Nanoparticles and cells: good companions and doomed partnerships

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Engineered nanoparticles are emerging as useful tools for different purposes in life sciences, medicine and agriculture. Nanomedicine, an emerging discipline, involves the application of nanotechnology (usually regarded within the size range of 1–1000 nm) in the design of systems and devices that can facilitate our understanding of disease pathophysiology, nano-imaging, nanomedicines and nano-diagnostics. Among the different nanomaterials used to construct nanoparticles, are organic polymers, co-polymers and metals. Some of these materials can self assemble, and depending on the conditions under which the self-assembly process occurs, a vast array of shapes can be formed. Frequently, the nanoparticle morphology is spherical or tubular, mimicking the shape, but thus far, not the functions of subcellular organelles. We discuss here several representative nanoparticles, made of block copolymers and metals, highlighting some of their current uses, advantages and limitations in medicine. Nano-oncology and nano-neurosciences will also be discussed in more detail in the context of the intracellular fate of nanoparticles and possible long-term consequences on cell functions.

Nanoparticles, their properties and applications

Engineered nanomaterials, in particular nanoparticles, are beginning to be widely used for different purposes in agriculture,[1] automobile and other industries, inks, bar coding, electronic devices,[2,3] cosmetic products,[4] clothing and medicine.[5] Nanoparticles and other nanotechnological products are becoming increasingly sophisticated; these nanoparticles can be presented as products of four generations: (i) passive nanostructures, (ii) active nanostructures for multiplexing, (iii) more sophisticated nanosystems with interacting components and (iv) hierarchical systems potentially mimicking cellular functions. The fourth stage nanoparticles are still emerging, however, several examples have reached stage (ii) and (iii) and these will be briefly discussed in the following sections.

Engineered nanoparticles have unique properties, which are different from their individual constitutive components.[6–8] An abundance of biological, chemical, toxicological and other basic scientific and clinical facts are available for metals of groups II, III, V, VI in the periodic table. However, much less information is available about the nanoparticles (*e.g.* quantum dots) whose cores are made of these metals. The novel properties of nanoparticles are being exploited in many imaginative ways and the number of new constructs is increasing exponentially. Several studies provide evidence for marked differences in usability of different types of tailor made and commercial Q-dots for live cell and animal imaging. They suggest that short-term imaging does not seem to damage the tissue locally at the site of administration, but that multiple exposures to external light (from lasers and/or ambient light sources) can induce phototoxic cell killing. This suggests that fluorescent nanoparticles could be favorably used for tumor elimination but should be used with caution since the Q-dots can be sequestered to healthy tissues where, if degraded, they may cause local tissue damage.

Remarkable advances in the synthesis of Q-dots, design of new surfaces and coatings as well as their integration with biomolecules (reviews on Q-dots[9–12]) place Q-dots as nanoparticle forerunners in terms of reaching clinical diagnostic laboratories.[13,14] Significant advances have also been made in the field of biosensors based on Q-dots. Other nanoparticle types that can respond to various

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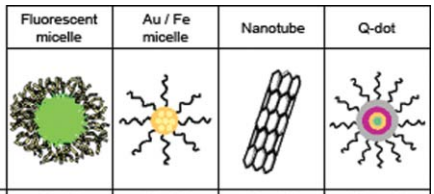
pathological or physiological stimuli are of particular interest for applied cell biology and medical investigations.[15–17]

Bioluminescent Q-dot conjugates for *in vivo* imaging were recently developed by Rao's group.[18] Instead of excitation from an external illumination source, these Q-dot nanocrystals are excited by bioluminescence resonance energy transfer and consequently significantly reduce the autofluorescence of tissue;[18] a lucrative approach using bioluminescent Q-dot conjugates, particularly in small animal imaging. Several types of nanoparticles employed for drug delivery and imaging are illustrated (Fig. 1).



	Fluorescent micelle	Au / Fe micelle	Nanotube	Q-dot
Diameter (nm)	20 - 50	20 - 80	20 - 100	20 - 100
Material core: / corona:	fluorescent dye / PEG	Au, Fe / PEG	carbon	CdSe, CdTe / PEG
Biomedical applications	drug delivery, imaging	imaging (TEM)	drug delivery	imaging (confocal, TEM)

Fig. 1 Nanoparticles used for drug delivery and imaging in medicine and some of their physical and chemical properties.

Nanoparticle types made of block copolymers containing fluorescent probes, gold, or super paramagnetic particles are among the most frequently employed in studies of subcellular distribution and the fate of nanoparticles. When covered with polyethylene glycol (PEG), they are usually innocuous; they enter cells passively and the core content can be visualized either by confocal (*e.g.* fluorescent micelles, quantum dots) or electron microscopy (gold and iron nanoparticles).

Dendrimers are one of the most beautiful and attractive classes of synthetic polymers with nearly perfect molecular structures. They were envisaged by Paul Flory in 1941,[19] but synthesized and characterized much later.[20,21] A wide range of dendrimers are used commercially and/or prepared in laboratories with modifications for specific applications.[22–24] Dendrimer characterization by various analytical techniques such as nuclear magnetic resonance, infrared spectroscopy, Raman spectroscopy, fluorescence, circular dichroism, X-ray diffraction, mass spectrometry, SANS, EPR, dielectric spectroscopy and others were reported.[25] Dendrimer–drug interactions[26] with an emphasis on drug entrapment within the dendrimer structure, dendrimer-based transfection agents[27] and dendrimers as carriers of contrast agents were also recently discussed.[28]

Among the most extensively studied and commercially available dendrimers are the poly(amido) amine PAMAMs. They were used for delivering anticancer agents, vaccines and genes. Depending on the type of dendrimer, route of administration, dose and cell type, they could be well tolerated when in contact with living cells. The biocompatibility and toxicity of dendrimers can be regulated by synthesis, particularly through judicious choice of functional groups at the periphery.[29] However, even these particular chemistries can only yield intrinsically "safe" dendrimers related to a specific application.[30] Some *ex vivo* applications of dendrimer formulations will most likely enter the market rather rapidly, but their approval for clinical use is much slower. Nevertheless, recent progress in the development of dendrimer-based nanocarriers combined with their superior physico-chemical properties are a strong impetus for considering these tree-like polymers as promising carriers of drugs and imaging agents as well as versatile materials for the development of sensitive bioassay systems.[22]

Nanoparticles for imaging and drug delivery in oncology and neuroscience

Promising applications of nanotechnology in oncology seem to be for passive and targeted drug delivery, tumor imaging and possibly biodiagnostics. Several examples are given here to illustrate these possibilities, but let us first consider "cancer nanotech" as defined by Ferrari, "cancer nanotechnology as a vast and diverse array of devices derived from engineering, biology, physics and chemistry, including nanovectors for the targeted delivery of anticancer drugs and imaging contrast agents and those detection systems such as nanowires and nanocantilever arrays under development of the early detection of precancerous and malignant lesions form biological fluids."[31]

The most advanced area of nanomedicine is the application of polymer-based nanomedicines for the diagnosis and treatment of cancer. These encompass polymer–drug conjugates and polymer–protein conjugates, polymeric micelles and multi-component polyplexes.[32–34] Q-dots conjugated with specific antibodies, ligands, small molecules or mimetics, which interact with specific cancer cell surfaces, are attractive options for tumor imaging. There are reasons why polymer–drug conjugates should be considered as viable approaches in cancer therapy:[35] first of all, linking the anticancer drug to a polymeric carrier can limit cellular uptake to the endocytic route and provide long-circulating drug reservoirs. Conjugates, which also contain targeting ligands such as antibodies, peptides or sugars, targeting particular cancer cells, could further promote tumor targeting by receptor-mediated endocytosis. Many polymers are not suitable for drug delivery, even if cell death is the ultimate goal, because although polymers may enhance the killing effect, target specificity is still lacking. A polymer moiety in a drug–polymer conjugate must be non-toxic. Several binding sites on the polymer is desirable so that it is able to carry an adequate payload, and the link between the two must be adequately strong, and not be broken *en route* to the tumor, but rather within the tumor. Lastly, intracellular delivery must be achieved if the target is a pharmacological intracellular receptor. A good example of such a delivery system is a conjugate containing doxorubicin linked by Gly-Phe-Leu-Gly, which releases the drug within 24 to 48 hours. In many cases, an intracellular delivery can be achieved only by polymers smaller than 100 000 g mol^{-1}, which is small enough to be internalized by many different tumor cells. Significant contributions from a number of laboratories were made in this regard.[32–39] Today, polymer–protein conjugates are used routinely as anticancer therapeutics, as an adjunct to chemotherapy and a component of combination therapy. There are several such examples: SMANCS (styrene maleic anhydride-neocarzinostatin), PEG-asparaginase, PEG-granulocyte colony stimulated factor, PEG-interferon 2alpha, 2beta, and others.[35] Polyglutamate-paclitaxel (PGA-paclitaxel) has already reached phase III clinical trials and is used for various cancers, particularly non-small cell lung cancer and ovarian cancer. Paclitaxel is linked

to PGA (Mwt 17 000 g mol^{-1}) and the conjugate contains a high drug loading (37 wt%). Enhanced permeability and retention (EPR)-mediated tumor targeting and the greater efficacy of PGA-paclitaxel was obtained in preclinical tumor models.[35] In phase III clinical studies, PGA-paclitaxel was compared with gemcitabine or vinorelbine as a first-line treatment for poor performance status in non-small cell lung cancer and the studies showed that some side effects were reduced. An ongoing study should provide comparative data for PGA-paclitaxel conjugate and non-conjugated paciltaxel in women with non-small cell lung cancer. Several comprehensive reviews on polymer conjugates as anticancer nanomedicines provide a useful platform for further directions in exploring polymeric drugs in oncology.[35,36,38]

Polymeric micelles, self-assembled nanoparticles from amphiphilic block copolymers, provide a unique core-shell architecture wherein the hydrophobic core serves as a natural carrier environment for hydrophobic drugs and the hydrophilic shell provides particle solubilization and stabilization in an aqueous environment. Improved drug delivery and cancer specificity can be achieved by active targeting whereby the corona contains a cancer-specific marker. Integrin alpha v beta3 is a molecular target highly expressed in angiogenic endothelial cells in many solid tumors. Gao's group has recently developed multifunctional polymeric micelles as cancer-targeting, MRI-ultrasensitive drug delivery systems.[40] The study presents an elegant approach to imaging and therapy of tumors. The micelles are composed of three components: (i) chemotherapeutic agent doxorubicin: released from the core in a pH-dependent manner; (ii) RGD ligand that recognizes alpha v beta3 integrins on the tumor endothelial cell surface and (iii) superparamagnetic iron oxide nanoparticles (SPION) within the micelle core for magnetic resonance imaging (MRI) detection. High loading density of SPION (up to 50 w/w%) allows detection of micelles at nanomolar concentrations. The uptake and intracellular distribution of these micelles was assessed by flow cytometry and confocal laser scanning microscopy in SLK endothelial cells, which express a high number of integrin receptors. These *in vitro* studies are promising and require further testing of RGD-doxorubicin-SPION micelles in *in vivo* animal models. The approach is worth testing for other chemotherapeutic agents in other tumor types in combination with non-invasive tumor imaging by MRI.

Nanotubes and nanowires are being developed and tested for biomolecular nanosensing.[41,42] Carbon nanotube properties range from semi-conducting to conducting, single or multi-walled, and they can change their properties by aggregating and forming different heterogeneous forms. An example of cancer biomarker PSA using microcantilevers was reported[43] but nanosensing devices in cancer based diagnostics are only developing and are not ready for routine use.

Unique photophysical properties of quantum dots make them promising candidates for molecular imaging of tumor and other cells and tumors in whole animals and eventually humans. Their sustained luminescence, high fluorescent yield, small size and functional flexibility are only some of the key features that indicate the potential of Q-dots as contrast agents for *in vivo* cancer detection and imaging (Fig. 2). Nie's group's report was among the first examples of *in vivo* cancer targeting and imaging with semiconductor quantum dots.[44] The study shows encapsulated luminescent Q-dots in an ABC triblock copolymer and functionalization of the corona with a tumor-targeting ligand, PSMA monoclonal antibodies. As a model system *in vitro*, the authors used human prostate cancer cells and then grew them in nude mice. Q-dot-PSMA specific uptake and retention, non-specific uptake, blood clearance and organ distribution were examined. The results showed that few or no Q-dots were detectable in the brain, the heart or kidney and that the presented multicolor fluorescence imaging of cancer cells *in vivo* was clearly possible. However, further modifications and improvements of Q-dot properties are required to be suitable for imaging in large animals. One of the reasons for relatively poor uptake of one class of Q-dots was an excessive negative charge on the probe surface, *i.e.* carboxylic groups on the polymer coating. In contrast, when compared to the photophysical properties of common organic dyes, Q-dots had significantly longer excited state lifetimes (20–50 ns) than organic dyes (about 1 ns) and pointed towards the usefulness of Q-dots in longer imaging sessions.[45]

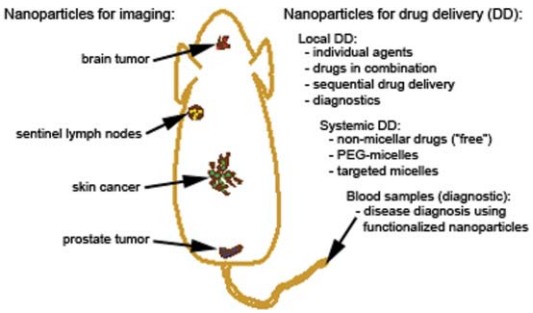


Fig. 2 Nanoparticles for tumor imaging and drug delivery in oncology.

In summary, recent studies point towards the application of multimodal Q-dots for molecular cancer diagnosis and therapy. In addition, multifunctional nanoparticle probes could enable surgeons to visually identify small tumors or other small lesions and facilitate removal of the undesirable growth more effectively. Nanoparticles such as micelles could deliver a cocktail of anticancer drugs in a more controllable manner than individually injected components. Finally, a combination of photo- and MRI imaging could provide an elegant non-invasive tool for localization of small tumors, guidance for surgical procedures and evaluation of chemotherapeutic efficacy. The realization of practical applications of these multi-functional nanodevices requires careful research of a multidisciplinary nature and is ongoing in several laboratories.

Neuroscience is another emerging area where nano-technology is beginning to make marked progress. Due to the complexities and intricacies of the nervous system, nanoparticles have tremendous potential to be exploited in order to address some of the key biological questions, such as those related to neuronal repair and degenerative processes. To date, there are only few such examples. The application of nanotechnology provided an insight into fundamental processes such as single molecule trafficking (*e.g.* NMDA and glycine receptor[17,46]), very basic information on fluorescent labeling of model neural cells (PC12 cells) and primary neural cells from cortical cultures (neurons and glia).[47,48] Attempts have been made to track nanoparticle fate in neural cells[47–50] and nanofiber scaffold for axon regeneration with the aim to return functional vision[51] has been reported.

Although currently available Q-dots provide excellent (though primarily non-functionalized and non-specific) tools for molecular imaging in real-time in cells, their application for real-time imaging in animals is limited because they require excitation from an external illumination source to fluoresce, resulting in considerable autofluorescence; and at high laser powers there could be non-selective tissue damage at the illuminated site. To avoid these obstacles So *et al.* have devised nanoparticles which self-illuminate, thereby avoiding the need for an external light source.[52] The authors took advantage of *Renilla* luciferase, which is constitutively active (when substrate is provided) as it can show exactly where Q-dots are located (if the enzyme has not detached or been cleaved on its way to the desired site). Although this approach is a very appealing tool for basic research, the major limitation for *in vivo* imaging is the uneven tissue distribution of coelentrazole (the luciferase substrate needed for generating photoluminescence), which can cause errors in interpreting signal intensities. Hence, this approach can be used for small animal imaging to qualitatively assess the sites of Q-dot distribution and serve for real-time imaging with minimal background because the excitation/emission wavelengths are outside the range of strong absorption/emission of tissue biomolecules (*e.g.* hemoglobin). Since luciferase-functionalized Q-dots are still not a satisfactory tool for real-time animal imaging and eventually clinical studies, alternative approaches must be developed. Multifunctional nanoparticles providing optical detection together with MRI or PET are emerging tools complementing the existing armamentarium of luminescent quantum dots.

Nanoparticles and their intracellular fate

Despite the remarkable development of nanoscience, relatively little is known about the interaction of nanoparticles with biological systems. This is an emerging area of research bridging chemistry, physics, biology and medicine. An example of intracellular location of Q-dots and labeled organelles is shown in Fig. 3.

The internalization of block copolymer micelles and Q-dots involves endocytosis.[53–55] Endocytotic pathways involve pinocytosis, caveolae, clathrin, and caveolae–clathrin independent processes. The best characterized pathway is clathrin-dependent endocytosis involving a number of accessory factors,[56] their regulation of specific proteins and their phosphorylation status. Other approaches used to explore nanoparticles and macromolecule endocytosis are genetic mutations and pharmacological manipulations.

Physical stability of nanoparticles is a fundamental requirement for an effective drug delivery system. In contrast to low molecular mass surfactants, and natural micelle-forming biomolecules, polymeric micelles exhibit significantly lower values for the critical association constant (CAC), indicating greater thermodynamic stability. The rate at which the micelles tend to dissociate is related to their composition, their physical state and the cohesion of the micelle core. Micellar stability correlates well with the length of the hydrophobic segment in the amphiphilic copolymer, with higher proportions of hydrophobic polymer conferring greater thermodynamic stability. Recent studies by Savić *et al.* demonstrated marked differences in PCL-b-PEO micelle stability depending on the microenvironment.[57] Fluorogenic dye incorporated into micelles was rapidly converted into the fluorescent agent in serum-containing medium and *in vivo*. These studies are the first to show PCL-b-PEO micelle instability in live animals.

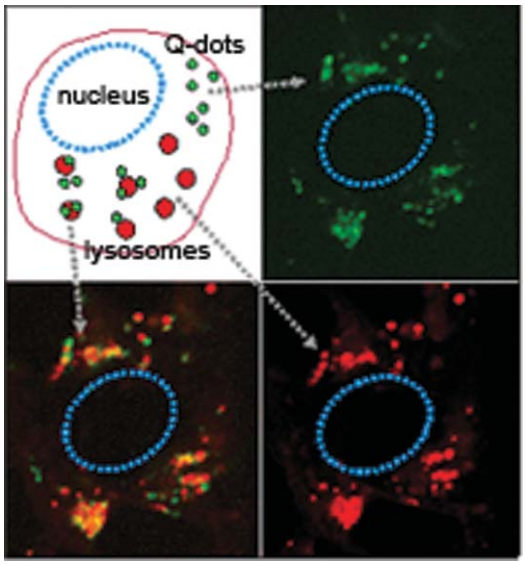


Fig. 3 Multiple labeling of cell and intracellular location of Q-dots: confocal micrographs of MCF-7 cells treated with green Cys-CdTe QDs (10 μg mL^{-1}). Top left: a schematic representation of the cellular compartments observed. Top right: visualization of green QDs in the intracellular compartment. Bottom left: staining of lysosomes with red fluorescent lysotracker dye. Bottom right: overlay of Q-dots and lysosome micrographs.

Intracellular trafficking and fluorescence imaging of micelles has been recently reviewed.[58] Ultrasensitive and high-resolution microscopic techniques are beginning to provide insights into the real-time dynamics of cellular components and macromolecular pharmacological agents as they are delivered into and travel within single cells. By combining genetic manipulations of cells with fluorescent markers and labeling of individual cellular organelles, the journeys of nanoparticles and the intricacies of their interactions with cellular components within the single cells are being elucidated.

Uncapped or inadequately capped Q-dots are not very stable and can produce reactive oxygen species (ROS). Consequently, ROS can damage multiple organelles (Fig. 4). Electron spin resonance spectroscopy, which is suitable for *in vitro* studies, can be cumbersome with tissues and cells, therefore, a battery of fluorescent dyes is used for ROS detection. All of these dyes have limited specificity and must be used with caution. Dichlorodihydrofluorescein diacetate detects nearly all ROS nonspecifically and is useful in preliminary screening. Generation of superoxide can be detected by dihydroethidium (mainly detects superoxide anion), and singlet oxygen as detected by singlet oxygen sensor green. Extracellular and intracellular cadmium concentrations are difficult to measure but they can be determined by using ion selective electrodes, fluorescent kits or atomic emission spectroscopy.

Nanoparticles in biological fluids (*e.g.* plasma) become coated with proteins and as such can exert biological effects. Therefore, comprehensive studies will be needed to explore protein–nanoparticle interactions and their consequences. In this context we need to gather information regarding the binding affinities and stoichiometries for different protein–nanoparticle combinations.

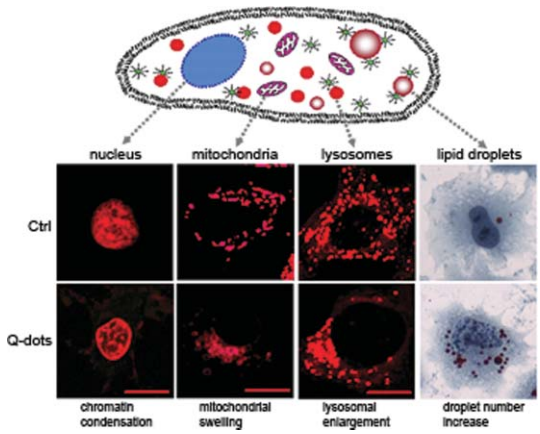


Fig. 4 Organelle states and functions under normal conditions and under oxidative stress. Top: schematic representation of the cellular compartments observed. Top row: organelles under normal conditions. Bottom row: changes in organelles exposed to quantum dots for 24 hours. Column 1: nuclei are deformed and often have condensed chromatin. Column 2: mitochondria are swollen and often localized in the perinuclear region. Column 3: lysosomes are swollen in Q-dot treated cells. Column 4: lipid droplets are more abundant and enlarged in CdTe Q-dot (10 μg mL^{-1}) treated cells than in the untreated controls. Cells were stained with Draq5 (0.5 uM, nucleus), MitoTracker deep red (0.5 μM, mitochondria); LysoTracker DND 99 (0.5 μM, lysosomes and oil red O (1 uM, lipid droplets).

Kinetics of nanoparticle association and dissociation with proteins and the concurrent exchange processes with free proteins play an important role in determining the interactions of protein-modified nanoparticles with biological receptors and the resulting biological effects. The lifetimes of typical protein–protein complexes range from microseconds to weeks, and protein–ligand complexes typically have lifetimes spanning microseconds to days. To gain quantitative data for the lifetimes of nanoparticle–protein complexes, several methods have been employed (*e.g.* isothermal titration calorimetry, surface plasmon resonance or gel filtration[59]); and many others are currently being developed.

Relatively little is known about the fate of fluorescent nanoparticles in different cell types and in the whole animal. This area is relatively new, and many fluorescent dyes or nanoparticles do not have sufficient stability and are not resistant to photobleaching upon multiple exposure to lasers. The synthesis of fluorescent polymers is not a trivial matter, and some dyes simply cannot be conjugated to the polymer. Additional problems include the autofluorescence of the tissue and a limited resolution of available instrumentation for *in vivo* imaging. An additional factor complicating the determination of the fate of biodegradable block copolymer micelles and Q-dots *in vivo* is the interference associated with components from blood and other biological molecules. One of the first studies with fluorogenic dyes incorporated into micelles demonstrates how the increasing complexity of the biological environment impacts on the micelle disintegration.[57] Further studies with fluorogenic dyes may yield a product emitting in the near-infra region, and might provide an important tool in the quest for new information on micelle fate *in vivo*.

The first studies on Q-dot pharmacokinetics were done by Ballou *et al.*[60] and more recently by Fischer *et al.*[61] The latter studies showed that Q-dots with lysine and bovine serum albumin (BSA) have considerably different distribution patterns, uptake in different organs, and clearance. In contrast, the volume of distribution was comparable for both types of Q-dots (66–68 mL kg^{-1}). Half-lives for the two types of Q-dots were deduced from inductively coupled atomic emission spectroscopy (ICP-AES) by measuring cadmium concentration correlated to the Q-dot concentration. This approach provided an indirect measure of Q-dot tissue *vs.* plasma concentrations; better approaches are needed for such studies.

Q-dots that can emit infrared or near infrared light are particularly suitable for deep tissue imaging because autofluorescence of hair and tissues in this range is minimal. Q-dots emitting within 650–800 nm conjugated with polyethylene glycol (PEG) and specific ligands recognizing receptors could be particularly useful. If administered intravenously, the liver does not immediately eliminate PEG-Q-dots; their protracted circulation permits adequate time so that their fate may be followed using different imaging set-ups. Injected Q-dots with PEG coatings or functionalized Q-dots can be detected with standard *in vivo* imaging systems for several days when administered subcutaneously or intravenously. *In vivo* monitoring of Q-dots is appealing because it can provide information required on time-dependent Q-dot distribution and accumulation in tissues, which is important in the evaluation of potential therapeutic applications. Data for the biodistribution and pharmacokinetics of Q-dots is emerging and more systematic studies are needed to demonstrate how rapidly these particles can be eliminated from the body, where they accumulate, and what non-specific tissue damage they may eventually cause.

Nanoparticles and their adverse effects on cells

Nanoparticles can exert a multitude of effects in biological systems and individual cells. The kind and extent of these effects depend on physico-chemical properties of nanoparticles, their concentration, duration of contact with cells, cellular and subcellular distribution and cell/tissue types. In addition, the cell status before and during the nanoparticle exposure also plays an important role. For example, the same type highly luminescent, purified nanoparticles in relatively low nanomolar or even picomolar concentrations suitable for bioimaging in a short contact with cells may not cause any noticeable change on or in these cells. In contrast, the same cells exposed to the same nanoparticles but for a prolonged time period or preconditioned so that they are more vulnerable to such a mild stress, could result in marked impairments of cellular functions. The most common mechanisms associated with nanoparticle-induced cell death are apoptosis and necrosis,[62] but there are many more forms of cell death. In addition, a degree of damage can lead from one type of cell death to the other. For instance, short term exposure to uncapped CdTe Q-dots (5 ug mL^{-1}) in the presence of serum does not kill almost any cell type but in the long-term presence of these unprotected CdTe Q-dots, there are multiple intracellular damaged sites[63] showing both apoptotic and necrotic cells. Cells in a nutrient enriched medium and not in a fast metabolizing state will generally deal well with small insults, and once the nanoparticles are removed they will recover. In contrast, these same particles under starvation/serum deprivation are sensitized to Q-dot insult and die in different ways, including apoptosis and necrosis. Common characteristic changes in cells undergoing necrotic cell death are: mitochondrial swelling

and clustering in the perinuclear region, calpain and cathepsin activation, lysosomal and plasma membrane rupture. Necrosis was long considered as an uncontrolled process. However, evidence is emerging that mitochondrial dysfunction, enhanced generation of reactive oxygen species, ATP depletion and membrane ruptures do not occur haphazardly. By looking at the morphological changes in Q-dot-induced cell death, one can often notice a transition from apoptosis to delayed necrosis. Nondividing cells such as neurons, in complete chemically defined medium supplied with trophic factors, vitamins, antioxidants and serum, usually cope with pegylated nanoparticles well and do not die, at least not within a few days (unpublished observation). In contrast, if cells are "preconditioned" thereby made more vulnerable to an additional insult (*e.g.* malnourished, hypoxic), they will be severely damaged in a concentration- and time-dependent manner. Oxidative stress is considered an important contributor to nanoparticle-induced cell death and several organelles respond to it in different manners.

An interesting example of organelles "sensing" stress induced by nanoparticles is a lipid droplet. This organelle has been mainly considered as an energy storage site and it was extensively studied in adipocytes. However, most cells produce lipid droplets at some point in their life. Lipid droplets seem to play multiple roles in different cell types and under different conditions. In Q-dot-treated cells, exposed to oxidative stress, their number and size changes (unpublished observation). An example of oil red O stained lipid droplets in PC12 cells treated with Q-dots is shown (Fig. 3). Studies on the role of lipid droplets in the physiology of various cell types is already a blossoming area of research[64] and may well become an attractive new direction in nanomedicine.

Due to their multiple roles in cell death and survival, mitochondria and individual mitochondrial proteins are also targets for drug development in different areas of medicine, *e.g.* cancer and arthritis therapies, cardiovascular, and neurodegenerative diseases.[65–68] A summary of the effects of Q-dots on mitochondria and other organelles is illustrated (Fig. 5) and discussed in several studies.[56,63]

Lysosomes are organelles commonly associated with paradigms of necrosis and apoptosis.[69,70] Lysosomes and lysosomal hydrolases participate in the engulfment and digestion of dying and dead cells and in cellular/tissue autolysis during necrosis. Christian de Duve was the first to define lysosomes as "sacks, suicide bags" in 1955, and ever since then their role was repeatedly confirmed in different cell death paradigms. Among the signaling pathways, PI3K plays a prominent role as demonstrated by using wortmannin; which caused the swelling of the perinuclear lysosomes and mis-sorting of cathepsin D in secretory granules.[71] The lysosomal compartment is a target dealt with in drug development of anticancer therapies. In this context, Rabs, Sigma 2-receptors, microtubules, and HSP70 are the proteins of particular interest as targets.[72] The limited availability of drug delivery systems of polypeptide, DNA or RNA therapeutics is a big problem in targeting lysosomes. Nanoparticle-based drug delivery systems with target-peptidomimetic moieties may offer novel ways of tackling this problem. Our limited knowledge about the mechanisms of nanoparticles impacting on living cells and in the whole body raises concerns about possible adverse effects on biological systems. We are beginning to explore their effects at local sites and in individual cells and how interactions with subcellular organelles could mimic or cause changes in cellular functions (Fig. 5).[73]

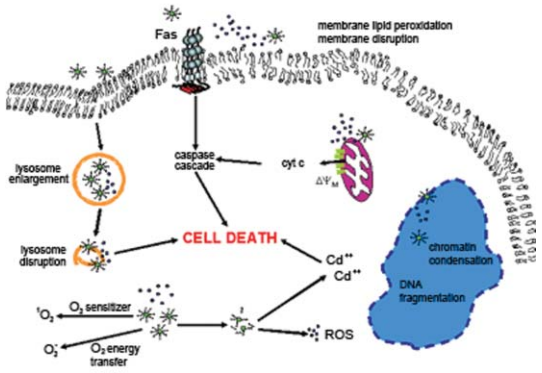


Fig. 5 Proposed mechanisms of Q-dot induced cell death. Exposure of cells to Q-dots (*e.g.* uncapped and/or destabilized CdTe or CdSe) often results in the generation of ROS. ROS can be detected both extracellularly and intracellularly. They can affect plasma membranes leading to lipid peroxidation and to Fas upregulation. Apoptotic cell death is induced by activation of Fas and its downstream effectors (*e.g.* caspases). Depending on the cell status, duration of exposure to nanoparticles and physico-chemical characteristics of the nanoparticles, *e.g.* positively charged Q-dots, lysosomes can become enlarged, destabilized and eventually disrupted. Lipid peroxidation also occurs at the mitochondrial membranes, degrading cardiolipin, changing the mitochondrial membrane potential, eventually leading to the release of cytochrome c, and promoting apoptotic cascades. Low nanomolar concentrations of Cd ions or ROS interacting with nuclear components can induce epigenomic changes. These are particularly relevant for the chronic exposures to barely detectable intracellular concentrations of nanoparticles and their integral components.

An intriguing question in nanomedicine and the nano-environment is that of a nanohazard. Wide disagreement between the two extreme views, *i.e.* they are safe and they are dangerous, is exacerbated by the lack of knowledge of nanoparticle–biological system interactions, especially by those which cannot be easily detected by robust techniques. For instance, our most recent studies strongly suggest that even small, hardly detectable or even undetectable concentrations of quantum dots can cause epigenomic changes.[74] Epigenetic changes may have long-term effects on gene expression, programmed long after the initial signal has been removed, and if these changes remain undetected they could lead to long-term untoward effects in biological systems. Nanoparticle or metal ion-induced oxidative stress leading to cell death has been previously reported at the genomic level, but has not yet been investigated at the epigenomic level.[75] Cells undergo chromatin condensation in response to insult by cadmium and selenium, both common elements constituting Q-dot cores. Epigenomics, a new scientific discipline merging epigenetics and genomics, provides new insights into our understanding of genetic regulation and its role in cellular growth, differentiation, cell death, diseases and aging.[76] Epigenetic variations operate through methylation of cytosine nucleotides in DNA or post-translational modification of histones such as acetylation, phosphorylation, methylation and sumoylation, all of which may be involved in modulating gene activation and expression.[77] Epigenetics can also be understood as the mediator of interactions between the environment and genetics.[78] Q-dot-induced oxidative stress has been well established in different cell types,[63] however, their effects on the epigenome, including histone modifications (acetylation and methylation) and DNA methylation is only beginning to be

examined. Our studies with easily degradable cadmium telluride quantum dots show that aside from genotoxic effects, nanoparticles can cause more subtle, epigenetic changes and that these merit thorough examination of environmental nanoparticles and novel candidate nanomaterials for medical applications.

In summary, the novel and unique properties of nanoparticles, which have been enthusiastically explored for their advancement as potential therapeutics and diagnostics, could also be the source of undesirable effects on biological systems.[79,80] The factors that play important roles include nanoparticle size, chemical composition, surface structure, shape, solubility and aggregation. Our most recent studies showed that both gold nanoparticle-containing micelles and indium-galium phosphate (InGaP) nanoparticles aggregate in the presence of serum proteins and these can be detected by confocal and electron microscopy both in the intracellular compartments and outside the cells. Most of the non-functionalized and PEG-nanoparticles end up in lysosomes. However, if functionalized they can easily reach other intracellular locations. For example, Hoshino *et al.*, achieved subcellular targeting with luminescent Q-dots conjugated with nuclear and mitochondria-targeting ligands.[81]

Are we there yet? Current status and future directions

Nanoparticles have a great potential for diagnosis, therapy and biosensing in medicine.

A current problem is how to establish a testing platform for predicting and assessing the potential hazard of new and available nanomaterials: shall we just apply common sense and apply toxicological tests as for any other drug? Or shall we treat them with additional rigor to increase the probability of correct predictions?

My personal view is that first and most importantly we should not exaggerate the good or bad properties of nanoparticles. Secondly, there is no general approach on how to treat different types of nanoparticles. Surely, they should be screened for biocompatibility but we do not need excessive and unnecessary additional tests if they are either applied in extremely low picomolar concentrations and for a short time. In contrast, stringent testing should be applied to diagnostic nanoparticles if they are to be repeatedly introduced into the human body. Cumulative effects of multiple administered small doses of nanoparticles cannot be ignored due to their possible epigenomic effects and sequestration in some organs.[61] Lastly, the most promising application of nanomaterials, including fluorescent, luminescent, superparamagnetic and other types of nanoparticles, functionalized or not, may be for diagnostic and analytical purposes by using biological fluids and easily accessible tissue samples in combination with functionalized nanoparticles for biosensing.

A partnership between nanotechnology, medicine and the environment is in many ways similar to others, beginning with fascination and high hopes. We have embraced nanotechnology with enthusiasm and we are also becoming aware of the potential hazards by some, but not all, of the new nanomaterials. The examples of the current uses, advantages and limitations of nanomaterials in medicine discussed herein, show that our increasing understanding of the possible adverse effects of nanoparticles in the human body and in our environment will help us to overcome hurdles and gain benefit from the companionship between nanomedicines and humans rather than witness their doomed relationship with live cells.

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