread application of nano-sized drug formulations emerged at the early 1990's along with the flourishing of nanotechnology.[23] Thus far, numerous nanomaterial-based drug delivery systems have been developed, including liposomes[24], polymers[25], metal and metal oxide[26] and composite nanomaterials[27]. More than 40 nanomaterial-based products have been approved by the Food and Drug Administration (FDA) for clinical use. (**Figure 3**) For example, Doxil®, a polyethylene glycol (PEG) functionalized liposomal formulation of doxorubicin, was approved in 1995 for the clinical treatment of cancer and sarcoma.[28] Unfortunately, although the first generation of nanomedicines (e.g. Doxil®) achieved commercial success, these formulations utilizing EPR effect to passively target tumor only showed limited improvement of cancer therapy in the clinical practice.[20, 24] Recently, a polymer-based site-targeted nanomedicine, BIND-014 demonstrated positive phase II result for the treatment of non-small cell lung cancers.[29, 30] As the first example of a site-targeted nanomedicine, BIND-014 actively targets prostate-specific membrane antigens expressed on prostate cancer cells and the neovasculature of most non-prostate solid tumors. The promising clinical trials of BIND-014 clearly demonstrate the bright future of site-targeted nanomedicines due to their improved ability to target tumor. In this section, we will highlight the mechanisms, methodologies and recent research progress of nanomaterial-based site-targeted drug delivery strategies.

2.1 Mechanisms and concept of nanomaterial-based site-targeted drug delivery

As mentioned above, the first generation of nanomedicines utilized EPR effect to target tumors. The earliest report of an EPR effect showed that the accumulation of anti-cancer protein at a tumor site is caused by the enhanced permeability in tumors due to the abnormal blood vessels and lymphatic drainage.[31] Such angiogenic blood vessel gaps, as large as 600 nm between adjacent endothelial cells, allow nanoparticles (NPs) to preferentially accumulate in tumors rather than spread into healthy tissues. In addition, cancer cells use high rate of glycolysis to obtain extra energy, resulting in a relatively acidic micro-environment when compared with normal tissues.[32] These characteristics of tumor cells provide a strong rationale for releasing drugs as a function of their pKas and the cellular pH gradient. In this passive-targeting protocol, the anti-cancer drug loaded on nano-carriers are engineered to be stable within microenvironments at physiological pH (i.e. during transport), while the release of drug can be triggered at the tumor site, where the pH is lower than that of normal cells.

EPR effect provides several benefits when compared to free diffusion of drug molecules into tumor tissues. However, EPR-based drug delivery systems as a passive targeting approach also face challenges. For instance, EPR effect is limited to specific stages and types of tumors;[33] In addition, the complexity of the tumor micro-environment offers many barriers (e.g., high interstitial fluid pressure, dense collagen matrix, etc.) that hamper the effect of passively delivered drugs into the tumor. Besides, the longer circulation times of the PEGylated liposomal doxorubicin is associated with several chemotherapy-induced side effects, such as stomatitis and palmar–plantar erythrodysesthesia.[20] A more promising way to overcome these limitations is to use site-targeted delivery systems. In these systems, nano-carriers functionalized with a homing molecule, such as ligand and monoclonal antibodies, are used to deliver drugs to solid tumors or cross biological barriers by a specific molecular recognition process. (**Figure 4**)

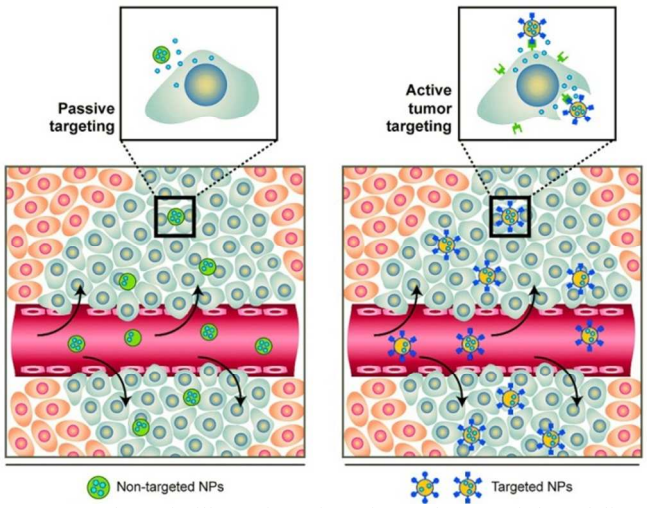

Figure 4 Schematic illustration of passive and targeted drug delivery. (Left) Non-targeted nanoparticles (NPs) end to passively extravasate through the leaky vasculature, which is characteristic of solid tumors and inflamed tissue, and preferentially accumulate through the EPR effect. (Right) Targeted NPs containing surface ligands can recognize the receptor located on tumor cell surfaces.[8]

The internalization of nanomaterials occurs via endocytosis pathways, which are initiated by the formation of the endosome through invaginating plasma membrane to envelope the conjugates formed by cell receptors and nanomaterials. Subsequently, newly formed endosomes are transported through endosomal–lysosomal-autophagy pathways. Besides entry into endosome, lysosome and autophagosome compartments, it is believed that nanomaterials can escape endosome through the so-called "proton sponge hypothesis"[34], which is the key for NPs to intracellular deliver therapeutics to their target and avoid the drug degradation inside acidic lysosome. (**Figure 5**) Meanwhile, the receptor released from the conjugate returns to the cell membrane to start a second round of transport through endocytic recycling pathways.[35] To increase the specificity, the corresponding antigen or receptor should be expressed exclusively on all tumors cells while not present on normal tissues. For example, the folate receptor is overexpressed on tumor cells to increase the nutritional uptake, and thus folate is widely used as the homing molecule. But folate is also supplied by food, which might compete with ligands present on nano-carriers.[35] To date, tumor-associated biomarkers such as transferring receptors, growth factors and other overexpressed proteins have been widely used as cancer targets.[36] The application of novel biomarkers is the way envisaged for increasing the therapeutic efficacy and specificity of nanomaterial-based site-targeted therapeutics.[29]

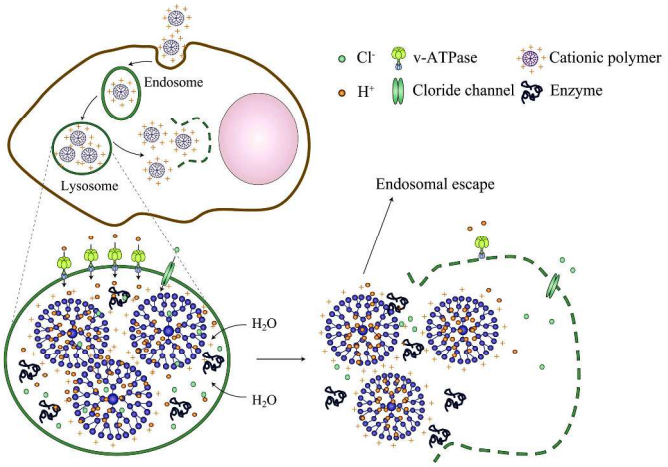

Figure 5 Schematic of the proton sponge effect by cationic nanoparticles. Cationic nanoparticles with positive surface charges are capable of sequestering protons when internalized into acidic lysosomal compartment. This function keeps the v-ATPase (proton pump) functioning and leads to the continuously reduction of pH and passive entry of chloride ions. Such high osmotic pressure subsequently causes the swelling and rupture of endosomes.[10]

2.2 Recent advances of nanomaterial-based site-targeted drug delivery

2.2.1 Intracellular targeting strategies

Intracellular targeting is of great interest for gene therapies, molecular imaging and treatment of organelle-specific diseases. Previous understandings of intracellular dynamics and cell uptake of nanomaterials showed that NPs enter the cell through various endocytosis pathways, including macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin/caveolin-independent endocytosis and phagocytosis. (**Figure 6**) Specific organelle targeting can be realized by utilizing the endosomal escape of nanoparticles after cell uptake. The first intracellular delivery system was created and driven by the development of synthetic vectors for gene delivery.[37] Cationic NPs are ideal nano-carriers to deliver and accumulate cargos (e.g. genes and drugs) into the peri-nuclear region. For example, recently Zhou *et al.* developed a co-delivery system of doxorubicin and siRNA for *in vivo* preclinical breast cancer treatment. siRNA was efficiently loaded onto polystyrene nanoparticles through layer-by-layer deposition.[38] The siRNA loading was optimized to be 3500 siRNA molecules per particle in order to overcome the loss of siRNA during endosomal escape. The nanoparticles achieved long circulating time with a half time of 28 hs without triggering inflammatory response, and significantly reduced the target gene expression (luciferase gene) in tumors by four-fold. In a combinatorial approach, tailored siRNAs that target multi-drug resistance proteins were loaded onto the surface of doxorubicin-loaded liposomes for achieving synergistic effect. This novel system successfully induced an eight-fold decrease in tumor volume within 15 days as compared to the control treatment.

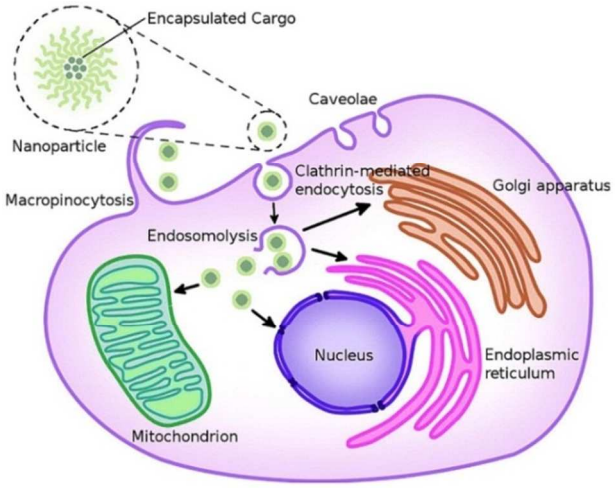

Figure 6 Schematic representation of nanoparticles-meditated delivery of cargo. After endosomal escape, the nanoparticle can be engineered to target various cellular compartments such as mitochondria, endoplasmic reticulum, Golgi, nucleus, and cytoplasm.

In contrast to passive nuclear targeting, active targeting strategy relies on the selective functionalization of NPs with nuclear localization signals for targeting the nuclear pore complexes (NPCs).[39] NPCs are large proteinaceous structures, which act as selective gates for nucleoplasmic transport of macromolecules. The transport is mediated by recognizing nuclear transport receptors and nuclear localization signals. Utilizing this feature enables the translocation of NPs into the nucleus with maximum size of up to 39 nm.[39-42] Similarly, other targeting peptides that are recongized by the cytosolic transport systems, such as mitochondrial localizaiton signal, endoplasmic reticulum (ER) signal peptide and ER retrieval sequence have also been used for translocation of nanoparticles. (**Table 1**) For example, mitochondriotropic triphenylphosphonium functionalized drug-loaded liposome exhibited enhanced uptake and cancer cell killing both *in vitro* and *in vivo* due to the efficient mitochondria-targeting capability.[43] Entrapping an ER-insertion signal sequence into poly(γ-glutamic acid) NPs exhibited an enhanced cellular immune responses as a result of the elevated antigen transport to ER, which is responsible for the antigen presentation process.[44] In addition, cytoplasmic targeting is of critical importance to overcome the MDR in cancer cells. It is known that MDR is mainly caused by the complex interplay of cell survival pathways, which facilitate cell survival by various mechanisms, including enhanced drug transport[18], over-expression of anti-apoptotic proteins[19], increased DNA damage repair[45] and autophagy[46]. So far, various strategies have been employed to design drug delivery systems for effective transport of anti-cancer drugs into the targeted intracellular compartment.[16] The chemotherapy-induced up-regulation of P-glycoprotein (P-gp), a broad-specificity trans-membrane drug efflux pump is considered the major event in the establishment of MDR in cancer cells. Inhibition P-gp by anionic liposome/ lipids[47, 48], conjugating NPs with P-gp antibody and P-gp inhibitors[49] have shown promising preclinical and clinical results for reversing MDR. Another feasible approach is to enhance the cellular uptake (i.e. facilitate endocytosis to bypass P-gp) to achieve the rapid accumulation and controlled intracellular release of cytotoxic drugs.[47,50-53] In this approach, anti-cancer drugs, such as doxorubicin can be conjugated on the surface NPs through a stimuli-responsive linker so that the drug release can be activated by changes of physiological microenvironments.[54]

Table 1 Examples of nanoparticles for intracellular targeting delivery

Targeting moieties	Target organelle	Reference
Mitochondrial localization signal	Mitochondria	39, 41, 43, 55
ER-insertional sequence	ER	44, 56, 57
Nuclear localization signals	Nucleus	39-42
P-gp inhibitor or bypass P-gp	Cytoplasm	44-52

2.2.2 Cell signalling targeting strategies

Delivery of specific proteins, peptides and molecules that influence signaling pathways and manipulate cell functions is another approach for cancer killing. Such strategies not only require a good understanding of the cell survival mechanisms, but also an optimal design of drug cocktails and the pharmacokinetics of drug delivery systems. However, the transition of this approach is still rare, and a systematic review on this strategy is yet to come. Herein, we summarize the latest research results on cell signaling targeting strategies that have been demonstrated in recent years.

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR) pathways are important intracellular signaling pathways that regulate cell cycle, proliferation and longevity. These signaling pathways are frequently overactive in many types of cancer cells, and thus make the inhibitors of PI3K, Akt and mTOR useful candidates for molecular-targeted therapy. Rapamycin is a clinically available mTOR inhibitor (trade name: Rapamune) for chemotherapy, but the intrinsic drug resistance of rapamycin in cancer cells hampered the therapeutic effect.[58] Combination delivery of rapamycin with other drugs can reverse such resistance and achieve synergistic therapeutic effects *in vitro*. Unfortunately, such synergy was not always translated into good clinical performances because of the unfavorable pharmacokinetic parameters of drug combinations. To address this challenge, Elvin *et al.* developed a polymer micelle-based combination system for the co-delivery of rapamycin and paclitaxel *in vivo* by using poly(ethylene glycol)-block-poly(D,L-lactide) polymer micelles. After carefully optimizing the drug loading ratio and release profile, the nanoparticles could accumulate in tumors within 24 hours and effectively suppressed tumor growth due to the inhibition of mTOR and Akt downstream signaling. (**Figure 7**) A similar approach aiming to manipulate Akt signaling is to deliver Akt inhibitors for blocking the phosphorylation of Akt, which can activate apoptosis signaling to kill cancer cells.[59] In this study, anti-pAkt was conjugated with silica nanoparticles followed by the delivery to MCF-7 breast cancer cells. *In vitro* results confirmed the enhanced apoptosis and cell death after 24h treatment.

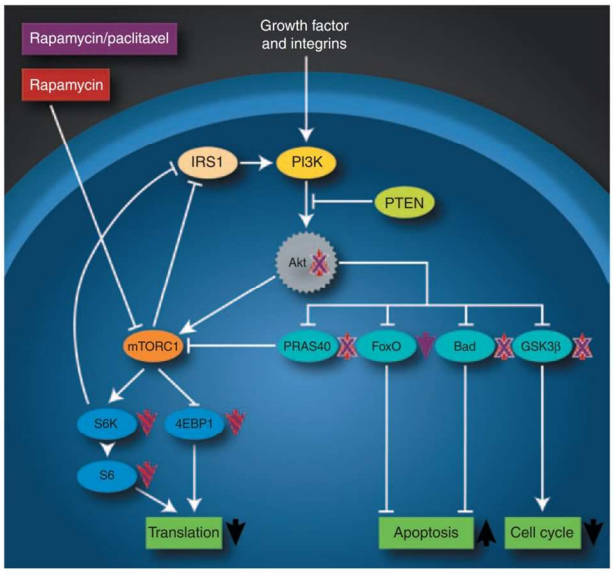

Figure 7 Rapamycin and paclitaxel nanoparticles synergistically target the PI3K/Akt/mTOR pathway by suppressing feedback loop Akt-phosphorylation.[60]

Recent cell signalling studies demonstrated that the sequential delivery of different drugs for targeting signaling networks has a significant effect on cancer cell killing. Mitogen-activated protein kinase (MAPK) signalling and epidermal growth factor receptors (EGRF) signalling are both critical for regulating cellular functions.[61,62] Dysregulations of these signalling pathways are correlated with tumor progression, invasion and metastasis in a variety of cancers. Previous research showed that delivery of the MAPK inhibitor PD98059 conjugated with poly(lactide co-glycide) (PLGA) NPs inhibited tumor growth *in vivo* and enhanced the anti-tumor efficacy of cisplatin-based chemotherapy.[63] Note that in this research PD98059-loaded NPs were administrated before cisplatin to achieve the scheduled inhibition and sensitization of tumor cells. Time-staggered inhibition of epidermal growth factor receptor (EGFR) was also shown to dramatically sensitise cancer cells to genotoxicity drugs such as doxorubicin.[64] Translating this knowledge into drug delivery systems, Stephen *et al.* developed a liposome-based combination delivery system to sequentially deliver a hydrophobic EGFR inhibitor and a DNA-damaging agent-doxorubicin.[45] By using the lipid shell for storage of the hydrophobic drug and the aqueous interior to load the hydrophilic drug, liposomes enable incorporation of high concentrations of both therapeutics for their sequential release: the hydrophobic EGFR inhibitor on the shell is released first followed by doxorubicin from the core of the liposome. (**Figure 8**) The nanocarrier functionalized with PEG and folic acid had enhanced cell uptake and cancer killing *in vitro* and effectively produced tumor regression *in vivo*. More importantly, these investigators explored a number of drug cocktails with EGRF inhibitors in this drug delivery system, which further strengthened their preclinical results for the future clinical transition.[45]

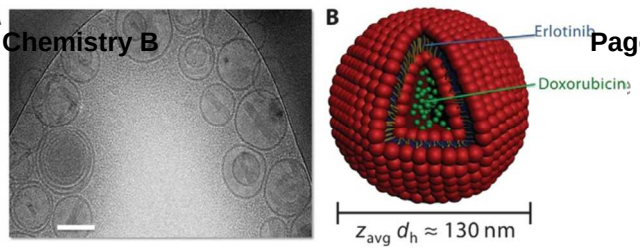

Figure 8 Characterization of the combination therapeutic–loaded liposomal system. (A) Cryogenic transmission electron micrograph of dual drug–loaded liposomes. Scale bar, 100 nm. (B) Schematic of dual loading of a small-molecule inhibitor (erlotinib, blue) into the hydrophobic, vesicular wall compartment and of a cytotoxic agent (doxorubicin, green) into the aqueous, hydrophilic interior.[45]

In another interesting work, a designed inhibitor of glycogen synthase kinase-3b (GSK-3b), so-called GFP-FRATtide was delivered into stem cells for the manipulation of Wnt–β-catenin signalling pathway.[65] This pathway is an evolutionarily conserved pathway that regulates crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development.[66] GSK-3β is a key component of the Wnt signalling pathways and has long been treated as a target for molecular therapy.[67] In this work, the delivery of the GSK-3β inhibitor by hydrophobic silica NPs was tested in human and rat stem cells. The delivery of GFP-FRATtide induced Wnt signalling, resulting in the elevation of β-catenin levels due to GSK-3b inhibition. Accumulation of β-catenin up-regulated the transcription of Wnt target genes, which manipulated cellular proliferation and maintained cells in an undifferentiated state. Taken together, the emerging development of nanomaterial-based cell signalling targeting strategies as novel molecular therapeutics presented new possibilities for precisely manipulate cellular behaviour, which is ideally envisaged for providing personalized and dynamic cancer therapies. (**Table 2**)

Table 2 Examples of nanoparticles for cell signaling targeting delivery[1]

Targeting signaling[2]	Reference
PI3K-AKT-mTOR signaling	60, 68, 69
EGFR signaling	45, 70, 71
Wnt signaling	65
MAPK/ERK signaling	63, 72, 73

[1] Note that nanoparticles as a cytotoxic paradigm can intrinsically regulate cell signalling. The related work will be discussed in nanotoxicity section to distinguish the concepts.
[2] This table only summarizes the delivery of signalling regulators by nanoparticles. Delivery of cytotoxic drugs also activates various signaling pathways such as apoptosis signaling, which is not included here.

2.2.3 Vasculature targeting strategies

The transport of therapeutic agents from the systemic circulation to cancer cells requires three steps: 1) the drug molecules or NPs need to reach the tumor tissue via the blood vessels, 2) then cross the vessel wall and 3) penetrate through interstitial spaces to target cancer cells. (**Figure 9**) However, many barriers exist in each step for tumor targeting. Unlike normal tissue, blood velocity in tumors is independent of vessel diameter and unevenly distributed. This heterogeneous micro-environment creates poorly perfused or even un-perfused

regions, which makes NPs, or even small molecules, difficult to reach the tumor homogeneously.[20,74] Furthermore, the lack of functional lymphatic vessels and the vascular hyperpermeability inside tumors results in interstitial hypertension (elevated interstitial fluid pressure), which in turn reduces the convective transport of drugs and NPs.[20]

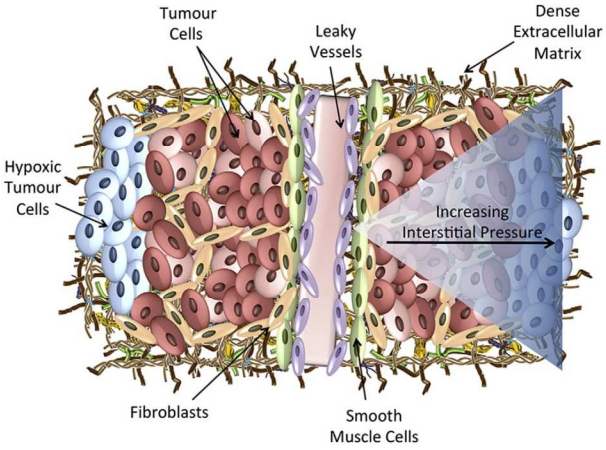

Figure 9 Complexity of the tumor microenvironment prevents nanoparticles from effectively penetrating deeply into and effectively accessing tumor cells. 1) The leaky nature of the endothelial can be variable thereby restricting access to certain areas of the tumor. 2) Once nanoparticles have exited the vessels, they usually have to pass through other cellular layers including smooth muscle cells and fibroblasts before gaining access to the tumor cells. 3) Interstitial pressure increases with increasing distance from the vessel which can prevent nanoparticles from penetrating deeply into the tumor. 4) Dense extracellular matrix can present an additional barrier to movement of nanoparticles into the tumor with stiffer tumors more difficult to penetrate. 5) High cell density of tumor cells is difficult to penetrate with most chemotherapy drugs only able to travel 3-5 cell diameters into the tumor and larger nanoparticles hindered to an even greater extent. Heterogeneity in tumor cells creates challenges for actively targeting as they can possess highly varied cell surface molecule expression.[75]

Tumor vasculature targeting is a promising strategy to circumvent the barriers encountered by nanoparticles. All solid tumors depend on angiogenesis--the formation of new blood vessels--to support tumor growth.[76] Tumor blood vessels tend to express or overexpress extracellular matrix proteins on endothelial cell surface when compared to normal blood vessels, making them ideal as potential targets.[77] For example, vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) as major angiogenic regulators have long been considered as tumor imaging and cancer therapeutic targets.[78,79] To translate this strategy into a nanomedicine approach, quantum dots were conjugated with VEGF and dual labelled with ^{64}Cu for VEGFR-targeted PET/near-infrared fluorescence imaging.[80] The nanoparticles exhibited high VEGFR-2-specific binding affinity *in vitro*, and *in vivo*, which a tumor accumulation of ≈4% ID/g (injected dose per gram of tissue) at 24 h post-injection, which was significantly higher than that of NPs without VEGF conjugation (<1% ID/g). Besides VEGFR, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which are overexpressed in angiogenic vessels, are also frequently used as targets. Ruoslathi *et al*. demonstrated the strategy of using iRGD peptide conjugated doxorubicin and NPs for targeting vasculature, in which the drugs / NPs could bind specifically to integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ via the RGD motif.[81, 82] Upon binding, the sequence undergoes proteolytic cleavage of the peptide exposing a new binding motif specific for neutrophilin-1 and allowing for deep penetration of the tumor tissue. Similar protocols have also been demonstrated by conjugating RGD with quantum dots[83], single-wall carbon nanotubes[84], zinc oxide nanowires[85], superparamagnetic iron oxide nanoparticles[86], gold nanorods[87], dendrimer[88] and other NPs.

In addition to the modification of moieties for targeting vasculature, the physiochemical properties of NPs, such as size, shape and surface charge also affect the ability of nanoparticles to penetrate tumors. As an example, *in vivo* experiments with gold NPs have demonstrated that only those NPs with sizes smaller than 10 nm can efficiently penetrate tumors.[89] (**Figure 10**) Nonetheless, the "size dilemma" becomes a concern when facing the fact that small sized NPs rapidly go through renal clearance, and thus are unlikely to effectively accumulate within the tumor.[90] To address this challenge, Wong *et al.* developed a 100 nm "multistage" gelatin quantum dots (QDGelNPs), which were designed to be broken down into smaller 10 nm nanoparticles for efficient tumor penetration by utilizing tumor-associate proteases to degrade gelatin.[91] These particles consist of a gelatine core with amino-PEG functionalized quantum dots conjugated onto the surface. The larger NPs can be cleaved by a matrix metalloproteinase (MMP), which is a protease present in high abundance within the tumor microenvironment. They demonstrated that only 25 ng of proteases (MMP-2) were required to release 50% of quantum dots *in vitro*. Furthermore, they found that the serum half-life of QDGelNPs (22.0 ± 3.4 h) is two-fold higher than the control group (silica NPs, 12.9 ± 2.4 h), which successfully extended the half-time of quantum dots. After 6 h post-injection, the QDGelNPs had penetrated up to ≈300 μm from the injection site while the silica NPs exhibited little or no dissemination from its initial location.

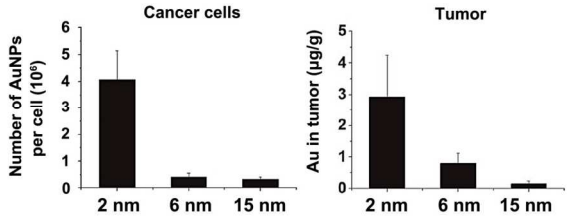

Figure 10 Uptake of 2, 6, and 15 nm gold nanoparticles in MCF-7 monolayer cells and in vivo tumor tissues. 2 nm gold nanoparticles showed significantly enhance tumor penetration as compared to larger nanoparticles.[89]

3. Nanotoxicity：Mechanisms and Implications for Safety Design of Nanomaterials

Bio-safety of nanomaterials is a critical pre-requisite to be considered when developing nano-carriers. An increasing attention has been focused on nanotoxicity since 2000. (**Figure 1**) It should be noted that the purpose of nanotoxicological research is to provide evaluations of adverse effects associated with a nanomaterial for its safe use in the workplace, especially to establish the safe working dosage in different exposure pathways such as ingestion, inhalation or skin absorption.[9,92,93] According to this definition, however, most of the previous research were mechanistic studies rather than nanotoxicological assessments. Nonetheless, these studies provide meaningful guidance and implications for the safe design of nanomaterials.

Among numerous synthetic NPs, several types of inorganic nanomaterials (e.g. quantum dots, gold, silver, iron oxide NPs) and polymeric NPs have gained particular interest for their clinical applications. However, conflicting results of these and other types of nanomaterials have been found when analysing the effect of physiochemical parameters of NPs on their toxicities. Besides the issues of reproducibility and analysis techniques, the major problems in nanotoxicity studies are the intrinsic complexity of NPs' physiochemical factors on their toxicological consequence. The identified major physiochemical factors are size, surface chemistry, surface charge, aspect ratio and compositions. (**Figure 11**) Each of these parameters has a specific impact on the toxicological consequences associated with a given nanomaterial.

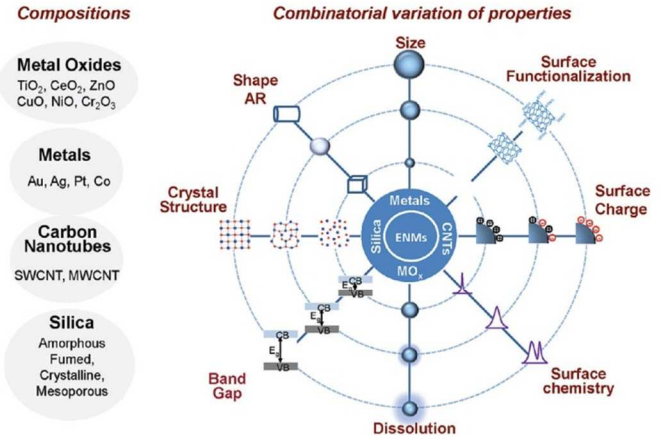

Figure 11 Key paradigms of nanotoxicity of the library of nanomaterials, including metals, metal oxides, carbon nanotubes, and silica-based nanomaterials. [94]

As the one of the most important factors, size plays a critical rule on nanotoxicity. The surface area and volume ratio of NPs increases exponentially with size reduction at nano-scale. Such "quantum size effect" leads to complex interactions between nanomaterial and biomolecules (i.e. protein, DNA) inside the biological milieu. It is reasonable to conclude that the size difference of NPs leads to variations of toxicological patterns. Such size-dependent effects were confirmed in cell-culture and animal experiments using various materials, such as gold[95] and silica NPs[97-99], in which the smaller sized NPs are more toxic than bigger size. However, this conclusion is not true for all types of nanomaterials as other toxicity paradigms, such as crystal structure and surface reactivity also change with the NPs' size. For instance, Warheit and co-workers have shown in a pulmonary instillation study that the toxicity of TiO_2 is independent of their size.[96] Concurrently, Karlsson *et al*. found out that Fe_2O_3, Fe_3O_4 and TiO_2 NPs with nano and micro-size showed similar toxicity with no size-dependent effect.

Aspect ratio, the ratio of the nanomaterial' size in two dimensions, also directly impacts the toxicity associated with one dimensional materials (e.g. nanowires) since it dramatically changes the fate of the cell-uptake, biodistribution and the subsequent toxicity patterns of nanoparticles.[100] One-dimensional nanomaterials with high aspect ratios, such as magnetic nanowire[101], alumina nanotubes[102,103], CeO_2 nanorods[104], silica NPs[106], silicon[107] and silver nanowires[108] displayed length-dependent acute toxicities (i.e. inflammatory response, lung fibrosis and organelle damage) shown in both *in vitro* and *in vivo* model systems as compared to their sphere forms. However, the impact of the aspect ratio on toxicity is hard to determine individually due to the interference factors generated from nanofabrication process. For example, the toxicity of gold nanorods comes from the use of growth-directing surfactant cetyltrimethylammonium bromide (CTAB);[105] while carbon nanotubes contain catalyst NPs as impurities, which also, at least in part, contribute to the length-dependant toxicity. In that respect, studies on other bio-inert inorganic fibre-like nanomaterials would make it possible to determine role of the aspect ratio of nanotoxicity. For example, we recently reported the toxicity study of anodic alumina nanotubes with aspect ratio ranging from 7.8 to 63.3, in which confirmed the toxic paradigms of high aspect ratio nanomaterials such as changes in cell morphology, pro-inflammatory responses and induction of apoptosis/necrosis.[102]

Surface chemistry and surface charge are two key parameters that determine the interactions of NPs with physiological environments. Considering the effect of surface chemistry, the reduced size of NPs down to nano-scale increases the number of atoms and crystal lattice defects on the surface of NPs, and thus enhances the surface energy and reactivity. The high surface energy can be released again by the formation of radicals such as reactive oxygen species (ROS) that causes DNA and protein damage.[118] The dissolution of toxic ions from NPs' surface, such as Zn^{2+} [113], Cu^{2+} [114], and Ag^{2+} [115] also generates serious organelle damage and cellular dysfunction. Nonetheless, the surface generation of ROS and dissolution of toxic ions are also dependent on the composition nature of nanomaterials. For example, CeO_2 NPs can suppress the ROS toxicity through surface oxidation of Ce^{3+} to Ce^{4+}.[109] Surface charges mainly contribute to the colloidal stability of nanomaterials, which is dominated by the colloidal forces (i.e. attractive van der Waals forces and repulsive electrostatic forces) at the solid–liquid interface. It is worth stressing that the charged surface with high surface energy adsorbs serum proteins, which constitutes a primary bio-nano interface determining the fate of the nanomaterials.[97] In cell culture experiments, adsorption of serum proteins facilitates the particle dispersion and changes the cell uptake of NPs from serum-independent to serum dependent; while animal studies prove that the adsorption of proteins contribute to the rapid clearance of NPs as well as the toxicity in major organs depending on the doses. [111,112]

Compared to single parameter analysis, more complicated is the combination of these factors (e.g. size, surface charge, etc.) together on a certain nanomaterial. Since each nanomaterial has its distinct toxicity pattern, it is difficult to generate a general principle for nanotoxicity evaluation. Furthermore, conflicting results between nanotoxicity studies are produced due to the inconsistency of experimental procedures employed in different laboratories. Therefore, the optimization and standardization of toxicological analysis is crucial to establish a comprehensive and coherent methodology for nanotoxicity studies. This problem has been reasonably addressed in the recent years by improving *in vitro* and *in vivo* high throughput sequence technologies and platforms, which have enabled a more coherent methodology to analyse complex nanotoxicity scenarios. These technologies provide powerful tools for the rapid establishment of the hieratical toxic levels of each toxic paradigm on a given nanomaterial.[94,116,117] In this section, we focus on the elucidation of mechanisms of nanotoxicity, aiming to shed light on the safe design of nanomaterials for clinical

applications. In addition, the recent researches utilizing intrinsic nanotoxicity for strategic cancer therapy will also be highlighted.

3.1 Mechanisms of Nanotoxicity

3.1.1 Oxidative stress, inflammation and genotoxicity

Oxidative stress, defined as an imbalance between production and elimination of intracellular ROS, can lead to chronic inflammation and genotoxicity, which in turn mediate most of chronic diseases including cancer, diabetes, and cardiovascular and pulmonary diseases.[118] As the product of a normal cellular metabolism (i.e. O^{2-}, H_2O_2, $OH^{\cdot}$ and NO), ROS play vital roles in signalling pathways of plant and animal cells in response to the intra- and extracellular environmental conditions.[119] ROS toxicity is one of the first identified predictive toxicity paradigms of nanomaterials, which has been extensively studied in the last two decades. Four different ROS generation by nanomaterials have been identified so far: 1) ROS are generated directly from the surface of NPs;[120] 2) transition metal ions catalyse oxygen metabolic products into more reactive hydroxyl radicals (i.e. $OH^{\cdot}$);[121-124] 3) NPs trigger mitochondria dysfunction, leading to an imbalance of the respiratory chain and disturbed ROS signalling;[125,126] 4) macrophages and neutrophils produce ROS when activated by NPs.[127] To eliminate the harmful pre-oxidant, cells utilize a complex system constituting of enzymatic antioxidants (e.g., glutathione peroxidase, glutathione reductase, catalase) and non-enzymatic antioxidants (e.g., glutathione, vitamins C and D).[118,128] However, unbalanced capability of intracellular ROS scavenging unavoidably leads to inflammatory reactions and DNA damage. (**Figure 12**)

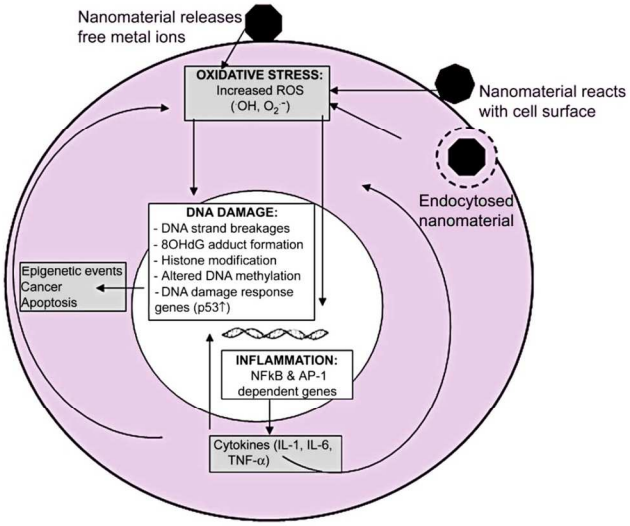

Figure 12 Schematic of cross-talks of mechanisms of oxidative stress, inflammation and geonotoxicity. Nanomaterials may result in oxidative stress or inflammatory responses that in turn have the potential to damage DNA and alter transcriptional patterns.[137]

Inflammation is a physiological process in response to cell / tissue injury and it is mediated by immune cells (i.e. macrophages, neutrophils and dendritic cells) that secret signalling cytokines, reactive nitrogen species. Although inflammation is an important protective defence against infection and injury, mechanistic studies revealed that the immunotoxicity of nanomaterials can trigger the activation of the inflammasomes (a group of intracellular multi-protein complexes that respond to exogenous stimuli) and immune responses.[129,130] Receptor meditated immune responses, including toll-like receptors (TLRs), NOD-like receptors (NLRs) and their related downstream signalling are also involved in the inflammation induction by various nanomaterials such as graphene oxide [131]. Once activated, inflammation response is characterized by the increased production of a number of cytokines such as tumor necrosis factor-α and interleukins, which lead to a cascade of immune reactions.[132-134] Severe consequences such as fibrosis and bronchial granulomas have been observed in test animals after instillation or inhalation of toxic nanomaterials such as carbon nanotubes at high doses due to the inflammation response.[127,135,136]

Genotoxicity is another critical toxic paradigm of nanomaterials. NPs with small sizes (<10 nm) can directly enter the cell nucleus and influence the function of DNA, which can cause genotoxic responses, such as chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts and alterations in gene expression profiles.[138] Larger NPs may access the during mitosis when the nuclear membrane dissolves. In addition, ROS generation induced by nanomaterials also contributes to DNA damage when the DNA repair machinery cannot counteract the ROS damage.[137] Recent comprehensive reviews have systematically documented the methodologies and research process of nanomaterial-associated genotoxicity.[137,139,140]

3.1.2 Dysfunction of Major Organelles: Autophagosome, Lysosome, Mitochondria and Endoplasmic Reticulum

The elucidation of the intracellular fate of nanomaterials shows that the transport of NPs is initiated by endocytosis pathways followed by the fusion of endosome with lysosomes for digestion. Lysosomal degradation pathway plays a vital role in balancing cellular homeostasis and cell function in that the hydrolytic enzymes and acidic environment in lysosome degrade intracellular pathogens (i.e. nanomaterials), damaged organelles and long-lived proteins.[141] Lysosomotropic agents, such as primary amine-based chemicals used to target lysosome have long been envisaged as chemotherapeutic agents due to their capability to rapture lysosome through proton sponge effect.[153] In the context of nanotoxicity, however, nanomaterials with proton buffering capabilities can serve as lysosomotropic agents to induce non-selective toxicity when uptaken by healthy cells. As a major toxic paradigm, a number of nanomaterials were documented to induce lysosomal dysfunctions, so-called lysosome membrane permeabilization (LMP). The release of lysosomal hydrolases such as cathepsin B, D and L are harmful to cells by initiating indiscriminate degradation of cellular components, which potentially leads to apoptosis. The lysosomal breakdown may also induce cytosolic acidification, which in turn induces cell death by necrosis.[142,143] Many studies have observed nanomaterial-induced LMP, including carbon nanomaterials[98,144], metal /metal oxides nanoparticles[145,146] and cationic NPs[147].

After endosomal escape or LMP, the released nanomaterials and lysosomal hydrolases can further generate inflammation, ROS and subsequently damage other major organelles

including mitochondria and endoplasmic reticulum (ER). (**Figure 13**) Mitochondria as the intra-cellular ROS generators and mediators play critical roles in many cell functions including ROS signaling, ROS generation/detoxification and programmed cell death[148]; Nanomaterial-induced mitochondrial dysfunction can cause the release of cytochrome c and activates caspase (caspase-9), which eventually lead to cell apoptosis. Different from the function of mitochondria, ER as the machinery of biosynthesis is responsible for intracellular calcium homeostasis, lipid synthesis and protein secretion. Interruption of ER leads to the accumulation of unfolded protein on ER, which activates a cell-rescue pathway so-called ER stress. The biomarker of ER stress was first identified in the early 2000, which generated great interest on the study of ER stress-related disease. However, it was not until in 2011 when researchers realized about the importance of ER stress on nanotoxicity. So far, ER stress has been treated as a biomarker of nanotoxicity in several nanomaterial models, including silver, gold, zinc oxide, polymeric NPs, and anodic alumina nanotubes.[95,102,126,151,152] Similar to the dysfunctions of lysosome and mitochondria, the interruption of ER can also lead to the disruption of cellular homeostasis such as mitochondrial-dependent apoptosis due to the up-regulation of cytosolic Ca^{2+} and ROS levels.

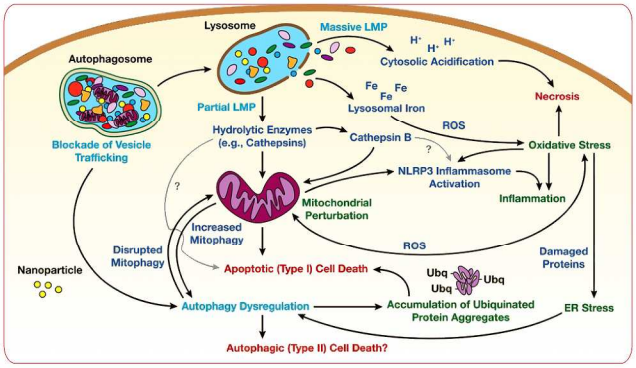

Figure 13 Mechanisms of autophagy and lysosomal dysfunction toxicity. The initiators of autophagy and lysosomal dysfunction toxicity, displayed in **light blue text** in the figure, include blockade of vesicle trafficking, lysosomal membrane permeabilization (LMP), and autophagy dysregulation. Toxic effectors (ROS, cytosolic acidification, hydrolytic enzymes, reactive oxygen species, and the NLRP3 inflammasome) are displayed in **dark blue**. Conditions resulting from effector-mediated loss of homeostasis (oxidative stress, inflammation, ER stress, disrupted mitophagy, accumulation of ubiquitinated protein aggregates, and mitochondrial perturbation) are displayed in **green**. Finally, this loss of homeostasis can result in the cell death pathways necrosis, and apoptotic (type I) and autophagic (type II) cell death; displayed in **red**.[150]

Finally, macro-autophagy, herein referred to as autophagy, is a homeostatic, catabolic degradation process which regulates the degradation of cytoplasmic material in response to various stress signals including those culminating from nanomaterial internalization.[154,155] The role of autophagy in nanotoxicity, however, can be either cyto-protective or cyto-destructive, since autophagic and apoptotic machineries share common signaling pathways. The formation of autophagosome can isolate toxic nanomaterials from other organelles. Nonetheless, the accumulation of non-degradable nanomaterials inside autophagosome may lead to autophagy dysfunction, defined as excessive autophagy induction or blockade of autophagy flux. Excessive cyto-protective autophagy turnover into autophagic cell death, which is characterized by the accumulation of autophagosome and caspase-independent cell death pattern.[156] Recent studies have identified that the outcome of nanomaterial-induced autophagy (cyto-protective or cyto-destructive) is highly depended on the physiological properties of the nanomaterials (i.e. size and surface chemistry) and experimental factors such as cell models, working dose and treatment time.[147,150,155,157] Therefore, autophagy research should be carefully optimized to minimize the experimental variation. A comprehensive review of nanomaterial-induced autophagy can be found elsewhere.[150,155,158]

In summary, the overall mechanism of nanotoxicity is related with ROS production, inflammation, geotoxicity and major organelle dysfunctions. Once the cellular homeostasis cannot counteract with the hostile impact of nanomaterials, a "domino effect" of cell signaling cascade and organelles' dysfunctions will follow, which will eventually lead to programmed cell death. The major mechanisms of nanotoxicity that we discussed above are summarized in **Table 3**.

3.2 Design of Materials for Safe and Efficient Drug Delivery Application

3.2.1 Strategies of making non-toxic nano-carriers

The toxicity of nano-carriers influences their maximum tolerance doses (MTDs) that can be used in practice. The elucidation of nanotoxicity paradigms provides direct benchmarks for designing non-toxic nanomaterials as drug carrier. A successful design of safe nanomaterials requires fully consideration of nanotoxicity paradigms, such as surface charge, surface chemistry, etc. (**Figure 11**) It is worth stressing that since nanomaterials have distinct physical and chemical properties, strategies to reduce the toxicity of nanomaterials should be discussed on a case-by-case basis.

In general, non-degradable inorganic NPs (e.g. gold, iron, silica and quantum dots) generate toxicities mainly due to three paradigms: 1) organelle dysfunctions caused by the intracellular accumulation of NPs, 2) ROS generation / oxidative stress and 3) dissolution of toxic ions. A recent review has systematically compiled the key effects of cytotoxicity induced by inorganic NPs, including gold, silver, iron oxide, zinc oxide and quantum dots.[207] After summarizing numerous results of nanotoxicity studies, the methods developed to overcome the cytotoxicity of inorganic NPs are virtually similar. Firstly, to avoid the unfavourable accumulation of non-degradable NPs inside cells and organs, working doses of NPs should be optimized according to their corresponding MTDs or median lethal doses. In addition, the physical sizes and hydrodynamic diameters of NPs are recommended to be less than 10 nm to permit complete and rapid elimination from the body.[167,168] Secondly, to prevent ROS generation, NPs' aggregation and dissolution of toxic ions, surface coatings are essentially required to cap the surface of NPs with a biocompatible layer via surface functionalization approaches. For example, PEGylation (the process of attaching PEG chains) is a well-established protocol used for surface functionalization. PEG polymers are FDA approved polymers formed by ethylene oxide in linear or branched structures.

PEGylation of NPs can efficiently prevent the direct contact of biological interfaces with reactive surfaces of inorganic NPs. Other surface functionalization strategies, including covalent and non-covalent functionalization, have also been widely used in the past decades. (**Table 4**) These versatile approaches not only make nanomaterials biocompatible, but also enabled the attachment of other functional chemical groups for preparing multimodal nanomaterials, in which the nanomaterials exhibit several functions such as bio-imaging, disease diagnose, cell targeting (selective binding /uptake through functional groups) and stimuli-responsive drug release.[169]

Unlike inorganic NPs, polymeric NPs contribute to the majority interest for gene and drug delivery due to their excellent biocompatibility and biodegradability. Biodegradable polymeric NPs (e.g. PLGA) can degrade into lactic acid and glycolic acid after administration. These degradation by-products can be eliminated by metabolic reactions cycles without inducing significant toxicity, although ROS generation and pro-inflammatory response were found at high working doses *in vitro*.[208] Note that biodegradable cationic NPs, such as cationic lipids, liposomes and polymers can trigger toxicities by acidifying endosomal-lysosomal compartment through the proton sponge effect (**Figure 5**). The dysfunction of liposome structure has potential to cause other cellular organelles' stresses, trigger inflammation as well as programmed cell death. Another cytotoxic paradigm associated with lipid is that some of lipids, such as derivatives of cholesterol, are protein kinase C (PKC) inhibitors, which may interfere normal PKC enzymes functions.[209] To overcome these problems, strategies have been developed to reduce the cationic charge density and replace the small molecule end-groups of lipid chain.[209,210] Non-degradable cationic polymers, such as polyethylenimine (PEI) are also promising materials as non-vial vectors for gene delivery. However, the non-degradable nature significantly hampered their practical application.[211] To facilitate the clearance of non-degradable cationic polymer, the size of polymer can be programmed to reduce by uing biodegradable linkers (i.e. acid labile ester) to bind short polymer chains into a longer chain.[212] Modifications of cationic polymers by addition of hydrophilic monomers and/or polymers, such as PEG is also an efficient way to reduce the excessive surface charges and prevent unfavourable interactions with serum proteins and red blood cells.

3.2.2 Optimization of pharmacological factors to enhance drug delivery efficiency

Our understanding of nanotoxicity in the last decade enabled the fabrication of non-toxic nano-carriers for drug delivery. However, the clinical success of nanomedicines requires further engineered designs for the optimization of pharmacological performances. The *in vivo* performance of nanomedicines has been mainly limited by their low stability and rapid clearance.[213] These two issues consequently lead to short circulation half-life, low efficiency of targeting and toxicity.

Driven by the motivation of improving pharmacokinetics of nanomedicine, numerous studies have been conducted to explore the impact of various physicochemical factors of nanomaterials on their biodistribution.[214] Recent studies have made key findings about the identification of protein corona to the biological fate of nanomaterials. Currently, it is well-accepted that the interactions of nanomaterials with biological barriers (e.g. reticuloendothelial system (RES)) are dominated by the bio-nano interfaces between the surface of nanomaterials and physiological environment. The surface of nanomaterials will be modified by proteins that existed in biological fluids (e.g. blood plasma) once nanomaterials are administrated into the blood stream.[215] Since nano-sized materials have high free energy on the surface, their surfaces tend to adsorb biomolecules in order to reach an equilibrium low-energy state. The formation of protein shells, so-called protein corona directly medicates establishes the dispersion and distribution of nanomaterials *in vivo*. Bio-analytical studies have revealed that protein corona contains a hard corona and a soft corona depending on the affinity and binding strength of proteins present in plasma. Proteins with high affinities bind tightly the nanoparticle surface, forming a relatively stable hard shell, while the proteins with loose affinities will form soft corona on the top of the hard one. Current studies of protein corona have revealed that the protein identities of the corona are dynamic in nature, which depends on the environments through which the nanomaterials are transported (e.g. blood streams and cell membranes).[216] As a result, the patterns of protein corona significantly differ from various types of plasmas and species. In addition, the features of protein corona are also distinct based on the physiological properties of nanomaterials (e.g. size, surface chemistry and surface charge etc.). The impact of these parameters on protein corona has recently been systematically reviewed.[217] The complexity of protein corona, similar to the patterns of nanotoxicity creates difficulties to generate a general rule to control the biological consequences associated with nanomaterials, since each type of nanomaterial in each biological fluid will have a distinct pattern of corona. The inconsistent reproducibility of nanofabrication and bioanalytical results are the major obstacles for the fundamental study of protein-corona and bio-nano interactions. Therefore, high-throughput and high sensitivity characterizations such as nuclear analytical techniques[218] will be required in the future to establish a complete library of protein coronas as a function of different nanomaterials and biological milieus. Such understanding will provide a powerful guidance for the precise manipulation of protein corona in order to better control the biological responses (e.g. cell uptake, targeting and immune response) at the bio-nano interfaces.

Although protein corona may favour the stability and biocompatibility of nanomaterials within biological fluids, it may have a negative impact on the nanomaterials' biodistribution.[219,220] Binding of complement and immunoglobulin promotes macrophage phagocytosis and activates immune system.[217] The complement system is part of innate immune system that assists phagocytic clearance of pathogens through a number of small proteins served as opsonin. Complement proteins, such as C3 and C5 will identify nanomaterials as foreign pathogens when adsorbed on their surface, resulting in the high rate of hepatic uptake and clearance. In addition, protein corona may also inhibit the recognition of legends (i.e. antibodies, proteins and peptides) that graft on the surface of nanomaterials as homing agent for active site-targeting. Therefore, reducing the formation of protein corona is required for the long time circulation of nanoparticles *in vivo*.

Overall, PEGylation is the most widely used approach for improving serum half-life of therapeutics and nanomaterials. The concept of PEGylation was first introduced in the late

Table 3 Summary of major mechanisms of the nanotoxicity-associated paradigms

Paradigms	Cause	Consequence	Reference
Oxidative stress	Nanoparticle (reactive surface, dissolution of toxic ions); LMP; mitochondria dysfunctions; activation of immune cells	ROS toxicity; damage of other organelles; induce inflammation and geonotoxicity; apoptosis	120, 124, 159, 160
Inflammation	Activation of TLRs and NLRs; uptake by immune cells; release of alarmins	NLRP3 inflammasome activation; release of cytokines	132-134
Genotoxicity	Nanoparticle interruption; ROS accumulation; Dissultion of toxic ions; inflammation	chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts and alterations in gene expression profiles	137-139
Lysosome dysfunction (LMP)	Proton sponges hypothesis; ROS toxicity; Increase of lysosomal pH; Distruption of lysosomal trafficking	NLRP3 inflammasome activation; release of ROS, ions and hydrolytic enzymes; induce other organells dysfunction; apoptosis	145, 150, 161
Mitochondria dysfunction	Mitochondria outer membrance depolarization; release of ROS;	NLRP3 inflammasome activation; autophagy induction; apoptosis	95, 125, 151, 162
ER stress	Unfolded protein accumulation of ER	Activation of ER stress signaling pathway and autophagy to balance homeostasis; apoptosis	152, 163-165
Autophagy dysfunction	Blockage of autophagy reflex caused by particle overloading; excessive autophagy induction	Apoptotic and autophagic cell death	150, 155, 166

Table 4 Representative surface chemistry strategies for modifying carbon nanomaterials

Functionalization Type	Methods	References
Covalent	polyethylene glycol	170, 171, 172
	polyacrylic acid	173 174
	polyethylenimine	175-177
	poly(N-isopropylacrylamide)	178
	chitosan	179-181
Non-covalent	Van der Waals force	182, 183
	Electrostatic	184 185
	Hydrogen bonding	182, 186-188
	Coordination bonding	189

1970s, and reached widespread application in the 1990s for liposome and polymeric NPs-based drug delivery systems. Besides the benefits of reducing toxicity of nanomaterials as we discussed above, the non-ionic hydrophilic property of PEG can provide stealth behaviour of NPs by minimizing the adsorption of opsonin and consequently diminish RES uptake. Grafting PEG brushes onto the surface of NPs also prolongs blood circulation by increasing hydrodynamic diameters of nanomateials for slowing renal clearance.[213] Many solid results have identified that the chain length/molecular weight of PEG and grafting density considerably influence the formation of protein corona and particle biodistribution. For example, one of the earliest reports from Gref *et al*. studied the influence of PEG layer thickness and grafting density on three different biodegradable nanoparticles, including PLGA, poly(lactic acid) (PLA) and poly(o-caprolactone). Their pioneering results showed that PEGylation significantly reduced protein adsorption (> 50%) especially apolipoprotein (A-IV and E) and complement protein C3. In addition, a steep decreasing in protein adsorption was observed when increasing the molecular weight of PEG from 2000 to 5000. When discussing the impact of PEG density, they found that even 0.5 wt% PEG on the surface was able to reduce protein adsorption by half as compared to the PLA reference particles. The most significant reduction of protein absorption was found in particles containing 5 wt% PEG.[221] Ten years later, Walkey *et al*. systematically studied the effect of macrophage uptake on gold NPs with differing particle size and grafted PEG densities. By using label-free liquid chromatography tandem mass spectrometry, they mapped over 70 most abundant proteins from protein corona. Their results showed that increased PEG density led to a monotonic decrease in total protein adsorption as well as a shift of cell-uptake pathway from serum dependent to serum independent.[222] Recently Lee *et al.* studied the impact of PEGylation on bio-distribution of filamentous NPs by using plant virus filamentous NPs as models. In their studies, PEGylation of high aspect ratio virus-like NPs effectively reduced RES clearance, minimized inflammation and improved overall half-life.[223] In summary, PEGylation as the most successful surface modification method have been applied in the majority of nanomaterial-based drug formulations used in clinical practice. No other synthetic polymer has reached this status.[224] A deeper insight of the pros and cons of PEGylation can be found in a review paper written by Knot *et al.*[224] Since the biodistribution and pharmacokinetics of nanomaterials directly impacts the toxicity and therapeutic outcomes, the physiochemical parameters of nanomaterials need to be carefully engineered on a case-by-case basis according to the specific requirements.

3.3 Engineered nanotoxicity for advanced disease therapy

Uncontrolled nanotoxicity leads to side-effects. To induce nanotoxicity in a selective manner by engineering nanomaterials, in contrast, may serves as advanced therapeutics. As we discussed above, autophagy as an intrinsic catabolic degradation pathway plays important roles on the development of MDR, cell longevity and programmed cell death. Precise regulation of autophagy behaviour by nanoparticles enables novel therapeutic concepts. Ling *et al.* demonstrated that the induction of autophagy can be manipulated by controlling the surface chemistry of carbon nanotubes. Autophagy induced by mTOR dependent and independent pathways has been realized by using a full library of 81 surface-modified carbon nanotubes.[111] Wei *et al.* recently reported the in vitro regulation of autophagy by europium hydroxide [$Eu^{III}(OH)_3$] nanorods for reducing protein aggregation. It is known that autophagy dysfunction contributes to neurogenerative diseases due to the lack of clearance of protein aggregates.[190] Their result, although not supported by *in vivo* studies, provide a proof-of-concept evidence that nanomaterials can regulate the longevity of cells for the therapy of neurodegenerative diseases such as Alzheimer's and Parkinson's disease.[149,162] Note that the nanomaterial-induced autophagy is not always of benefit for the longevity of brain cells. As an example, Chen *et al.* reported that CdSe/ZnS quantum dots induced autophagy-dependent synaptic dysfunction in mouse brains after intrahippocampal infusion. Treatment with autophagy inhibitors (wortmaninnin and chloroquine) can reverse toxicity by suppressing nanoparticle induced-autophagy flux and down-regulating synapsin impairments.[157] In addition, nano-alumina was found to trigger cerebrovascular toxicity by up-regulating of autophagy activity in the brain and elevating blood-brain barrier permeability.[191]

Autophagy also gained increased attention in the area of chemotherapy. Autophagy is intrinsically activated by cytotoxic drugs, and is one of the origins of MDR. The application of autophagy inhibitors such as chloroquine (CQ) in combination with other therapies is currently in clinical trials and has recently been translated into nanoparticle-based drug delivery systems.[192-194] In one study, nano-sized manganese (II) oxide (MnO) nanocrystal was utilized for magnetic resonance imaging (MRI) and combination chemotherapy. (**Figure 14**) The MnO nanoparticles can induce significant T_1-MRI contrast enhancement as well as high levels of autophagy, which is shown to be independent to tumor suppressor p53. In addition, suppression of autophagy by an inhibitor (i.e. 3-methylamphetamine), or inhibition of apoptosis by z-VAD-fmk reduced the cytotoxicity of MnO, providing evidence that autophagy is responsible for the MnO nanoparticle induced-cytotoxicity. More importantly, autophagy triggered by MnO was shown to have a synergistic effect when co-administrated with doxorubicin *in vitro* and *in vivo*. Such asynergistic effect of nanomaterial-medicated autophagy and anti-cancer drugs provides new concepts for cancer therapy. In another study, drug loaded PLGA nanoparticles were co-administrated with the autophagy inhibitor CQ for cancer therapy. PLGA nanoparticles were found to localize in the autophagosome for degradation after internalization. After autophagy inhibition, the drug delivery system showed significantly enhanced cancer killing ability *in vitro* and tumor suppression *in vivo*.[193,195] Recently, an interesting study demonstrated the anti-tumor effect of graphene oxide when injected intratumorally *in vivo*. Such effect was shown to be due to the cytotoxic autophagy induction through TLR signalling, in which the activation of TLR-4/9 up-regulates the autophagy machinery including

Beclin-1 and microtubule-associated protein 1A/1B-light chain 3 (LC3).[196]

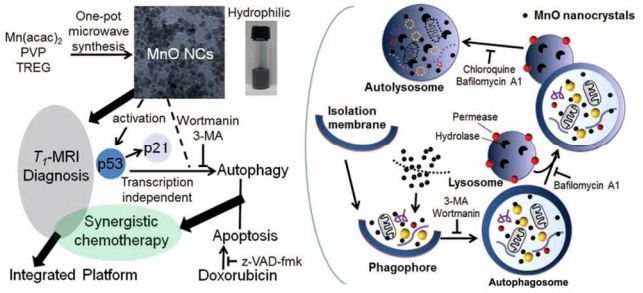

Figure 14 Schematic illustration of the preparation of MnO nanocrystals for the integration of MRI and autophagy induction for chemotherapy.[194] **Left**: MnO prepared by microwave synthesis showed enhanced MRI and induce cytotoxic autophagy, which is synergistic with doxorubicin for chemotherapy. **Right**: the process of autophagy consists of the formation of autolysosome to engulf nanoparticles and damaged organelles, which can be inhibited by 3-MA, wortmanin (inhibit the formation of autophagosome) and bafilomycin A1 (inhibit fusion of autophagosome and lysosome).

Finally, autophagy plays a critical role in immunotherapy by nanoparticle-induced tumor vaccination. Tumors escape defensive immune attack by a variety of mechanisms of immunosuppression, which inhibit the activity of antigen presenting cells, mainly dendritic cells (DCs) and cytotoxic T lymphocyte (CD^{8+} T cells). Anti-tumor immune responses must go through a process, so called cross-presentation, in order to generate protective T-cell responses to kill cancer cells. Cross-presentation of DC requires three distinctive steps to trigger adaptive immune responses, including antigen internalization, protein degradation and loading of antigen-derived peptides into major histocompatibility complex class I molecules (MHC I) presented at the surface of DCs. Autophagy as a protein degradation process facilitates MHC I presentation; fusion of autophagosomes with the MHC II–containing compartment, such as late endosomes and lysosomes, also enhances MHC II presentation of cytosolic proteins and viral antigens.[197,198] (**Figure 15**) Based on this mechanism, a novel therapeutic vaccine was development by utilizing alumina nanoparticles to transport conjugated antigen (ovalbumin) to autophagosomes of DCs.[199] Nano-alumina with 60 nm diameter triggered significant CD^{8+} T cell production as compared to TiO_2 and Fe_2O_3 nanoparticles. Importantly, autophagy suppression by 3-MA or wortmanin, knockdown of Beclin 1 and autophagy gene silencing (Atg 12) nearly abolished the cross-presentation of OVA, proving the essential role of autophagy for antigen-presentation. Mice injected with nano-alumina-OVA completely rejected tumors and remained tumor-free for more than 40 days. This study further demonstrated the advantage of using nano-alumina conjugated autophagosome to suppress tumor growth due to the higher containing of antigens than nano-alumina-OVA. Note that nanomaterial-based tumor vaccination has been extensively reported in the last ten years. Numerous nanoparticles have demonstrated the capability for effective immune activation by utilizing nanoparticle-induced immunotoxicity. Comprehensive strategies and mechanism of this application can be found elsewhere.[200,201]

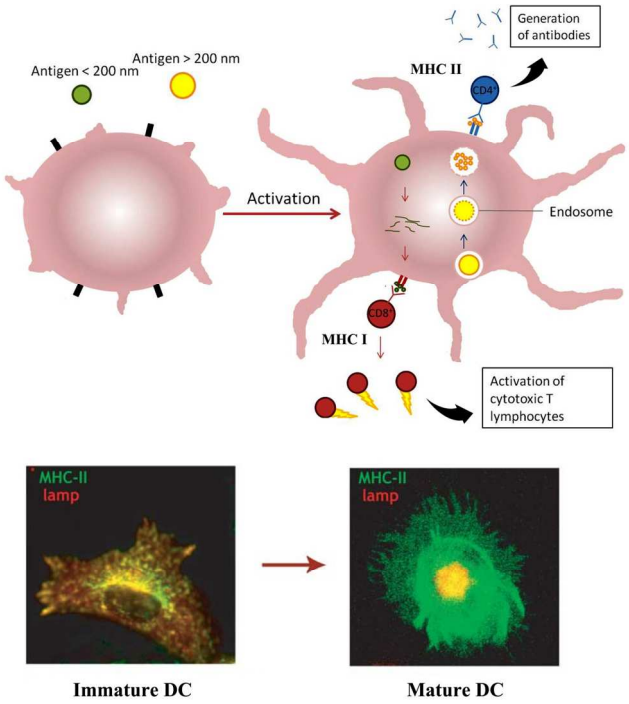

Figure 15 Scheme of introduction of immune response by dendritic cells. **Top**: Antigens which enter cells via endosomal pathways (blue arrows) are typically degraded within a vesicle before the contents is displayed on the cellular surface by MHC II receptors and recognized by CD^{4+} T cells. Alternatively, antigens present in the cytosol (red arrows) are broken down and presented on MHC I receptors, which are recognized by CD^{8+} T cells. **Bottom**: Immunofluorescence images of the mutation of DC, which showed a dramatic reorganization of cell formation and function. MHC-II-GFP is shown in green, the lysosomal membrane protein Lamp is shown in red. Immature DCd are highly endocytic and accumulate most of MHC-II molecules inside the cell in lysosomal compartments. Once maturation, MHC-II is transported to the plasma membrane to activate T helper cells (CD^{4+} T cells).[202]

4. Conclusion and Future Perspective

This review has summarized the concepts, mechanisms and recent progress on drug delivery and nanotoxicity research. From proof-of-concept to commercialization, our increasing understanding of drug delivery has dramatically promoted the commercial transition of nanomaterial-based drug delivery systems into the market for improving our well-being. After the success of the first generation of nanomedicines, the next generation of nanomedicines with improved site-targeting, stimuli-responsive drug release, and multimodal capacities are currently undergoing clinical trials.[24,54,203] However, the biosafety issue and the complex of bio-nano interactions in the human body could hamper the development of nanomedicines, and thus will continually gain interests from both researchers and pharmaceutical business in the future. With the maturity of nanofabrication and drug delivery techniques, personalized nanomaterial-based cancer therapy may address the future requirements of individual cancer patients such as drug cocktail selection, dynamic dose schedule and specialized nano-carrier

design according to disease symptoms. Furthermore, nanoparticles as a nano-sized tool provide new opportunities in dealing with biological science. Besides fighting cancer and disease, recent findings showed remote manipulation of glucose homeostasis in mice by using 5 nm iron nanoparticles to controlling ion channels through radio-wave heating with major implications in the fight against diabetes.[204-206] It can be envisioned that controlling advanced animal behaviour by bioengineered nanoparticles will be possible and highly influential in the future.

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