

**NanoPharmaceuticals Online Journal, Vol 1, Oct, 2006**

# Death by Nanoparticles

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**Received on 22<sup>nd</sup> Sept 2006, Published on line on 1st Oct 2006**

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## **1. Abstract**

Nanoparticle technology has been put to a staggering variety of uses. The ever-accelerating technology, however, is generating an important cause for concern,

namely that some nanoparticles, such as those already used in the manufacture of electronic equipment, luminescent paints etc., could become environmental hazards. Only relatively recently, however, nanoparticles have begun to catch the interest of life scientists. They are expected to have an impact in basic research and in the development of new technologies for medical diagnosis and drug delivery. To date, nanoparticles such as luminescent quantum dots, iron nanoparticles and block copolymer micelles have been studied as nano-containers for controlled and/or sustained drug delivery or as imaging tools for cells and whole organisms. Our objective has been to determine the impact of nanoparticles on cell function, with a focus on those particles which contain metals, fluorescent labels and selected drugs. A brief overview of the currently available data on the potential hazards and usefulness of selected classes of nanoparticles will be provided, with some comments on possible strategies for optimizing the latter. Induction of cell death by nanoparticles will be discussed in the context of physico-chemical properties of selected nanoparticles, cell status and the cellular environment.

## 2. Introduction

As the exploitation of nanoparticles accelerates we are beginning to understand some of their possible hazards as well as their enormous potential for economic growth, improved quality of life, and medical diagnosis. However, nanoparticle research driven by rational applications must also address the management and elimination of hazardous particles in order to protect our health and our environment. A word of caution must be noted particularly because of the seductive quality of some intellectually very attractive nanoparticles. Nanoparticles with different morphologies made of non-biodegradable materials provided invaluable information on their self-assembly and physicochemical properties, but were rarely tested *in vivo*. Unfortunately these nanoparticles can constitute threats to living cells, animals or humans. Working as multidisciplinary teams [1] we will be better poised to select appropriate materials, modify nanoparticle surfaces, identify optimal routes of administration, and understand the pharmacodynamics which will allow a safe use of nanoparticles [2]. Currently, metal-containing nanoparticles, including quantum dots, are still largely an attractive chemical, pharmacological and medical toolbox but not a clinical solution for diagnosis or therapy.

Recent reviews have provided instructive examples of nanotechnology application in basic neurosciences and its use in addressing interesting biological questions [1-13].

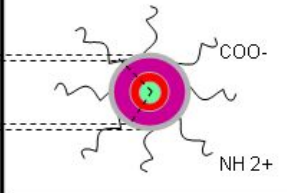
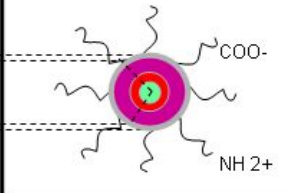
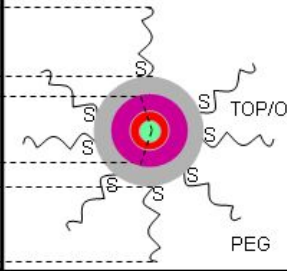
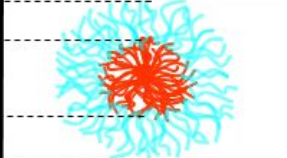
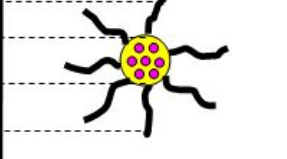
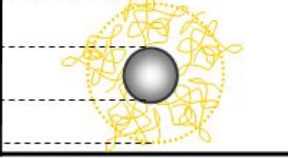
	QD "unmodified"	Core material	Surface molecules	seize (nm)	$\lambda$ em (nm)
bandgap material		PbSe	kationic: core + cystenamide	~ 2-5	green
		CdTe		~ 5-10	red
bandgap material		ZnSe		~ 5-10	red
		CdSe	anionic: core + 3-mercapto propionic acid	>10	deep red NIR
<b>QD "modified"</b>					
surface molecules		CdTe	TOP/O ZnS (20 layer) + Trioctyl phosphine/ oxide	~ 20-30	green
		CdSe		~ 20-30	red
			- OH (Hydroxy) - COOH (Carboxy) - PEG (Polyethylen- glycol)	~ 20-30	deep red NIR
surface molecules		PCL goly (caprolactone)	- PEG - PAA goly (acrylic acid)	~ 20-30	variable
surface molecules		Au 4VP (vinylglyridine) (3-5 nm)	- PEG - PAA	~ 20-30	>540
surface molecules		Fe (9-10 nm)	- PEG - dextran - PVA goly (vinyl alcohols)	~ 30	~594

Figure 1. Summary of different classes of nanoparticles and their characteristics

### 3. Nanoparticles

#### 3.1 Nanoparticles containing metals

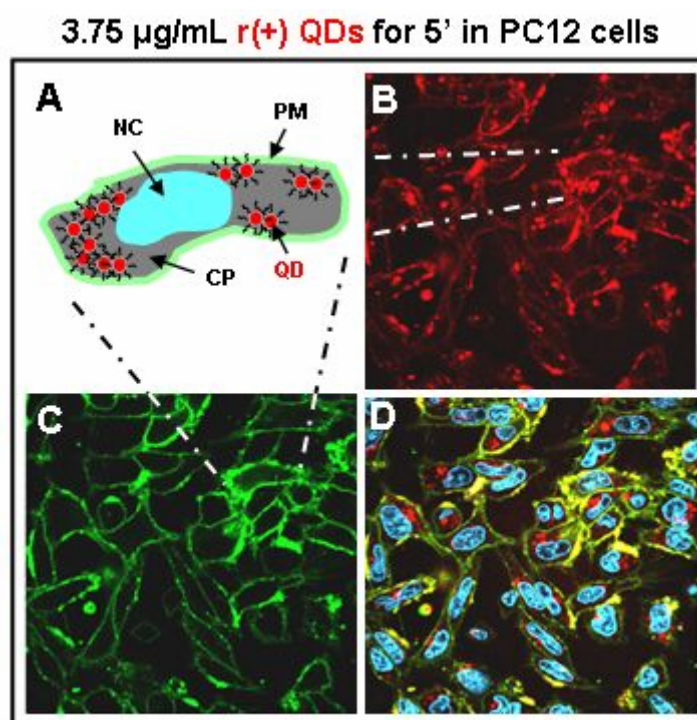
Nanoparticles made of metals e.g. quantum dots, QDs or metallic particles linked with polymers endows them with unique optical properties. This section reviews some of their specific photo-physical and optical characteristics which are exploited for long-term and multicolor imaging [14, 15] and provides examples of metallic nanoparticles used as research tools in biology, pharmacology and medicine (Figure 1).

QDs can be excited at a single wavelength far removed from their emission maxima, which are tunable by the nanoparticles composition and size. This allows for the simultaneous detection of multiple color QDs upon illumination with single light source. QD absorption spectra are broad, but emission spectra are narrow without the red tail characteristic of organic dyes. QDs are rather resistant to photo bleaching [4], which is one of the major drawbacks of currently used fluorescent dyes. The electron dense QD core allows their detection by electron microscopy. Combined fluorescence and electron microscopy analyses using the same multipotent probe provide both temporal dynamics and high-resolution intracellular localization [16]. Furthermore, QDs have an efficient multiphoton absorption cross-section making them suitable for *in vivo* imaging [17]. An example of a multicolor imaging of live cells with organelle-selective dyes and QDs is shown in Figure 2. QDs' wide variety of emissions allows the use of numerous combinations of fluorescent dyes and QDs for nanoparticle tracking within cells. QDs 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 [18, 19]. Several QDs emitting within 650-800 nm are commercially available (e.g. QD 655, QD 705; QD 800) and some of them can be obtained as conjugates with polyethylene glycol (PEG). Such conjugates seem particularly useful; when administered subcutaneously, they remain localized at the site of injection for several days. If administered intravenously, PEG-QDs are not immediately eliminated by the liver and their protracted circulation time allows their fate to be followed using different imaging set-ups. Injected QDs with PEG coating or functionalized QDs can be detected with common *in vivo* imaging systems for several days when administered subcutaneously or intravenously [14-16]. *In vivo* monitoring of QDs is appealing because it can provide needed information on time-dependent QD distribution and accumulation in tissues, important in the evaluation of potential therapeutic applications. An accumulation of QDs due to the enhanced permeability and retention (EPR) effect in tumor tissue could enhance efficiency of phototherapy and, if the luminescence is satisfactorily high, could coincidentally allow for monitoring of tumor-size reduction or spread of metastases. Such studies have been already initiated in several laboratories [20, 21, 22]. Data for biodistribution and pharmacokinetics of QDs are not currently extensive 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.

A variety of ligands have been attached to QD surfaces, including thiol-containing molecules [23, 24] peptides [25], polymerized silica shells with polar groups, amphiphilic diblock or triblock copolymers and phospholipids [26]. For tumor targeting, multiple specific ligands such as folic acid and others especially suitable for different types of tumors can be conjugated to the QD surface [27, 28]. Bioluminescent QD conjugates for *in vivo* imaging were recently developed by So's group [19]. Instead of fluorescent excitation light, these QD nanocrystals are excited by bioluminescence resonance energy transfer and consequently tissue auto fluorescence is significantly reduced [19]. This complementary approach using

bioluminescent QD conjugates could be especially useful for small animal imaging.

There appear to be no studies on the stability of QDs either at the immediate site of administration or after longer times post injection at remote sites. Due to its inorganic character the QD core is relatively resistant to photo degradation, but prolonged exposure to UV light can affect its integrity [29, 30]. In general QDs with ligands, which are resistant to photo oxidation are better suited for long-term cellular or *in vivo* imaging. Photo oxidation of surface ligands takes place upon prolonged UV illumination of QDs. However, increasing the thickness and packing density of the ligand shell can delay the onset of photo oxidation [30]. Taken together, the QD stability is essential to optimize their performance as imaging tools and to ensure their compatibility with living cells (see sections 3 and 4 for discussions).



**Figure 2. QDs *in vitro* - principle of cell labeling**

Photomicrograph of PC12 cells treated with QDs and stained with organelle-specific fluorescent dyes. Schematic of cell organelles stained with Hoechst 33342, DAF and internalized QDs (red) (**A**) Photomicrograph of QDs obtained by confocal microscopy (**B**) Plasma membrane (green) labeled with DAF (**C**) Superimposed signals from QDs (red) and plasma membrane (green) are seen as yellow suggesting a presence of QDs associated with plasma membrane (**D**). There are no QDs within nuclei (blue).

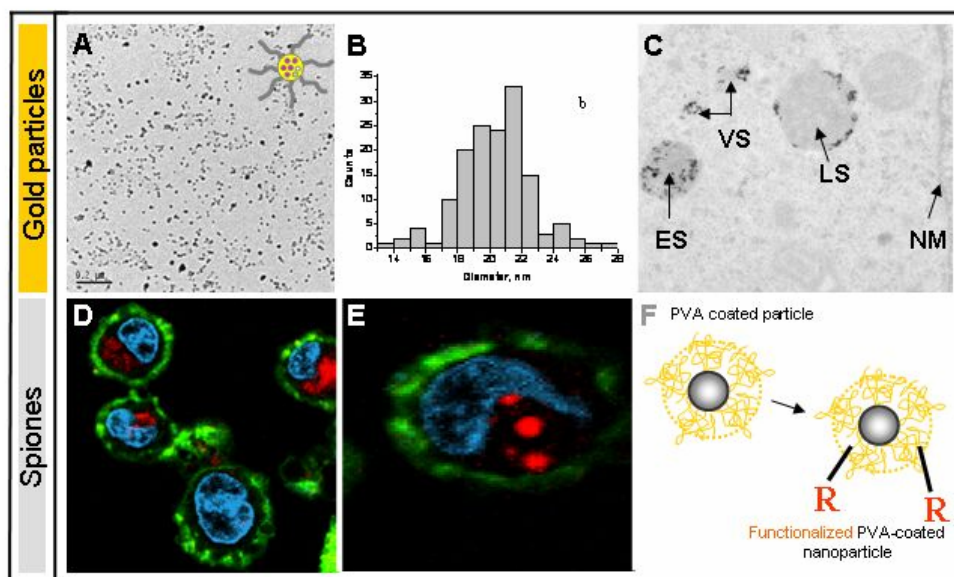
Abbreviations: nucleus, NC; cytoplasm, CP; plasma membrane, PM

Another class of metal-containing nanoparticles includes that made of gold. Gold nanoparticles have been traditionally used as labels for immunocytochemistry and in conjunction with electron microscopy they allow detection of selected proteins with high resolution. In such labeling studies the gold nanoparticles are integral parts of the secondary antibody. This labeling is usually achieved by attaching gold nanoparticles of different sizes to IgG molecules which bind to the primary antibodies. Although this approach can reveal specific intracellular location of up to three proteins

at the same time, the quantification of the small (5 nm), medium (10-120 nm) and large (30-50 nm) gold nanoparticles associated with the individual proteins of choice is rather tedious and therefore is not frequently used. In practice, single protein gold labeling is one of the “gold standards” in life sciences/cell biology.

We were interested in developing gold-labeled micelles that would allow us to assess the fate of block copolymer micelles in different cells (Figure 3). To this end we used block copolymer micelles containing covalently bound nanoparticles. [31, 32]. A schematic presentation of such a nanoparticle is given in Figure 1 and Figure 3. An advantage of gold-labeled micelles compared with other nonmetallic nanoparticles (e.g. silica or styrenes) is that their distribution can be detected with transmission electron microscopy (TEM), which provides information about their location within the cells with high resolution. The disadvantage of EM is that the tissues or cells have to be fixed. Moreover to obtain a dynamic picture of nanoparticle distribution using TEM is rather time consuming and tedious.

Supraparamagnetic iron nanoparticles (spions) labeled with fluorescent dye Cy5.5 (Figure 1 and Figure 3) [33] allow dual imaging. Live cells are imaged by confocal microscopy to gain a dynamic picture of cell-nanoparticle interaction and fixed cells are imaged by TEM to gain information on nanoparticle subcellular distribution at the high level of resolution. Thus, iron and QD nanoparticles serve as complementary tools to standard fluorescent dyes and provide a versatile means of assessing their fate both by confocal and electron microscopy [14, 26, 34]. Spions without conjugated fluorescent dyes have been examined *in vivo* [35, 36].

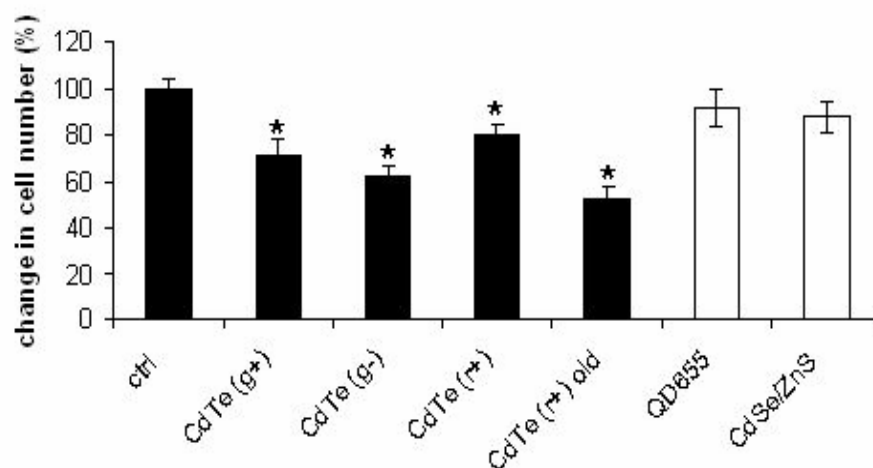


**Figure 3. Types of nanoparticles and their location in cells**

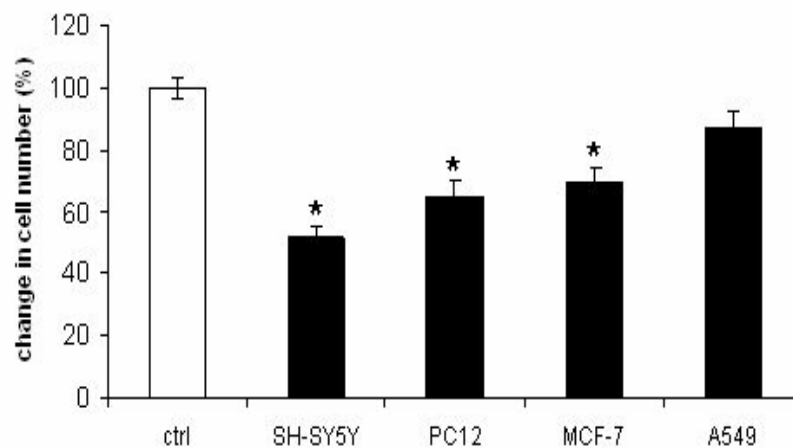
Gold and superparamagnetic iron nanoparticles in the cells. Transmission electron micrograph of gold nanoparticle (A) and their size distribution (B) Gold nanoparticles in lysosomes and endosomes (C) Iron nanoparticles labeled with Cy5.5 taken up by cells can be visualized by confocal microscope (D and E). Schematic of a functionalized spion (F). (For details see ref 31 and 33

Abbreviations: ES-endosome, VS-vesicle, LS-lysosome, NM-nuclear membrane.

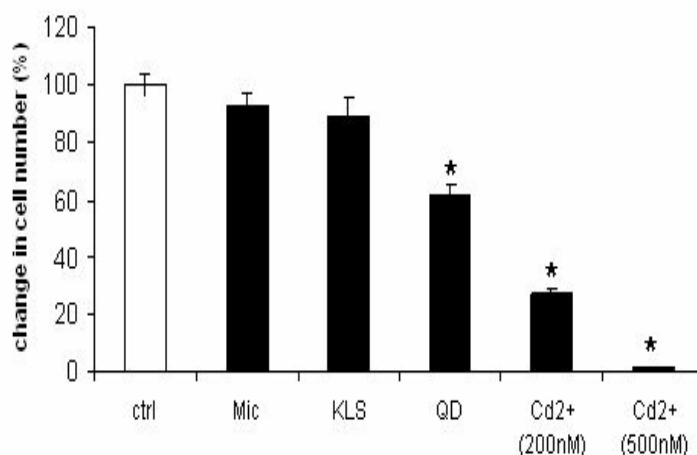
### A: PC12 cells - CdTe and Cd/Se QDs; biocompatibility



### B: Different cell types are differently susceptible to CdTe QDs



### C: Different nanoparticles are not equally tolerated by PC12 cells



**Figure 4. Types of nanoparticles and their location in cells**

Gold and superparamagnetic iron nanoparticles in the cells. Transmission electron micrograph of gold nanoparticle (**A**) and their size distribution (**B**) Gold nanoparticles in lysosomes and endosomes (**C**) Iron nanoparticles labeled with Cy5.5 taken up by cells can be visualized by confocal microscope (**D** and **E**). Schematic of a functionalized spion (**F**).

Abbreviations: ES-endosome, VS-vesicle, LS-lysosome, NM-nuclear membrane.

#### Fluorescent nanoparticles

To follow the fate of a nanoparticle alone or nanoparticle containing fluorescent dyes or drugs, several groups have used fluorescent labeled polymers in combination with organelle selective fluorescent dyes [12, 37-40]. Although, fluorescent dyes are useful for visualizing micelles at selected time points, the tracking of micelles, particularly in time-lapse experiments using confocal microscopy is considerably more difficult because of dye bleaching.

Relatively little is known about the fate of fluorescent nanoparticles in the whole animal. One of the reasons for this is that the quantities required for *in vivo* imaging are significantly larger than those used for *in vitro* experiments and some fluorescent dyes are expensive. Moreover 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 access to suitable instrumentation for *in vivo* imaging. Finally, a factor complicating the determination of the fate of biodegradable block copolymer micelles *in vivo* is the interference associated with components from blood and other biological molecules plus the polymer degradation products within individual organs and cellular compartments. One of the first studies designed to assess the fate of fluorogenic dyes incorporated into micelles in complex biological media was recently reported [41]. The findings showed a gradual increase in fluorescence at the site of micelle decomposition, which is due to the conversion of the fluorogenic dye to the fluorescent product. Fluorogenic dyes, which could yield a product emitting in the near-infra region, would be very useful in the quest for new information on micelle fate *in vivo*.

The problem of micelle stability can be partly circumvented by cross-linking the micelle shell. However, complete control of drug release from such micelles becomes an issue to be resolved for each individual drug. A significant contribution in development and utilization of shell-cross-linked nanoparticles for tumor imaging is has been made by Wooley's group [42]. Shell cross-linked knedel-like (SCKs) nanoparticles are core-shell nanospheres inspired by biological structures (e.g. virus capsids) and optimized for drug packaging. Stable SCKs are obtained by cross-linking the shell layers of micelles made of amphiphilic diblock copolymers. The cross-linking procedures yield SCKs with well-defined sizes, surface charges, shapes and functionalities. SCKs can be labeled with fluorescent, radioligand or dense core particles and decorated with different ligands for cell or tissue targeting [43-46].

In summary, using both fluorescently labeled agents and fluorescent polymers one can distinguish micelle-incorporated agents from free agents, and obtain information about their intracellular location [40]. Studies using new imaging approaches and tools are already beginning to emerge [41, 47-51]. It seems certain that the use of nanoparticles with high fluorescence/luminescence in combination with advanced imaging equipment will soon provide needed information relevant to the *in vivo* condition.

#### **4. Cell death induced by nanoparticles**

##### **4.1. Nanoparticle properties and cell death**

Once cells internalize nanoparticles they often undergo degenerative changes and eventually die. This is independent of the mechanism of their entry (e.g. non-specific endocytosis [4] or specific endocytosis [52]). Micelles with polyethylene glycol (PEG) corona, purified to satisfy the stringent requirements for work with biological systems, are mostly non toxic in low concentrations [12, 40]. In contrast, polyethylene imines are often toxic (PEI) [2]. Non-functionalized QDs mostly end up in the cytoplasm. However, functionalized QDs with the Nuclear Localization Signal (NLS) peptide or the mitochondrial localization signal peptide enter the nucleus and mitochondria [53]. Peptide modifications on QD surface make them relatively well tolerated by most cells.

The toxicity of QDs when tested *in vitro*, is dependent on numerous factors associated with QD properties and cell status. QD properties, which are critical for their biocompatibility, are: size, surface charge, type of ligand attached to the surface, stability, and concentrations. Properties determining the cell status are associated with the cells themselves and their environment. Thus, the fate of cells exposed to nanoparticles will depend on: their tissue of origin and differentiation state, their proliferative status (doubling time) and their redox state. In addition to this, plating cell density, composition of the medium, presence and absence of serum or defined trophic factors in chemically defined media will be also critical to the final outcome. The combination of these physical and chemical factors of QDs, the cellular environment, and the status of the cells determine cell fate: survival, death or differentiation (Figure 5).

Depending on the kind of nanoparticles, the type of cells, the duration of exposure, the concentration of nanoparticles and the conditions under which cell-nanoparticle interaction takes place, different cells will either "tolerate" nanoparticles or succumb to their "attack". In a brief contact with cells, particles with surfaces well-protected by polymers or proteins, are generally not very harmful or are totally benign [15].

However, the breakdown of protective coating (e.g. by irradiation, low lysosomal pH or possibly metabolic degradation) can lead to cell damage and death [29]. The cellular environment plays a critical role in influencing physiological status of cells and has an impact on their response to QDs. For example, cells exposed to QDs are more vulnerable when deprived of growth factors, whereas cells cultured in the presence of serum will be more resistant to QDs induced cell death. Moreover, certain types of QDs can interact with serum and other biological fluid components reducing or increasing their damaging effects on cells. In general, short-term exposure (1-4 hours) and low concentrations (nanomolar range) of nanoparticles is well tolerated by living cells. Under certain circumstances, nanoparticle degradation "on command" could be desirable and a number of different types of such nanoparticles have been prepared and tested. (e.g. nanoparticles responding to changes in pH, in temperature or in redox potential). Significant advances have been made in this field and a number of biosensors based on QDs and other nanoparticle structures exist that can respond to various pathological or physiological stimuli [54-56].

#### 4.2. Types of cell death and roles of individual organelles

Cells can die by apoptosis, autophagy or by necrosis ("accidental but aggressive" death) [57]. The mode of cell death depends on the type of insult, its intensity and duration. The most common mechanisms associated with nanoparticle-induced cell death are apoptosis and necrosis. For instance, the long-term exposure to unprotected CdTe QDs, which are internalized by cells, will cause damage at multiple cellular sites [58]. Cells in a nutrient enriched medium and in a low metabolic state will generally handle small insults well and once the nanoparticles are removed they will recover. In contrast, cells under starvation/serum deprivation conditions will be sensitized to the QD insult and will die by apoptosis, autophagy and/or necrosis. Looking at morphological changes during QD-induced cell death one can often notice a transition from one mode of death to the other (e.g. apoptosis to delayed necrosis).

Programmed cell death is most frequently associated with the term "apoptosis", but there is emerging evidence for "programmed necrosis". Several recent reviews cover different aspects of classical and "nonclassical" types of cell death [59, 60] and provide an overview of consensus terms to be used in research associated with cell death and dying [61]. A brief account of the damaging effects of some nanoparticle types (e.g. QDs) on different organelles is provided in the following section.

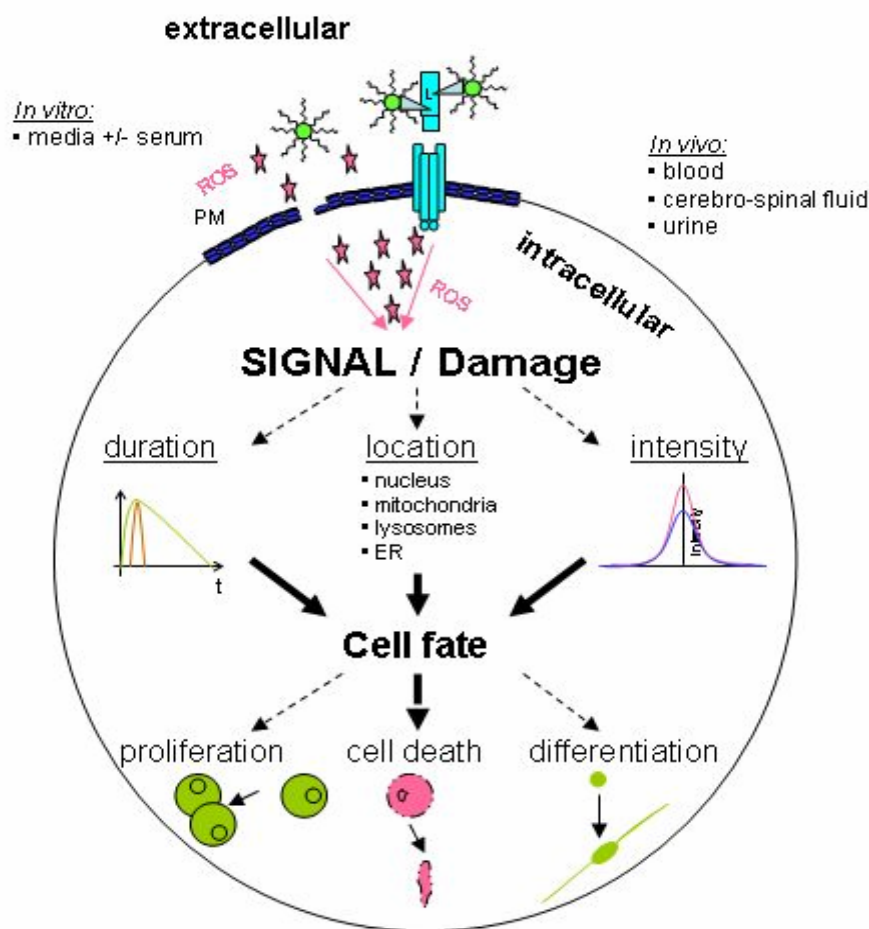
*Mitochondria* play a central role in energy metabolism and they are responsive to even small stresses in multiple ways [62]. When cells are exposed to nanoparticles (e.g. QDs) which can induce generation of ROS, mitochondria are among the first and most sensitive organelles affected. In QD-insulted cells, reduction of mitochondrial membrane potential and swelling of mitochondria can be detected [63] (Przybytkowski, unpublished observation). Mitochondria and their associated proteins, which serve multiple roles in cell death and survival, are emerging as potential targets for drug development in different areas of medicine, e.g. cancer and arthritis therapies, and cardiovascular and neurodegenerative diseases [64-67]).

*Lysosomes* are organelles commonly associated with cell death [68, 69 and cited in them refs]. Lysosomes and lysosomal hydrolyses play a role in the engulfment and digestion of dead and dying cells and in cellular/tissue autolysis during necrosis. Studies showing the involvement of lysosomes in cell death, induced by either

pharmacological or genetic manipulations, were recently reviewed [69 and refs cited in them]. Among the signal transduction pathways, communicating with lysosomes, PI3K seems to play a prominent role as demonstrated by use of wortmanin. This drug causes the swelling of perinuclear lysosomes and missorting of cathepsin D in secretory granules [70]. The lysosomal compartment is a theme in drug development of anticancer therapies. In this context, Rabs, Sigma 2- receptors, microtubules, and HSP70 are proteins of particular interest as targets [69].

*Endoplasmic reticulum* (ER) is another organelle at risk in cells exposed to certain types of nanoparticles. The ER is a sensor for oxidative stress [rev. in 71, 72, 73]. If the ER damage is extensive, programmed cell death is initiated by the unfolded protein response or by the release of calcium. The Bcl-2 family of proteins and cytoplasmic calcium orchestrate the cross talk between the mitochondria and the ER [74, 75].

It is important to emphasize, that even if cells are not killed during exposure to QDs and even if there is no apparent damage to the cellular structures, they may respond by a change in some cellular functions. One example of this is change in size and number of lipid droplets present in the cytoplasm (Przybytkowski, unpublished observation). Although lipid accumulation has been considered harmful, there is evidence that it could have adaptive functions and protect against lipotoxicity [76]. The role of lipid droplets in cell function is still not clear, but it is becoming a new and attractive area of research and may open unexpected possibilities for new therapeutics [77].



**Figure 5. Mechanism of cell death induced by nanoparticles**

The fate of cells exposed to nanoparticles depends on their extracellular and intracellular environment. Moreover, the signal duration, location and intensity will determine whether cells will proliferate, die or differentiate.

## 5. Mechanisms of nanoparticle-induced cell death

Different mechanisms can be involved in inducing nanoparticle cell death depending on nanoparticle properties, dose and duration of cell exposure (Fig. 4). [68, 78-81]. Two main causes for induction of cell death by nanoparticles (QDs) have been proposed: (i) formation of reactive oxygen species (ROS) [63, 82-84], (ii) release of metals ( $\text{Cd}^{2+}$ ,  $\text{Te}^{2+}$ , or  $\text{Se}^{2+}$ ) from the core of nanoparticles with subsequent direct cell damage [85].

### 5.1. ROS and cell death

QDs could generate reactive oxygen species by electron transfer to molecular oxygen. Cells are able to respond to even small changes of intracellular redox status which is

sufficient to inhibit proliferation and induce differentiation [86]. The type of ROS formed strongly depends on the material used to build QDs [87]. A number of fluorescent probes have been used to reveal ROS in live cells. Dihydroethidium has been used to detect superoxide anion [63, 82, 88], Singlet Oxygen Sensor green is used for detection of singlet oxygen species which can also be detected by electron spin resonance spectroscopy (ESR) [83, 88, 89]. Dichlorodihydrofluorescein diacetate detects nearly all ROS nonspecifically and it is particularly useful in the preliminary screening to detect if an insult causes ROS formation. ROS are well known inducers of damage to cellular proteins, lipids, DNA and carbohydrates, and can cause apoptosis or necrosis depending on the severity of damage. Recently, we have provided evidence for the production of ROS in live cells incubated with surface-unprotected CdTe QDs [63, 89].

## 5.2. Metal ion-induced cell death

Materials from which nanoparticles are often formed include metals such as lead, iron, selenium, tellurium, indium and gallium. Under certain biological conditions the protective layer on the surface of the QDs can be damaged and ions can leak out. Cadmium and selenium can interfere with the cell redox system. Analyses of thioredoxin in oxidized and reduced states by western blot analysis, using antibodies, which recognize these two states of thioredoxin, provided insights into the extent and time course of its conversion from one to the other in the presence of these ions [62, 90-94].

Cd cytotoxicity is generally not significant with multilayered ZnS capping, but it can occur with single layers or non-capped QDs in biological media. Released Cd ions, even at very low nanomolar to micromolar range, can cause cell damage. Defined concentrations and time of maximal damage by QDs depends on the cell type and cell status: healthy, non-starved, starved etc. We have determined extracellular and intracellular Cd concentrations in several cell types [89] after 24 hour treatment with different types of QDs (Figure 4). Mechanisms by which Cd can induce cell death are reviewed elsewhere [95, 96] and illustrated here (Figure 4).

In summary, cell death by nanoparticles is a relatively unexplored area of research. Further studies are required to understand the mechanisms involved in nanoparticle cell death, and to find the ways to prevent it. We should also keep in mind that nanoparticles could have an impact on cellular function even when they are not toxic. Studying all these effects will be important to the safe preparation, handling and utilization of nanoparticles in science and beyond.

## 6. Conclusions: Nanoparticles; prospects and perils

Developments of new nanotechnologies are gaining momentum and claims are made that they will dominate the economy of the 21<sup>st</sup> century. The application of these technologies already extends to solar technology, new means of transportation, telecommunications, medical diagnostics and devices. However, the clearly evident huge potential benefits of nanotechnology in our society will only be achieved if we apply it sensibly and learn how to reduce potential health risks associated with nanoparticle contamination of our environment. In the context of biological systems we must especially understand the mechanisms underlying the interactions between

nanoparticles with different physical and chemical properties and biological structure and function. Using the advantages of multidisciplinary teams that embrace chemistry, physics, and biology, we can anticipate a clear understanding of the problems and hazards in the application of nanoparticle technology, and find the ways to realize this technological promise without posing a threat to our daily life.

#### 4. Acknowledgments

We acknowledge the partial financial support of the Canadian Institutes of Health Research, the National Sciences and Engineering Research Council (Canada) and the Juvenile Diabetes Research Foundation.

#### 5. About Author



Dr. D. Maysinger earned her Ph.D. at the University of Southern California, Los Angeles, and she got her post-doctoral trainings at Max Planck Institute in Munchen, Karolinska Institut in Stockholm and Oxford University in UK. She is an associate professor at McGill University. Research activities in Dr. Maysinger's laboratory focus on investigating therapeutic interventions in diabetes and neurodegenerative disorders in conjunction with nano-delivery systems. Moreover, they are investigating the effectiveness of drugs from the nano-delivery systems in modulating signal transduction pathways in cell death and differentiation.

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