CHEMICAL REVIEWS

REVIEW

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Effect of Nanoparticles on the Cell Life Cycle

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1. INTRODUCTION

Nanoscience is often referred to as "key", "horizontal", or "enabling", since it can virtually encompass all technological areas.[1−4] Bringing together different areas of science, nanoscience benefits from a multidisciplinary approach in order to attain innovative solutions for many of the problems facing today's society.[5−8] A scientific and technological revolution has begun that is based on systematic organization, fabrication, or manipulation of matter in the nanometer length scale, where unique, distinctive material properties can be attained. To date, several nanotechnology-based products have been marketed including electronic components, scratch-free paint, sports equipments, wrinkle- and stain-resistant fabrics, sun creams, and medical products. From a chronological point of view, nanoscience and nanotechnology were first employed in materials manufacturing applications such as composites and coatings, followed by incorporation of nanotech in electronics and information technology applications such as advanced memory chips and displays. Subsequently, nanoscience principles were applied in the fields of healthcare and life sciences to produce new products such as nanostructured medical devices and nanotherapeutics.[9,10,12−15] Constantly increasing demand for nanoparticulate materials has resulted in the emergence and evolvement of many companies in the fields of synthesis, functionalization, and application of nanoparticles both in vitro and in vivo.[11] This is evidenced by the increasing number of nanoparticle-related publications in the past decade with most investigations reporting data on synthesis, characterization, and surface properties.[11]

Taking into consideration the rapidly growing interest in nanoparticulate functional materials, potential job-related and public exposure to manufactured nanoparticles will be dramatically increasing in the near future. However, currently, there is limited knowledge about the relative health and environmental risk assessments of the in use nanoparticles and nanomaterials. While the total number of nanoparticles-related publications has increased significantly over the past decade, most investigations exclusively pertained to synthesis, characterization, and surface properties of nanomaterials. In recent years, biological issues and toxicity of nanoparticles and nanomaterials have emerged as

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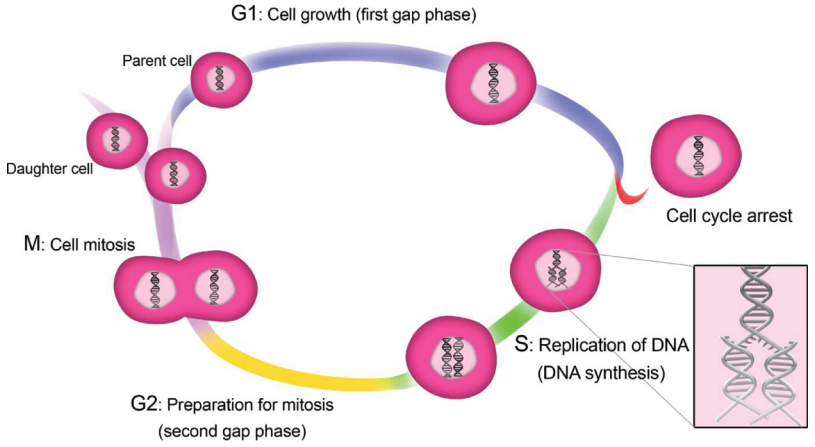

Figure 1. Schematic cell cycle.

troublesome barriers to the development of nanotechnology. Although information about the toxicity of nanoparticles continues to increase, safe application of these promising materials in numerous biomedical areas requires a greater knowledge about the toxicological profiles of different nanoparticles. Without adequate toxicity data, the risk assessment and regulation of nanomaterials will suffer significantly.[8]

Toxicants can lead to a wide array of cellular responses.[16] Scientists are particularly focused on the mechanisms by which cells die and therefore study the factors which lead to apoptosis or necrosis in various cell populations. These mechanisms of cell death can also be intricately linked with the cell cycle, as there are various check-points and controls in a cell's life cycle to ensure that appropriate division processes occur. Mechanisms by which toxicants induce cell death by necrosis and apoptosis have been the focus of many biomedical disciplines because they help us understand toxicity and also provide opportunities for drugs to impact dysregulation of the cell cycle in diseases such as cancer.

With the generation of an increasing number and diversity of nanoparticles, these same interests are emerging. That is, there is ongoing concern about the mechanisms by which nanoparticles produce toxicity as well as applied research to leverage nanotechnology for the targeted killing of cancer cells. Moreover, as regenerative medicine develops with nanotechnology, the regulation of cell proliferation secondary to nanostructure exposure will also be central to its success.

As nanotoxicological research continues to evolve, not only will apoptotic and necrotic experimental end-points be applied but more emphasis will be directed toward the mechanisms and pathways by which nanoparticles induce cell death as well as the dose−response relationships of these pathways. One of the emerging central concepts in nanoparticle toxicity is the induction of oxidative stress.[17,18] Oxidative stress has a spectrum of severity and can also guide a tiered testing approach whereby apoptosis would be considered a Tier 3 outcome in toxicity studies.[18] Indeed, it is well-known that nanoparticles can impair cellular functions leading to oxidative stress which can in turn lead to cell death via apoptosis. Specifically, some nanoparticles impair the mitochondrial function leading to cell death. Thus, insults from specific nanoparticulate formulations may abort the usual cell cycle as it is known that mitochondrial dysfunction from oxidative stress is linked to apoptosis.[19,20]

This review presents a broad overview of the effect of nanoparticles on the cell life cycle and their corresponding phase arresting data, as currently available. The reader will realize that the phase arresting data varies significantly depending on composition, size, size distribution, surface (including coating), and subsequent surface derivatization. In addition, results differ due to the varying experimental conditions. Also presented is a comprehensive description of the available assays for probing the effect of nanoparticles on the cell life cycle (e.g., flow cytometry, and laser scanning cytometry).

2. CELL LIFE CYCLE

The cell cycle corresponds to a series of events which lead the cell to its division and duplication.[21−25] In eukaryotes, the cell cycle can be divided into two brief periods: (i) the interphase, during which the cell grows and accumulates nutrients needed for mitosis and DNA replication and (ii) the mitosis (M) phase, in which the cell splits itself into two distinct daughter cells. This cell-division cycle is a vital process. Almost all of a cell's life is spent in interphase, which is split into three parts: G_1, S, and G_2 phases (see Figure 1). In the first gap phase (G_1), the cell grows and produces enzymes that are necessary for cell division. In the synthesis phase (S), the DNA is replicated. Finally, in the second gap phase (G_2), the cell continues to grow and carries out processes necessary for mitosis. In both the G_1 and G_2 phases, there are checkpoints that ensure that the cell is prepared for crucial steps in its division.

The mitosis (M) phase corresponds to the actual cell division and requires significant amounts of energy. Although there are many stages in mitosis (prophase, prometaphase, metaphase, anaphase, and telophase), it basically consists of the cell dividing its nucleus and cytoplasmic contents. The final division is called cytokinesis. After this division, the two daughter cells produced restart the cell cycle. Mitosis and cytokinesis together define the mitosis (M) phase of the cell cycle − the division of the mother cell into two daughter cells, genetically identical to their parent cell. The process of mitosis is complex and highly regulated. The sequence of events is divided into phases, corresponding to the completion of one set of activities and the start of the next. Errors in mitosis can either kill a cell through apoptosis or cause mutations that may lead to cancer. This entire cycle can take from 12 h to many days, depending on the type of cell.

Regulation of the cell cycle involves processes crucial to cell survival, including the detection and repair of genetic damage as well as the prevention of uncontrolled cell division. Each process occurs in a sequential fashion and it is impossible to "reverse" the cycle.

Two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), determine a cell's progress through the cell cycle.[26] When activated by a bound cyclin, CDKs perform a common biochemical reaction called phosphorylation that activates or inactivates target proteins to orchestrate coordinated entry into the next phase of the cell cycle. Different cyclin−CDK combinations determine the downstream proteins targeted. CDKs are constitutively expressed in cells, whereas cyclins are synthesized at specific stages of the cell cycle in response to various molecular signals.[27]

Upon receiving a pro-mitotic extracellular signal, G_1 cyclin−CDK complexes become active to prepare cells for the S phase, promoting the expression of transcription factors that in turn promote the expression of S cyclins and of enzymes required for DNA replication. The G_1 cyclin−CDK complexes also promote the degradation of molecules that function as S phase inhibitors by targeting them for ubiquitination. Once a protein has been ubiquitinated, it is targeted for proteolytic degradation by the proteasome. Active S cyclin−CDK complexes phosphorylate proteins that make up the prereplication complexes assembled during the G_1 phase on DNA replication origins. The phosphorylation serves two purposes: to activate each already-assembled prereplication complex and to prevent new complexes from forming. This ensures that every portion of the cell's genome will be replicated once and only once. The reason for the prevention of gaps in replication is fairly clear, because daughter cells that are missing all or part of crucial genes will die. However, for reasons related to gene copy number effects, possession of extra copies of certain genes would also prove deleterious to the daughter cells.

Mitotic cyclin−CDK complexes, which are synthesized but inactivated during the S and G_2 phases, promote the initiation of mitosis by stimulating downstream proteins involved in chromosome condensation and mitotic spindle assembly. A critical complex activated during this process is an ubiquitin ligase known as the anaphase-promoting complex (APC), which promotes degradation of structural proteins associated with the chromosomal kinetochore. APC also targets the mitotic cyclins for degradation, ensuring that telophase and cytokinesis can proceed.

2.1. Specific Action of Cyclin−CDK Complexes

Cyclin D, the first cyclin produced in the cell cycle in response to extracellular signals such as growth factors, binds to existing CDK4, forming the active cyclin D−CDK4 complex. Cyclin D−CDK4 complex in turn phosphorylates the retinoblastoma susceptibility protein (Rb). The hyperphosphorylated Rb dissociates from the E2F/DP1/Rb complex, activating E2F. Activation of E2F results in transcription of various genes such as cyclin E, cyclin A, DNA polymerase, and thymidine kinase. Cyclin E then binds to CDK2, forming the cyclin E−CDK2 complex, which pushes the cell from the G_1 to the S phase (G_1/S transition). Cyclin B along with cdc2 (cdc2 in fission yeasts and CDK1 in mammals) forms the cyclin B−cdc2 complex, which initiates the G_2/M transition.[28] Cyclin B−cdc2 complex activation causes the breakdown of the nuclear envelope and the initiation of prophase, and subsequently, its deactivation causes the cell to exit mitosis.[27]

Cell cycle checkpoints are used by the cell to monitor and regulate the progress of the cell cycle. These checkpoints prevent cell cycle progression at specific points, allowing verification of necessary phase processes and repair of DNA damage. The cell cannot proceed to the next phase until checkpoint requirements have been met. Several checkpoints are designed to ensure that damaged or incomplete DNA is not passed on to daughter cells. Two main checkpoints exist: the G_1/S checkpoint and the G_2/M checkpoint. G_1/S transition is a rate-limiting step in the cell cycle and is also known as a restriction point. An alternative model of the cell cycle response to DNA damage has also been proposed, known as the postreplication checkpoint.[29,30] Finally, the interaction between cyclins, CDKs, and cell cycle has been fully discussed elsewhere.[31]

2.2. Role in Tumor Formation

Dysregulation of the cell cycle components may lead to tumor formation. Specifically when key functional genes, such as the cell cycle inhibitors RB and p53, mutate, this may cause the cell to multiply uncontrollably, forming a tumor. Although the duration of the cell cycle in tumor cells is equal to or longer than that of a normal cell cycle, the proportion of cells that are in active cell division (versus quiescent cells in G_0 phase) in tumors is much higher than that in normal tissue. Thus, there is a net increase in cell number as the number of cells that die by apoptosis or senescence remains the same. The cells which are actively undergoing division are targeted in cancer therapy as the DNA is relatively exposed during cell division and hence susceptible to damage by drugs or radiation. This biology is exploited in cancer treatment by a process known as debulking, whereby the removal of a significant mass of the tumor pushes the relative number of the remaining tumor cells to go from G_0 to G_1 phase (due to increased availability of nutrients, oxygen, growth factors, etc.). Radiation or chemotherapy following the debulking procedure kills these cells which have newly entered the cell cycle.

The fastest cycling mammalian cells in culture, and crypt cells in the intestinal epithelium, have a cycle time as short as 9−10 h. Stem cells in resting mouse skin may have a cycle time of more than 200 h. Most of this difference is due to the varying length of G_1, the most variable phase of the cycle. M and S phases demonstrate much less variability.

In general, cells are the most radiosensitive in the late M and G_2 phases and most resistant in the late S phase. For cells with a longer cell cycle time and a significantly long G_1 phase, there is a second peak of resistance late in G_1. The pattern of resistance and sensitivity correlates with the level of sulfhydryl compounds in the cell. Sulfhydryls are natural radioprotectors and tend to be at their highest levels in S and at their lowest near mitosis.[32]

3. CELL CYCLE ANALYSIS METHODS

Nanoparticles could have various effects on cells. These effects range from interactions with cell membranes and cellular uptake, signaling pathways, ROS production to gene regulation, cell cycle dysregulation, and finally apoptosis or necrosis.[33] Monitoring the cell cycle changes is an important index of both the cytotoxicity of the nanoparticle, and its effectiveness in those cases in which targeting a dysregulated cell is the aim of the study. Nanoparticles have been widely used as vehicles for delivering antiproliferative drugs to cancer cells.[34,35]

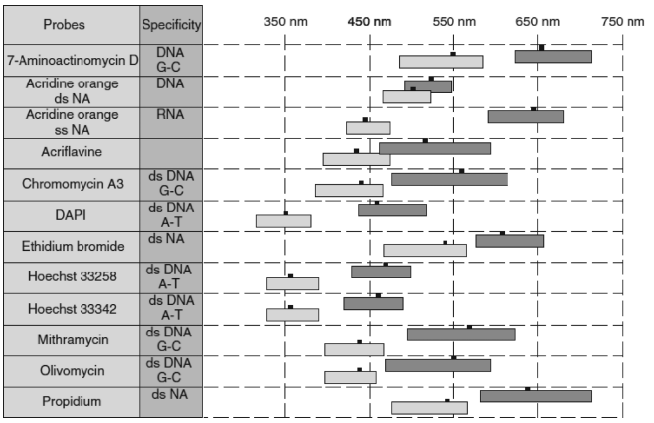

Figure 2. Fluorescence excitation (light gray bars) and emission (dark graybars) wavelengths of the most commonly used DNA fluorochromes. Abbreviations: ds, double-stranded; ss, single-stranded; NA, nucleic acid. Reprinted with permission from ref 47. Copyright 2001 John Wiley & Sons.

The cell cycle is controlled via different mechanisms, including genetic and epigenetic events.[35−37] Genes responsible for promoting cell division could be evaluated by different methods, mainly real time PCR and microarray analyses. However, these approaches have not been commonly used for evaluating the cell cycle itself due to their sophistication and difficulty of validation. DNA content measurements, in contrast, have been widely used to evaluate genotoxicity of different compounds, including nanoparticles,[34,35,38−43] because the techniques are more feasible and the measured outcome is the final consequence of any effect on cell replication. However, these techniques usually lack the specificity to elucidate which step has been damaged in the process, whereas monitoring the genes and proteins can give a more detailed picture of plausible causes of cell cycle dysregulation.

The DNA content of cells is well correlated with the stage of the cells in the cell cycle. Cells in the G_0/G_1 step have half the DNA of a G_2/M phase cell (corresponding to 2N and 4N chromosome phases), while the DNA content is in between these two during the synthesis phase. Cells with lower than 2N chromosome DNA are mostly cell garbage, or apoptotic cells which have lost different amounts of their DNA, usually in small fragments. Methods such as the Comet assay can help to detect and quantify the number of cells in this latter stage, but they cannot distinguish between other phases of the cell cycle.[44] Herein we review the two most commonly used techniques to perform cell cycle analysis by DNA content measurement and their application for the in vitro toxicity testing of nanoparticles.

3.1. Flow Cytometry

Flow cytometry is a relatively old technique developed in the 1940s. Its fascinating history has been reviewed by Shapiro in a paper published in 2004.[45] It is based on evaluating the cell or particle properties in a flow. Later, in the 1960s, the concept of sorting the particles after measuring their characteristics was developed. In the 1970s, the term fluorescent activated cell sorting (FACS) was introduced and was commercially adapted by Becton Dickenson in their machines. Since then, however, it has been interchangeably used for flow cytometry in many papers, although different manufacturers produce similar machines and despite the fact that many analyses have been done without actual *sorting* of the cells.

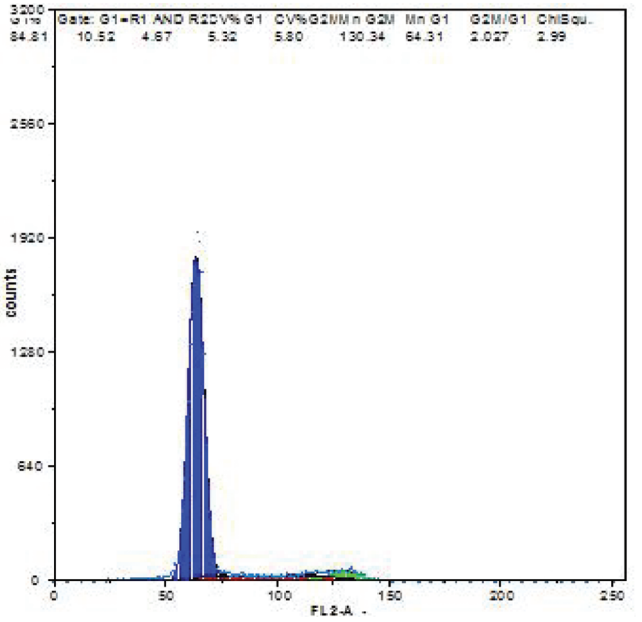

Figure 3. A typical cell cycle graph of a nonsynchronized cell line in culture using the PI staining method (unpublished work by K. Azadmanesh).

Uni- and bivariate cell cycle analysis using flow cytometry is based on the use of fluorescent dyes which interact with DNA. These dyes bind in different ways to the double-stranded DNA, which is mainly in the cells' nuclei.[46] Though some dyes have preferences to interact with some parts of DNA (such as GC-rich versus AT-rich regions), this differentiation is not usually helpful in cell cycle analysis. A list of the more commonly used dyes is given in Figure 2.[46,47]

The most commonly used dyes are propidium iodide (PI) and DAPI. DAPI has the advantage of not interacting with double-stranded RNA, which is a drawback of PI;[46,48] but since most presently used flow cytometers are not equipped with UV lamps or UV diode light sources, which are the light of choice for exciting DAPI, it is less common than PI. Besides, the blue channel for acquiring the emission of DAPI is also not a preinstalled detector in many currently available machines. On the other hand, PI needs the most common light source of flow cytometers, the 488 nm LASER or diode, and could be detected in yellow-orange detectors, which corresponds to FL2-FL3 channels in most flow cytometers. The problem of nonspecific binding to the double-stranded RNA is easily resolved by adding RNase A to the staining solution.[47,48]

For analyzing the flow cytometry data of a cell cycle analysis experiment, it is a common practice to exclude the doublets or aggregates of the ethanol-fixed and PI-stained cells in a FL2 width or height versus area scatter diagram, and then apply this gate to an FL2 area histogram.[49,50] A typical nonsynchronized cell culture gives a result like that in Figure 3.

The first prominent peak could be attributed to cells in the G_0/G_1 phase, while the second peak corresponds to G_2/M. The S phase lies between these two peaks. Since the amount of stain in the cells at each phase is a continuum rather than a fixed value, differentiating the number of cells in the border areas between G_0/G_1 and S or S and G_2/M is not so easy.[51] This problem could be partially overcome by adding labeled nucleotides to the medium during the cell culture and performing a dual color

analysis in addition to the simple staining by PI. This latter approach will be further addressed later. Software programs from different providers are available which tries to fit mathematical models to the data and estimate each region. The output of one of these models is given in Figure 3. However, sometimes it is not possible to fit such a model to a given data, so the most common and yet not so robust practice in such cases is to visually differentiate between different regions by the so-called "region gates".

Many basic flow cytometry operating software such as Cell Quest (BD) or FlowMax (Partec) provide primary tools for such a gating, but it is advisible to use more specific software to analyze the cell cycle raw data. ModFit LT (Verity Software House, Topsham, ME, USA) is an example of software dedicated to this purpose. FlowJo (Tree Star Inc., Ashland, OR, USA) is multipurpose software that also provides cell cycle modeling tools. However, each of these software programs has its advantages and disadvantages for univariate cell cycle analysis. ModFit has the option to fit its models for sub G_0/G_1 (mostly apoptotic) cells or cell debris. While Flowjo lacks this ability, it can report a crude sub G_0/G_1 ratio. Since the sub G_1 area is of special interest in many toxicity studies, including nanoparticles, this might be regarded as a disadvantage for Flowjo. On the other hand, the super G_2/M area might be interesting in those cases in which cytokinesis is damaged by the nanoparticle, leading to cells with greater than 4N chromosomes or cells with intrinsic tendency to become so, like mesenchymal stem cells (Azadmanesh, unpublished data). In any case, most of these software programs fail to automatically fit good models to cell toxicity data since the peaks are too deformed, thus requiring some manual adjustment and some degree of subjective interpretation.

Any dysregulation of the cell cycle usually results in accumulation of cells in one or more steps, leading to an increased area of the corresponding region in the histogram.[52,53] This accumulation is usually due to the forced arrest of damaged cells by the cellular checkpoint pathways (mainly in G_1/S and G_2/M junctions) or to the failure to separate the condensed chromosomes in the M phase.

There is yet another weakness of this technique in cell cycle analysis: it is not possible to differentiate between cells in the G_0 and G_1 phases, and more practically problematic between the G_2 and M phases.[47,48] Cell lines which are mostly used as in vitro models for testing toxicity of nanoparticles have a continuous cell division, which prevents them from entering the G_0 phase. But all of them should undergo the G_2 to M transition, while the DNA content is equal in both steps. Working with primary cell cultures might be trickier since not all of them are committed to dividing, so that many of them will be in the G_0 phase. It is not possible to distinguish between those two steps either by DNA content analysis or by evaluating incorporation of labeled nucleotides. If such a distinction is important for evaluating the effect of a given substance on the primary cells, it may be possible by evaluating other cell cycle markers, like specific proteins, rather than relying on DNA.

As in any other biological experiment, choosing the proper time after a manipulation is important for the cell cycle analysis. Since different cell types have different doubling times, which may also further vary by culture conditions, it is critical to give the cells enough time. It is a common practice to follow the cell cycle variations over time rather than relying on one time section evaluation. Besides, dosing the substance of interest is also important for obtaining reasonable results. Higher doses of a toxic nanoparticle or testing a long time after manipulation may lead to more cell death, either apoptosis which might be evident in sub G_1 peaks or necrosis, thus possibly masking underlying cell cycle dysregulation.

If the effect of the tested nanoparticles on the cell cycle is not prominent in a test setting, another approach to enhance the effect is synchronizing the cells before adding the test substance. Synchronizing the cells is achieved by arresting all cells in a specific phase, usually by depriving the cells from growth factors such as FBS, or adding mitosis inhibiting reagent for a while, then removing the arresting factor from the environment.[54] In this case, all cells move through their cycle almost in a synchronized way, so a possible dysregulation is not masked by cells which are in a different phase. However, this process does not really synchronize all cells as it is obvious that after a few cell divisions the cell mixture is not synchronized anymore. Besides, arresting the cells in one phase to synchronize them does not resemble any real biological process in vivo, so the actual added value of this technique could be questioned,[36,55] and it is not a common practice in this field.

3.2. Analyses for Cell Division Time

Doubling time of a cell line is another parameter affected by cell cycle dysregulation. Usually, foreign materials lead to an extension of the time a cell goes through its cycle. Since it is not practical to follow single cells to measure their doubling time, indirect measurements are used for this. The main approach is based on evaluating the dilution rate of a marker during time. The other approach is using the cell cycle analysis in a slice of time. A commonly used marker is the amount of unusual nucleic acids incorporated into the DNA of cells. Bromodeoxyuridine (BrDU) is a nucleoside analogue of thymidine.[56,57] If it is added to dividing cells, it can be incorporated into DNA along with other natural nucleotides during the S phase. Antibodies are available for BrDU bond DNA, which makes detection of such cells possible. By the time BrDU is removed from the cell culture medium, its content starts to dilute within the labeled cells. Mathematical models are available to measure the doubling time.[58−60] Another application of BrDU labeling is discrimination between G_0/G_1 and S phase cells in a conventional cell cycle analysis.[56,61] As it was discussed earlier, the margin between these two phases is not very clear in a univariate flow cytometric analysis. If a G_0/G_1 to S arrest occurs due to the toxic effect of a nanoparticle, the cells do not incorporate BrDU into their DNA. The use of this approach has been frequently reported for evaluating the behavior of cancer cells.

A technical problem with detecting DNA incorporated BrdU is the need to expose those molecules in the chromosome to enable the antibodies to reach them. This phenomenon includes partial denaturation of the DNA, which on the other hand reduces the incorporation of the binding dyes to the DNA thus decreasing the quality of cell cycle analysis. A recently adapted technique from a relatively old approach is using "click" chemistry to label the incorporated BrdU molecules with small fluorescently labeled molecules.[62,63] This approach precludes the need to denature the DNA, so that the quality of cell cycle analysis is maintained. Other halogenated dTTP analogues, such as iodide or salts of uracil have also been successfully used for this purpose.[63]

Another possible approach to evaluate the effect of a substance on the cell cycle is tracing the doubling of the cells by cytoplasmic incorporated dyes.[64−68] Though this method has long been used in immunology to evaluate the replication of lymphocytes in response to antigens both *in vivo* and *in vitro*, it has rarely been

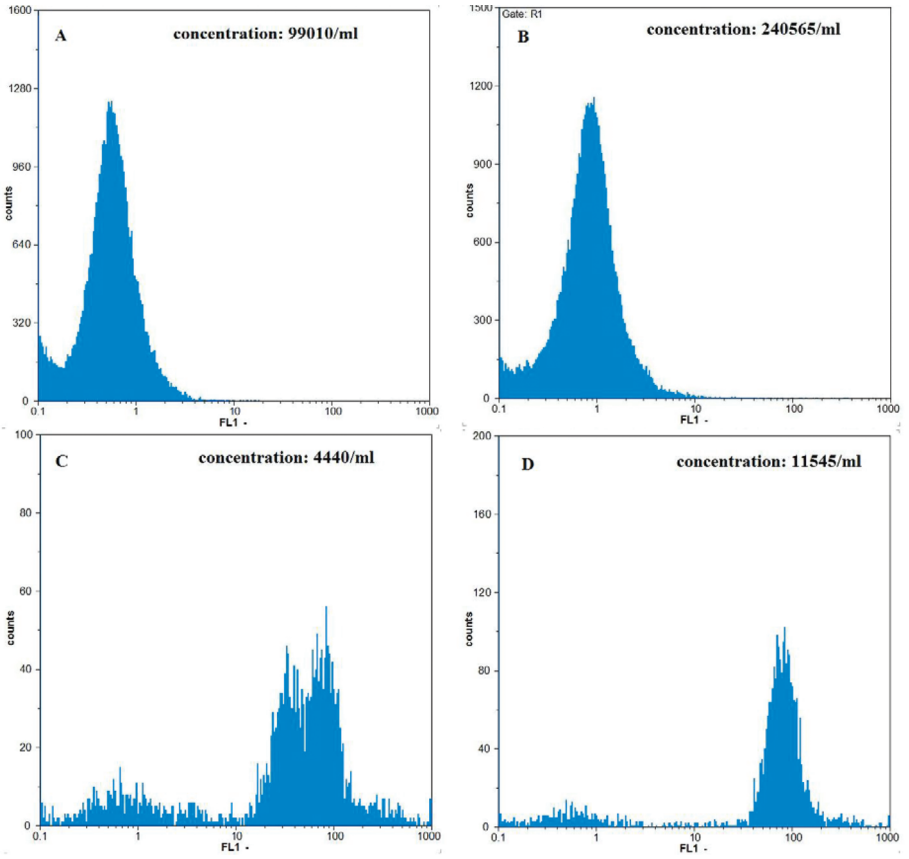

Figure 4. CFSE histograms in K562 cells treated with various doses of bare SPIONs for one week: (A) no SPION and no CFSE, (B) 1 mmol SPIONs; (C) 10 mmol SPIONs; and (D) 100 mmol SPIONs. The concentration of cells in each sample is shown in each graph. It can be seen in panel B that the cells have replicated so many times that the CFSE fluorescence has become almost as the autofluorescence of the cells (panel A), while in 10 mmol concentration of SPION some cells have divided once before the arrest, and in 100 mmol concentration almost all cells died before any division. The concentration of cells also shows that more divisions have taken place in the presence of 1 mmol SPION than in the negative control sample (unpublished work by M. Mahmoudi and K. Azadmanesh).

used in toxicological studies, especially for nanoparticles. However, in our experience it could be efficiently used for evaluating the effect of nanoparticles in cell culture; and, theoretically, it can be used for studying in vivo the effects of a nanoparticle on a specific cell type. Figure 4 shows an example of using carboxyfluorescein succinimidyl ester (CFSE) to study the effect of different concentrations of an SPIO nanoparticle on K562 cells. CFSE is absorbed by the cells and, upon cleavage inside the cell, produces a bright green fluorochore which then is equally divided between the two daughter cells upon cell division, leading to two half bright cells. The division can be monitored up to seven cycles, after which the concentration of the dye is too low to be discriminated from the autofluorescence.[69] It is evident in this example that adding the nanoparticle in lower concentrations not only did not block replication but enhanced it. On the other hand, higher concentration of the nanoparticle led to only one division, while the highest concentration killed almost all cells before any division (M. Mahmoudi and K. Azadmanesh, unpublished data). Another possibility of this method is taking the advantage of cell counting, if the option is available with the flow cytometer. If the protocol of labeling, adding the nanoparticle, trypsinization (if applicable), and resuspending the cells for flow cytometry is strictly followed with all test and control samples, it is possible to use the reading count of the flow cytometer as another parameter to replace the cell counting tests (like direct counting by microscopy, MTT, XTT, etc.). Partec machines (Partec GmBH; Germany), for example, have this volumetric measurement option, whereas for other machines such as BD (Beckton Dickenson, USA) the option can be used by adding a standard amount of calibration particles to the samples.

3.3. Analyzing Cell Cycle Related Protein Markers

The cell cycle progress is the result of an orchestrated interaction between several intracellular proteins. These proteins are mostly members of cyclins, cyclin dependent kinases (CDK), CDK inhibitors, and other similar proteins.[70,71] The expression of these molecules is time-controlled, so tracing them can further confirm a cell cycle analysis or discriminate between overlapping parts of a cell cycle histogram. Notably, cyclin A and B are mainly G_2 cyclins while D and E are G_1.[72] It is also possible to differentiate between different stages of the cell cycle in polyploid cells.[73] Figure 5 shows a typical pattern of cyclins A, B1, D1, and E correlated with DNA content.

Other proliferation related proteins such as proliferative nuclear cell antigen (PCNA)[74] or the target of K_i-67 antibody[75] have also been used for discriminating between nondividing (G_0) and dividing cells.

Since most cellular RNA is composed in ribosomes, whose increase is also an index of the need for protein production which could be an indicator of committing the cell to division, methods

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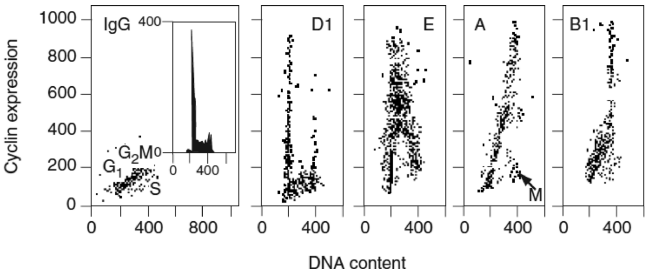

Figure 5. Typical bivariate cyclin versus DNA content distributions (scatter plots) showing expression of cyclin D1 in human normal fibroblasts and cyclins E, A, and B1 in phytohemagglutinin (PHA)-stimulated human lymphocytes. The IgG control in the left panel shows the level of fluorescence of control cells stained with the isotype IgG rather than the respective cyclin monoclonal antibody, prior to fluoresceinated secondary antibody. Reprinted with permission from ref 72. Copyright 2001 John Wiley & Sons.

have been developed as well to quantify the RNA content of the cells. Acridine Orange (AO) is an interesting dye in this regard. It reacts with both DNA and RNA and produces different light emissions upon excitation by a 488 nm beam. However, the process of staining should be well controlled otherwise the affinity of the dye to DNA or RNA may vary in different reaction conditions.[46] Pyronin Y is another fluorescent dye with affinities to both DNA and RNA, but its DNA binding can be easily blocked by previous staining of DNA with another dye such as 7-AAD.[76,77]

3.4. Laser Scanning Cytometry (LSC)

Despite several advantages of the flow cytometric approach for cell cycle analysis (e.g., power, ease of use, and availability), it also has disadvantages. First of all, since it studies the cells that pass in front of its detector in a flow, it is impossible to recheck the same cell. Second, though it provides indirect data on size or complexity of a cell or particle, it does not provide any actual visual data of the cell. The distribution of a fluorescent dye inside a cell cannot be deduced either from the flow cytometry data. To overcome these disadvantages (and others which are not in the scope of this manuscript), laser scanning cytometry (LSC), a similar technique, was developed in the 1990s.[78] LSC is based on the same staining principles as flow cytometry, that is, using fluorescent dyes to mark different components of cells, but unlike flow cytometry it studies the cells on a surface rather than in flow. The micrometer-scaled computerized movements of the cell stage in front of the optical devices of LSC enables it to locate any given cell on the stage separately, and link different fluorescent characteristics of a cell to its actual position. Besides, adding a light microscope to the fluorescent apparatus made the morphological studies of the cells possible. Higher resolution of this system compared to flow cytometry also provides the opportunity to study the distribution pattern of a given dye within the cells.[79]

The cell cycle data obtained from an LSC is basically similar to that provided by a flow cytometer. However, by adding a light microscope to the fluorescent apparatus, LSC enables us to study the morphology of the cells too. This is especially helpful in excluding artifacts or detecting apoptotic cells. It is noteworthy to mention that, despite common use of different flow cytometric techniques for detecting apoptotic cells, the standard criteria of apoptosis are still based on microscopic examination.[80] Another advantage of LSC over flow cytometry for cell cycle analysis is that its higher resolution offers the opportunity to distinguish different patterns of the DNA binding dye inside the cell.[81] Since the condensation of chromosomal DNA happens in the M phase and not in the G_2 phase, which has an equal DNA amount, the condensation of DNA binding dye is different between G_2 and M phase cells. This gives the chance to discriminate between G_2 and M phase cells and, thus, makes it possible to study different types of cell cycle arrests in these two phases.

The visual ability of LSC also provides the opportunity to microscopically study the distribution of the nanoparticles within the cells. If those particles are labeled with a different fluorescent dye, this technique can simultaneously study the distribution or amount of the intracellular nanoparticles and the cell cycle state. Another advantage of the ability of studying cells on a surface by LSC rather than flow is the possibility of studying histological sections of test animals.[82] Though criteria for in vivo toxicity of nanoparticles are less defined in cellular or subcellular scale,[83] LSC provides researchers with an option to study subcellular or cellular effects of a given compound after in vivo administration. Labeling the nanoparticles with high efficiency fluorescent dyes (like quantum dots) will make it possible to study the distribution of a hypothetical cancer specific nanoparticle in its target cancer tissue, as well as its effect on the microscopic pathology of the tissue and the cell division state within the cancer tissue. Since most LSC samples can be stored, it is possible to safe keep the analyzed cell cycle sample for future testing of other markers.

4. TOXICITY OF NANOPARTICLES

Magnetic nanoparticles are used in a large variety of biomedical applications, such as cell labeling, cell isolation, hyperthermia, and controlled drug delivery, and as MRI contrast agents.[8,9,84−93] These biomedical applications require controlled interactions between the nanoparticles and living cells.[8]

Nanotoxicology is an important emerging subdiscipline of nanotechnology that studies the interactions of nanostructures with biological systems and the adverse effects of such particles on living organisms.[8] It is an interdisciplinary technology which employs chemistry, physics, material sciences, pharmacy, medicine, and molecular biology. Nanotoxicology attempts to understand the relationship between the physical and chemical properties (size, shape, surface chemistry, composition, and aggregation) of nanostructures with the induction of toxic biological responses. Conversely, this field aims to establish favorable characteristics of nanomaterials which render them more amenable for use in biological environments.

The majority of nanotoxicity research has focused on cell culture systems; however, the data from these studies could be misleading and will require verification from in vivo animal experiments. In vivo systems are extremely complicated and the interactions of the nanostructures with biological components, such as proteins and cells, could lead to unique biodistribution, clearance, immune response, and metabolism. An understanding of the relationship between the physical and chemical properties of the nanostructures and their in vivo behavior would provide a basis for assessing toxic response and more importantly could lead to predictive models for assessing toxicity.

Fischer and Chan[94] suggested that the development of predictive models of nanostructure toxicity requires a systematic mapping of the fate, kinetics, clearance, metabolism, protein coating, immune response, and toxicity parameters and that these factors need to be correlated with the nanostructure's physical properties within a life cycle model. They emphasize the importance

of the in vivo activities of nanostructures and a thorough understanding of the overall toxicity of specific nanostructures.

The overall behavior of nanostructures can be summed only in general terms due to particle diversity and inadequate toxicity data. Key factors implicated in toxicity studies include: (i) nanostructures can enter the body via six principle routes: intravenous, dermal, subcutaneous, inhalation, intraperitoneal, and oral; (ii) absorption can occur where the nanostructures first interact with biological components (proteins, cells); (iii) afterward they can distribute to various organs in the body and may remain the same structurally, be modified, metabolized, or excreted; and (iv) they can enter the cells of the organ and reside in the cells before leaving and being excreted. The importance of nanotoxicology resides not only in the advancement of nanotechnology in a wide-array of applications but also in that studies in this area could provide the required information to make responsible regulatory decisions.

Recent nanotoxicity studies revealed that the physicochemical characteristics of nanomaterials play an important role in the interactions with living cells.[95−101] Safi et al.[23] reported on the toxicity and uptake of cerium and iron oxide nanoparticles (coated with citric acid or polymers such as poly(acrylic acid) , M_W = 2000 g mol^{-1}) by NIH/3T3 mouse fibroblasts. Most particles were biocompatible, as exposed cells remained 100% viable. Only the bare and the citrate-coated nanomaterial led to a slight decrease in mitochondrial activity at very high cerium concentrations (>1 g/L). The citrate-coated particles were internalized/adsorbed by the cells in large amounts, typically 250 pg/cell after 24 h incubation for iron oxide. In contrast, the polymer-coated particles were taken up at much lower rates (<30 pg/cell). The authors concluded that the uptake of nanoparticles by living cells depends on the coating and on its ability to preserve the colloidal nature of the dispersions.

One factor that can contribute to nanotoxicity is the size of the particles. Smaller particles have a greater reactive surface area than larger particles, are more chemically reactive, and produce greater numbers of reactive oxygen species (ROS) that include free radicals.[100] The production of reactive oxygen species has been found in a diverse range of nanomaterials including carbon fullerenes, carbon nanotubes, and metal oxides.[102] This is one of the primary mechanisms of nanotoxicity and it may result in oxidative stress, inflammation, and consequent damage to proteins, membranes, and DNA.[100] Extremely small sized particles are also much more readily taken up by the human body than larger sized particles. Nanomaterials can gain access to the bloodstream following inhalation[103] or ingestion.[104] Some nanomaterials can penetrate the skin,[105] especially if it is flexed.[106] Broken skin is an ineffective particle barrier,[102] suggesting that acne, eczema, shaving wounds, or severe sunburn may enable skin uptake of nanomaterials more readily. Once in the bloodstream, nanomaterials can be transported around the body and are taken up by organs and tissues including the brain, heart, liver, kidneys, spleen, bone marrow, and the nervous system.[102] Some nanomaterials have proved toxic to human tissue and cell cultures, resulting in increased oxidative stress, inflammatory cytokine production, and cell death.[103]

Unlike larger particles, nanomaterials may be taken up by cell mitochondria[107,108] and the cell nucleus.[109,110] Studies demonstrate the potential for nanomaterials to cause DNA mutation[109] and induce major structural damage to mitochondria, even resulting in cell death.[107,108] Size is clearly a key factor in determining the potential toxicity of a particle. However, it is not the only important factor. Other properties of nanomaterials that influence toxicity include chemical composition, shape, surface structure, surface charge, aggregation and solubility,[100] and the presence of functional groups of other chemicals.[111] The large number of variables influencing toxicity means that it is difficult to generalize about health risks associated with exposure to nanomaterials − each new nanomaterial must be assessed individually and all material properties must be taken into account in the toxicity assessment.

Several tests on animals have shown that no side effects occurred after a 7 day treatment with superparamagnetic iron oxide nanoparticles (SPIONs) according to the histology and serological markers.[112−114] The suitability of SPIONs for MRI and cell labeling has been investigated in several studies both in vitro and in vivo.[115,116] When injected intravenously, SPION are cleared rapidly from the circulation, predominantly by the liver Kupffer cells and the spleen macrophages.[117] Various coatings of SPIONs have also been shown to be metabolized.[118]

4.1. In Vitro Toxicity of Magnetic Nanoparticles

Mueller et al.[119] reported that SPIONs showed significant toxicities, by measuring the detrimental effects of nanoparticles on human granulocytes in vitro. Hilger et al.[120] observed significant drops in cell survival rates for human adenocarcinoma cells exposed to various SPIONs, especially those coated with cationic surfactants. Based upon the assumption of the SPIONs safety, the authors attributed the observed cytotoxicities to cellular membrane binding, chemical formulation components, or generation of a nonphysiological pH. Berry et al.[121,122] showed that both uncoated and dextran-coated SPIONs caused varying degrees of cell death and induced vacuole formation and clear disruptions in the cytoskeleton of dermal fibroblasts. Gupta et al.[123,124] confirmed the cytotoxicity and cytoskeletal disruption by uncoated SPIONs in fibroblasts. Other studies tried to prevent the apparent endocytosis-mediated cytotoxic effects by coating SPIONs with different proteins and showed that the cell response could be directly modulated by the type of coating.[123,125] Given the increasing number of studies on cells loaded with SPIONs, some nanotoxicity data have emerged. Table 1 summarizes some studies on the in vitro toxicity of SPIONs.

There are multiple other in vitro studies that have demonstrated little or more moderate toxicities of SPIONs; many of those are summarized in more broad nanotoxicity reviews.[136]

As mentioned previously, the chemical composition and coating can have drastic consequences for nanoparticle stability, agglomerate size, and the level and type of cellular interaction, significantly affecting the fate and extent of SPIONs internalization.[137]

Some other magnetic nanosystems have also been studied. Doiron et al.[138] have prepared polymeric particles of a small size with high loading of diethylenetriaminepentaacetic acid gadolinium (Gd-DTPA) and demonstrate their usefulness for MRI. A water-in-oil-in-oil double emulsion solvent evaporation technique was used to encapsulate the MRI agent in a poly(lactide-co-glycolide) (PLGA) or polylactide-poly(ethylene glycol) (PLA-PEG) particle. PLGA particles with two separate average sizes of 1.83 μm and 920 nm, and PLA-PEG particles with a mean diameter of 952 nm, were synthesized. Loading of up to 30 wt % Gd-DTPA was achieved, and in vitro release occurred over 5 h. PLGA particles had highly negative zeta potentials, whereas the particles incorporating PEG had zeta potentials closer to neutral. Cytotoxicity of the particles on human umbilical vein endothelial cells (HUVEC) was shown to be minimal. The

Table 1. Some Examples of Studies Showing in Vitro Toxicity of SPIONs

particle coating	observed toxic effects	refs
polylactide, lipids, PVA	dose-dependent reduced cell viability	9, 119
cationic, anionic starch	strong decrease in cell survival rates, abnormal subcellular structures	120
bare, dextran, albumin	apoptosis, alterations in proliferation, behavior, and morphology	121, 122
bare, PEG, lactoferrin, ceruloplasmin, pullalan	reduced cell adherence, altered behavior and morphology	123−125
dextran, liposomes	generation of free radicals, reduced proliferation and death	126
citrate	induction of oxidative stress	127
bare	cell specific toxicity, reduction of mesothelioma viability	9, 128−131
	reduction in cell viability	
	alterations in mitochondrial function at higher concentrations	
DMSA	dose-dependent reductions in adhesion, viability, and biological response to cue	132, 133
	decrease in mitochondrial activity at higher concentrations	
DMSA, citric acid, lauric acid	dose-dependent toxicity, alteration in morphology, apoptosis	134
glucose, maltose, lactose	Cell morphology, decrease in viability	135

Table 2. Studies Reporting in Vivo Toxicity of SPIONs

particle coating	observed toxic effects	refs
bare, tartrate, citric acid	cell death, apoptosis, mutagenicity, inflammatory reactions	113, 114
DMSA	particles agglomeration in blood vessels, inflammation, leukocyte infiltration	139
silica	particles crossed blood brain and blood testes barriers	140
oleic and pluronic acid	transient increase in oxidative stress, significant increase in iron levels	141, 142
Dextran	long-term increase in iron levels, alterations in neurobehavior, teratogenicity	143

results demonstrated the possible utility of the contrast agent-loaded polymeric particles for plaque detection with MRI. Riyahi-Alam et al.[97] have studied a novel emulsion composed of a silicon-based nanocomposite polymer and gadolinium oxide (Gd_2O_3) nanoparticles. No cytotoxicity in SK-MEL-3 cancer cells was observed. Nanoparticles including dendrimers, quantum dots, liposomes and carbon, gold and silica-based nanoparticles possess enormous potential as diagnostic imaging agents and hold promise for the development of multimodality agents with both imaging and therapeutic capabilities. Yet, some of the most promising nanoparticles demonstrate prolonged tissue retention and contain heavy metals. This presents serious concerns for toxicity. The creation of nanoparticles with optimal clearance characteristics will minimize toxicity risks by reducing the duration of exposure to these agents.

4.2. In Vivo Toxicity of Magnetic Nanoparticles

Weissleder et al.[112] published the first methodical study of SPIONs in vivo toxicity using dextran-coated nanoparticles. The pharmacokinetics (distribution, metabolism, bioavailability, excretion) and toxicity (acute and subacute toxicity, mutagenicity) were evaluated by ^{59}Fe radiotracer studies, relaxation rate measurements, ability to reverse iron deficiency anemia, and histological examination. One hour after administration of nanoparticles to rats, 82.5% of the administered dose was sequestered in the liver and 6.2% in the spleen. Peak concentrations of ^{59}Fe were found in liver after 2 h and in the spleen after 4 h. ^{59}Fe slowly cleared from the liver ($T_{1/2}$ 3 days) and spleen ($T_{1/2}$ 4 days) and was incorporated into hemoglobin of erythrocytes in a time-dependent fashion. The half-life of the T_2 effect on liver and spleen (24−48 h) was shorter than the ^{59}Fe clearance, indicating metabolism of iron oxide nanoparticles into other iron forms. No acute or subacute toxic effects were detected by histological or serological studies in rats or beagle dogs who received a total of 3000 μmol Fe/kg. This dosage was 150 times the dose proposed for MR imaging of the liver in humans. The results indicated that nanoparticles are a potential biocompatible contrast agent for MRI.

For the next 10 years, the literature was almost completely silent on the possibility of in vivo SPIONs toxicity, while subsequent studies on the in vivo toxicity of SPIONs have confirmed these initial conclusions that point to toxic effects. These are listed in Table 2.

Currently, the only SPIONs clinically used as MRI contrast agents are the superparamagnetic iron oxide (SPIO) and ultrasmall superparamagnetic iron oxide (USPIO) type dextran-coated iron oxides. Likewise, there exist in the literature many reports of their use in various MRI applications. Very few of these studies note any adverse side effects.[144,145] A notable exception is the report of preclinical safety studies of a USPIO formulation that showed low to moderate toxicity at lower concentrations but significant toxic effects at high doses.[143] The authors reported induction of neurobehavioral and neurovegetative effects at moderate doses, as well as reproductive toxicity including fetal malformations and teratogenicity in rats and rabbits, yet concluded that these findings should be considered acceptable within the overall biocompatible profile.

Despite the somewhat alarming observations described above, the number of studies further investigating potential SPION-mediated nanotoxicity has been markedly deficient. There still remains a significant body of work from various studies ranging from particle-cell interactions to particle toxicology that can be pieced together to form a better understanding of both the demonstrated and the potential nanotoxicity of SPIONs. In addition, the coating of the nanosystems is very important. Changes of surface by coating of various quantum dots have been shown to change their body distribution and their effects on biological systems significantly.[101]

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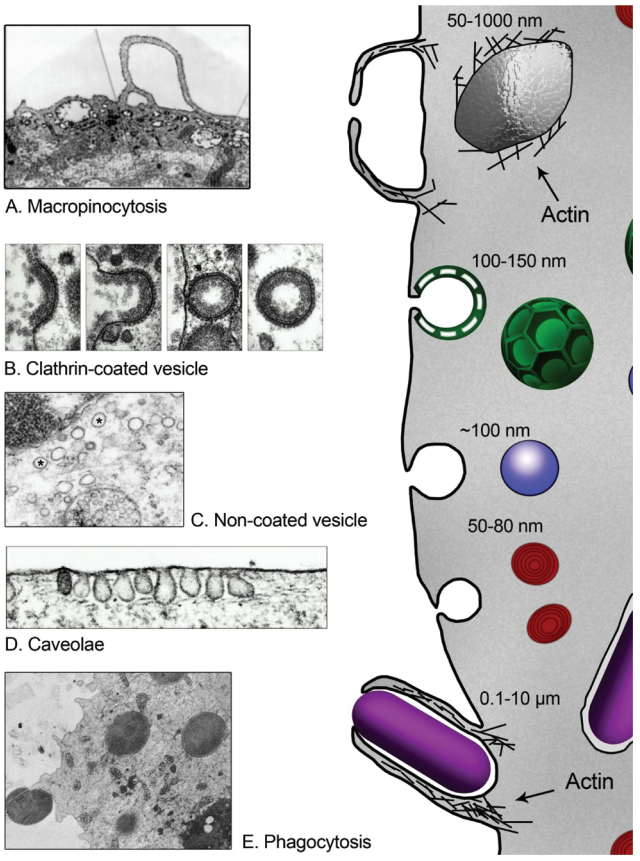

Figure 6. Drawings and electron micrographs illustrating endocytic pathways: macropinocytosis (A), clathrin-dependent pinocytosis (B), clathrin-independent pinocytosis (C), caveolae-dependent pinocytosis (D), and phagocytosis (E). Reprinted with permission from refs 24 and 146−149. Copyright 2007 Jones & Bartlett Publishers, 2002 Saunders, 2007 Nature Publishing Group, 1967 The Rockefeller University Press, 1995 Elsevier.

4.3. Interactions and Internalization of Nanoparticles in Cells

The term "endocytosis" is used to name the different cellular internalization mechanisms (Figure 6). Endocytosis is a process by which cells capture extracellular molecules in vesicles derived from the plasma membrane. It allows cells to feed themselves, to defend themselves, and to regulate homeostasis. Traditionally, according to the type of ingested substance and the size of the endocytic vesicle, two categories of endocytosis are described: phagocytosis and pinocytosis.[146]

4.3.1. Pinocytosis. Pinocytosis is performed by all eukaryotic cells except erythrocytes and concerns the cellular uptake of fluids, soluble material, or nanoparticles into vesicles (generally in the order of 100 nm for "micropinocytic" events, and until 1000 nm in the case of macropinocytosis).[137,146] This phenomenon is often called also "fluid-phase endocytosis" or "fluid-phase pinocytosis" when it does not involve specific receptors.

4.3.1.1. Macropinocytosis. Macropinocytosis is an actin-based process characterized by the formation of large vesicles: the macropinosomes (50−1000 nm). Macropinocytosis constitutively occurs in thyroid cells taking up thyroglobulin and in dendritic cells that need to sample large amounts of extracellular fluid for immune surveillance (Figure 6A).

4.3.1.2. Clathrin-Dependent Pinocytosis and Receptor-Mediated Endocytosis. Clathrin-dependent pinocytosis is used by all eukaryotic cells to internalize nutrients (iron, cholesterol) and degrade or recycle substances that briefly exert their action, such as hormones. Thus, this process is well-known for its role in the selective uptake of molecules through specific interactions with plasma membrane receptors. These receptors form complexes with a ligand and are concentrated at specialized areas of the cell membrane before endocytosis. The protein lattice underlying the patches of accumulated ligand−receptor complexes is made of clathrin and adaptor proteins. Clathrin is a three-legged structure consisting of three 190-kDa heavy chains associated with one of two 30-kDa light chains (LCa or LCb). This hexameric protein complex is named a triskelion. The adaptor protein is composed of two adaptin subunits, a medium μ chain, and a small σ chain. The heterotetramer is also known as an assembly protein. Indeed, adaptor proteins are recruited to docking sites on the cytoplasmic face of the plasma membrane. They concentrate the receptors carrying their ligand and assemble with clathrin to form protein coats.

Clathrin triskelions can aggregate into soccer ball-shaped cages. Adaptor proteins mediate this polygonal arrangement of clathrin that induces an invagination of the plasma membrane into coated pits and, consequently, a clustering of the receptors occupied by their ligands. (Figure 6B). Docking sites for adaptor proteins are still not well characterized, but some data suggest that they are short (4−6) amino acid sequences, containing an essential tyrosine residue, located on the cytoplasmic tail of the transmembrane receptors. This mechanism has been proposed for the low-density lipoprotein (LDL) receptor and the transferrin receptor.[146] The formation of a coated vesicle is regulated by a protein with GTPase activity, called dynamin, which, in association with amphiphysin bound to clathrin and adaptor proteins, self-assembles into a collar at the neck of deeply invaginated coated pits. The vesicle release ("budding") in the cytoplasm occurs after the constriction of the dynamin collar, which is dependent on the hydrolysis of a GTP molecule. The last step of the endocytic vesicle formation is the removal of the protein coat, catalyzed by Hsc70, a member of the heat shock protein. Specifically in the brain, Hsc70-mediated clathrin release is helped by auxilin (Figure 7).

The clathrin-dependent receptor-mediated endocytosis process forms vesicles of 100−150 nm in diameter and is, of course, accompanied by nonspecific fluid-phase endocytosis. Indeed, a volume of extracellular media proportional to the available internal volume of the endocytic vesicle is internalized together with the ligands bound to the concentrated receptors.[146]

4.3.1.3. Clathrin-Independent Pinocytosis. Uncoated pinocytic vesicles (~100 nm) can also be formed. In this poorly characterized process, an invagination of the cell membrane occurs and leads to a nonspecific uptake of surrounding bulk fluid after fission of the newly formed vesicle at the level of its apical pole (Figure 6C).

4.3.1.4. Adsorptive Endocytosis. Nonpermeant molecules contained in the extracellular fluid can be internalized by clathrin-dependent or clathrin-independent fluid-phase endocytosis. A phenomenon of adsorptive endocytosis can be induced by nonspecific interactions between a molecule and the cell membrane.[150] Lectins, such as wheat germ agglutinin (WGA), bind to sugars (sialic acid and N-acetylglucosamine) present on the membrane glycoproteins of brain endothelial cells.[151] The endocytosis of positively charged peptides was suggested to be adsorptive-mediated. Indeed, electrostatic interactions occurring between the peptide and the surface of brain endothelial cells,

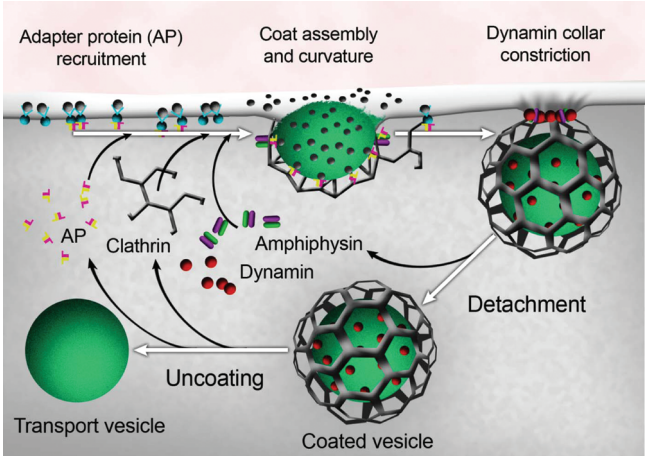

Figure 7. Receptor-mediated endocytosis, involving the formation of a clathrin-coated vesicle.

which is negatively charged due in part to sialylation, were inhibited by polycationic molecules such as poly-L-lysine or protamine sulfate.[152] Furthermore, the nephrotoxicity of aminoglycoside antibiotics (organic polycations) is thought to be due to their adsorption to membrane anionic phospholipids through electrostatic interactions, which induces their endocytosis by renal proximal tubular cells.[153]

Nanoparticles covered with an anionic coating were suggested to nonspecifically adsorb onto the membrane of cancer cells (HeLa) through electrostatic interactions.[154] As plasma membrane cationic sites are much scarcer than anionic sites, the adsorbed anionic nanoparticles are inferred to form clusters because of repulsive interactions with the large negatively charged domains of the membrane.

Anionic heme-peptide hybrids were also found to adsorb on the endothelial surface of mouse pancreas capillaries.[155] In the same context, electrostatic interactions between the cell membrane and nanoparticles carrying a (positive or negative) charge are thought to induce membrane bending and disruption, leading to the formation of invaginations and to the encapsulation of the nanoparticles in endocytic vesicles.[156,157]

Pinocytic vesicles, formed either by clathrin-dependent or clathrin-independent mechanisms, fuse with endosomes. They first deliver their content to tubulovesicular early endosomes in which pH is around 6.5. Acidification of the pH by proton pumps (ATPases) leads to the dissociation of receptor−ligand complexes. Membrane and free receptors accumulate in tubular portions of the endosomes that are detached and recycled, while free ligands are released in the lumen of the vacuolar part of the early endosome. Internal membrane accumulates in the endosome during its maturation. The early endosome becomes a multivesicular body in which pH decreases to 5.5 and receptor−ligand complexes continue to dissociate for the recycling of receptors. The multivesicular bodies mature to late endosomes and, because of the proton pump activity, pH is rendered more acidic (4.5). This maturation process prepares the endosomes for fusion with lysosomes containing hydrolytic enzymes that will degrade the initially endocytosed material.[146]

4.3.1.5. Caveolae-Dependent Pinocytosis. Caveolae are pinocytic vesicles (50−80 nm) coated with a self-assembly of caveolin, an integral membrane protein with high affinity for cholesterol. In fact, caveolin helps in forming and/or stabilizing the lipid rafts of the plasma membrane. These membrane subdomains, which are rich in cholesterol and glycosphingolipids, can become highly specialized because many plasma membrane proteins involved in signaling pathways are anchored to the lipid bilayer via glycosylphosphatidylinositol (GPI, extracellular leaflet), or via fatty acylation (intracellular leaflet). Thus, caveolae can bud into the cytoplasm from these plasma membrane-associated lipid rafts (Figure 6D). Then, through a dynamin-dependent process, transport vesicles are formed. This endocytosis process is abundantly performed by endothelial cells but is also found in virtually all animal cells. However, compared to clathrin-dependent endocytosis, the rates and extents of caveolae-mediated endocytosis are smaller and the physiological significance remains unclear. Potocytosis is an internalization mechanism involving caveolae. As clathrin-dependent endocytosis, potocytosis is induced by the binding of a ligand to its specific receptor. In this process, a caveolin-coated pit is formed but not separated from the cell membrane. Nevertheless, the invagination is pinched and the lumen of the nearly formed vesicle is acidified through H^+ ATPases, which facilitates the dissociation of ligand−receptor complexes. Folate is known to be endocytosed through this mechanism; after detachment from its receptor in the acidified compartment, the vitamin is brought into the cytoplasm by a specific transporter. Furthermore, the activity of the folate transporter is driven by the transmembrane proton gradient.[146,158]

4.3.2. Phagocytosis. Phagocytosis is the uptake of large particles into large phagosomes (0.1−10 μm). In higher organisms, this process is restricted to macrophages or neutrophils, which are called "professional phagocytes". These cells are involved in host defense and capture of invading pathogens such as yeast or bacteria. The initiation of phagocytosis is mediated by receptors that either recognize serum components (opsonins) bound to the particle, or directly recognize molecular determinants on the phagocytic target. Thus, phagocytosis is either opsonin-dependent or opsonin independent.

The in vivo opsonization of foreign particles induces their recognition by specific receptors situated at the surface of the phagocytes. For example, the immunoglobulin IgG (a type of antibody) is an opsonin that binds to foreign antigens of invading bacteria. The constant regions of the IgG molecules covering the bacteria are then recognized by the Fc receptors of macrophages, a process which initiates the ingestion of the foreign particle. Complement proteins tag infected or dying cells and allow their attachment to specific phagocyte receptors. For example, apoptotic cells are opsonized with iC3b. This fragment of the C3b fraction of the C3 complement protein binds to the complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18), which are β2 integrins expressed by macrophages. Thus, an opsonization with iC3b allows for the recognition and clearance of the apoptotic cells by macrophages.[146,159] Opsonin-independent phagocytosis is mediated by phagocyte receptors that directly recognize their phagocytic target. The class A scavenger receptor (SR-A), the vitronectin receptor, the mannose receptor, the asialoglycoprotein receptor, and the β2 integrins mediate a nonopsonic recognition of microorganisms or apoptotic cells before their phagocytosis.[160] The SR-A type I and II bind anionic polymers including the bacterial Gram-negative lipopolysaccharide (LPS), the bacterial Gram-positive lipoteichoic acid, and apoptotic cells probably by interacting with negatively charged phospholipids displayed on the surface of dying cells.[161] The β2 integrin CR3 can interact with the iC3b opsonin, but also, for example, with the *Bordetella pertussis* adhesin filamentous

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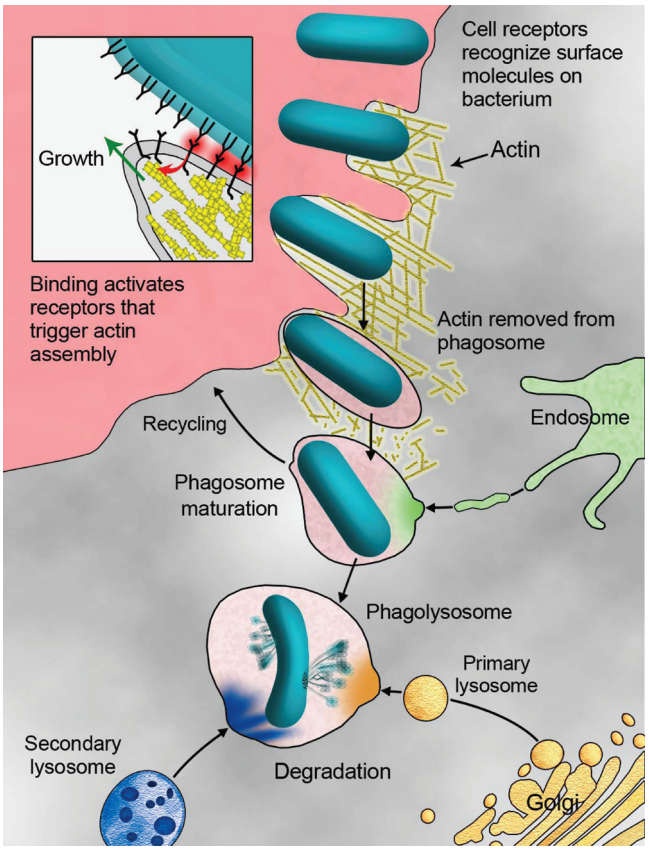

Figure 8. The mechanism of phagocytosis, from contact with the target to its intracellular digestion. Reprinted with permission from ref 146. Copyright 2002 Saunders.

hemagglutinin (FHA). Thus, the β2 integrins (CD11/CD18 integrins) are able to mediate both opsonophagocytosis and nonopsonic phagocytosis.[160,162−164] Attachment of the phagocytic target to the phagocyte receptors triggers an energy-dependent rearrangement of the actin microfilament network near the site of ligand−receptor binding, so that the plasma membrane starts to extend along the target sides forming pseudopodia. Thanks to new interactions between free receptors and available ligands along the extending "arms" of the membrane, the engulfment process continues until the formation of a phagosome is complete (Figure 6E). After dismantling of the actin microfilaments, the phagosome is directed more deeply into the cell by microtubules and is matured. Finally, a phagolysosome is formed by fusion with an acidic lysosome (vesicle coming from the Golgi apparatus) containing hydrolytic enzymes that will degrade the ingested target. The products of this digestion can be used by the cell for the synthesis of new molecules[146] (Figure 8).

Some studies have indicated that SPIONs undergo endocytosis via mediation through a different cell surface receptor, namely, Mac-1 (CD llb/CD18), which may imply that particle size determines with which receptors SPIONs interact.[165] SPIONs are endocytosed more readily by activated macrophages than by nonactivated ones, pointing out the fact that not only cell type but also cell phenotype and changes in membrane receptor expression can significantly influence the uptake. This corroborates the study of Rogers and Basu[166] in which activated macrophages exhibited different uptake profiles depending upon their exposure to various cytokines.

The lowest levels of intracellular delivery have been reported for SPIONs coated with polymers, particularly natural polysaccharides such as dextran and pullalan.[124]

Similarly to macrophages, even the modest uptake of dextran-coated particles in nonphagocytic cells has recently been shown to be dependent upon the nanoparticles size.[167] In other studies involving the use of synthetic polymers, such as polyethylene glycol (PEG), Zhang et al.[168] have observed reduced uptake in some cells and increased uptake in others via nonendocytic mechanisms, including passive diffusion through the plasma membrane. The increased uptake in these cells has not been well-characterized and has been attributed to either the membrane-soluble nature of PEG[153] or simply increased fluid-phase endocytosis.[123] In other applications (e.g., magnetofection), the polymeric coating may actually be endowed with charge in order to facilitate SPION−cell interactions and promote labeling into the cell.[169−171]

Studies of cell interactions and labeling involving proteins and antibodies as SPION coatings have shown that they typically enter through classical endocytic mechanisms, which can, in some cases, be modulated or blocked by the choice of protein coating. Berry et al.[172] observed reduced levels of clathrin-mediated endocytosis in albumin-coated SPIONs versus bare and dextran-coated particles. Specific receptors, such as folate receptors, have been targeted by SPIONs, resulting in the induction of receptor-mediated endocytosis.[164,173] Curtis et al.[125,172] demonstrated that targeting of certain cell surface receptors, such as the insulin receptor, or coating with iron binding proteins, such as transferrin, could act to prevent endocytosis. Other types of SPION coatings such as silica have been shown to be internalized by many cell types, including multiple organ systems in vivo and stem cells in vitro.[173] Using clathrin inhibitors, silica SPION−cell interactions[174] revealed clathrin mediated endocytosis as the likely route of access into mesenchymal stem cells.

The most successful delivery methods have been obtained with coatings of highly charged molecules such as the TAT peptide,[116,175] as well as anionic coating such as dimercaptosuccinic acid (DMSA) or citric acid.[127,134] While TAT-coated SPIONs would be expected to be electrostatically attracted by negative charge of the cell surface (due to sialic acid residues) and enter the cell via macropinocytosis,[176] it is interesting that anionic coatings also seem to be effective at coordinating the particles to isolated positively charged regions on the cell surface. Wilhelm and Gazeau[177] have shown that the anionic magnetic nanoparticles enter the cell through both clathrin-mediated endocytosis as well as nonspecific endocytosis. The observed efficiency of these and similar particles, however, may be somewhat of a double-edged sword, as highly electrostatic coatings have shown a disproportionately high toxicity compared with other coatings in multiple in vitro and in vivo studies

4.4. Models of Nanotoxicity

The field of particulate nanotoxicology has recently been the subject of several reviews.[178−181] Some of the major constituents of particles, including SPIONs, are transition metals, and it is the oxides of these transition metals which are thought to be primarily responsible for the oxidative stress they can cause. Much work has gone into the study of the mechanisms of health effects and the role that transition metals may play in the generation of free radicals.

There are thought to be at least four primary sources of oxidative stress in response to particles: direct generation of ROS

from the surface of the SPIONs, via leaching of iron molecules from the iron oxide particles, altered mitochondrial and other organelle functions, and induction of cell signaling pathways and activation of inflammatory cells resulting in the generation of ROS and reactive nitrogen species such as nitric oxide (NO).[182]

4.4.1. Cytotoxicity Assays. Viability studies require appropriate controls, since unwanted side reactions must be checked. In order to get a reliable result, several assays should be combined: cell membrane integrity (Trypan blue exclusion assay, lactate dehydrogenase (LDH) assay), mitochondrial metabolism (MTT assay), cell metabolism (Alamar Blue), lysosomal membrane integrity (neutral red) or nucleic acid staining (propidium iodide staining).[183]

The cellular uptake of iron oxide cores usually results in a transient induction of reactive oxygen species (ROS) that can impair the cell plasma membrane, mitochondria, and nucleus, resulting in severe cell dysfunction and finally cell death. ROS are caused by the presence of iron oxide moieties in the acidic lysosomal environment. The coating itself can also transiently induce ROS.[184]

As result, cells can suffer from DNA damage or initiate an apoptotic/necrotic cell death pathway. This has been described as being mediated by increased cytoplasmic calcium levels due to the altered redox equilibrium state of the cell.[185] The correlation between induced ROS and increasing cytoplasmic calcium levels has been observed for various types of nanoparticles, but the precise mechanism by which reactive species can lead to a Ca^{2+} influx is still elusive and appears to differ depending on the precise conditions.[184]

Gao et al.[186] reported that intact magnetic iron oxide cores exhibit an intrinsic peroxidase activity that can reduce the amount of hydrogen peroxide present in the cell. As H_2O_2 plays an important role in regulating cell growth, the decreased cellular levels of H_2O_2 were found to stimulate cell proliferation.[187]

Other than direct cytotoxic effects, cell labeling with iron oxide nanoparticles can lead to secondary or long-term effects. One of these secondary effects is a transiently decreased proliferative capacity of the labeled cells as compared with unlabeled control cells. The absorbance measured in metabolic assays, such as the MTT assay, reflects the total metabolic activity of a cell population and is therefore also an indirect measurement of cell proliferation.

Another method[188] is based on assessing DNA duplication, by incorporation of thymidine analogues such as 5-bromo-2deoxyuridine or 5-ethynyl-2′-deoxyuridine during the active synthesis.

Intracellular accumulation of nanoparticles can have profound effects on cell morphology, and high levels of internalized particles can invoke cellular stress and induce changes in the architecture of the actin cytoskeleton. An altered network displaying actin stress fibers can lead to reduced proliferative capacity and cell spreading and can influence migration and differentiation of stem cells.[189,190] Iron oxide labeling can impair cellular capacities.[191]

Transition metal ions such as Fe(II) and Fe(III) can generate ROS through Harber-Weiss reactions:

$$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$$

Superoxide, produced via normal metabolic processes and redox cycling within the cell, reacts with hydrogen peroxide to form the hydroxyl free radical. The catalysts are the iron ions that have been brought into the cell by the SPIONs. The hydroxyl free radicals are responsible for oxidative stress and damage to the cell. These reactions are explained by Fenton chemistry and can be described by the equations:

$$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

The potent hydroxyl free radical generated by these reactions is then free to attack any biomolecule within a diffusive distance.

Several studies already mentioned have shown direct evidence of ROS damage by SPIONs.[126,127,142,192,193] van den Bos et al.[126] showed a SPION dose-dependent increase in lipid peroxidation, while Stroh[127] measured significant increases in both lipid and protein oxidation. Jain et al.[142] noted considerable increases in lipid peroxidation in several tissues in vivo following SPION administration in rats. Alekseenko et al.[192] also studied the effects of SPIONs upon neuronal cells. They studied the effects of ferritin, the natural iron storage protein that contains a 7 nm iron oxide core, surrounded by a protein coat.[193] Ferritin was found to generate ROS directly in rat synaptosomes, and the authors surmised that it might lead to neurodegeneration in vivo.

The effects of SPIONs on the cell life phases have been studied also. Mahmoudi et al.[10] employed the effect of both bare and PVA coated SPIONs (with iron concentrations of 20, 40, and 80 mM) on the mouse tissue connective fibroblast (L929 cell line) cell life cycle in order to facilitate the identification of any phase changes. It is worth noting that the core sizes of the bare and coated SPIONs were 4.5 and 5 nm (hydrodynamic size of 48 and 85 nm), respectively. According to the obtained results, significant apoptosis occurred for bare SPIONs at the highest applied concentration (80 mM), whereas negligible apoptosis had been detected for the coated magnetic nanoparticles. In contrast, the treated cells with the coated SPIONs at a concentration of 80 mM indicated enhancement in the mitotic phase (G_2/M) while no improvement in this phase was observed for the uncoated SPIONs. Interestingly, the increasing granularity of the treated cells, with both the bare and coated SPIONs, together with the formation of gas vesicles indicated the domination of autophagy cell death rather than apoptosis or necrosis.[10,92] Considering apoptosis, the same observation on the treated L929 cells with both uncoated and coated-SPIONs has also been reported using a TUNEL assay at a nanoparticle concentration of 40 mM.[11] The authors claimed that the apoptosis in the sub G_0/G_1 phase is attributed to irreversibly damaged DNA,[10] which was later confirmed by comet assay as described in ref 175.

Chekhun et al.[194] probed the effect of a doxorubicin−magnetite (Fe_3O_4; with a diameter of 20−40 nm) combination on the cell life cycle in vitro and in vivo. It is noteworthy that doxorubicin is a phase-specific preparation and the biggest sensitivity to it is observed in the S-phase of cell life.[195] The cell cycle of tumor cells taken from the abdominal cavity of mice after intraperitoneal injections with doxorubicin, magnetite nanoparticles, or their combination was studied. For the in vitro assessments, the cell cycle of intact Ehrlich ascitic carcinoma cells was tracked after the cells were incubated with doxorubicin and magnetic nanoparticles. In this case, the concentration of magnetite was constant (iron ion concentration of 100 μM); in contrast, doxorubicin was employed at various concentrations (1 μM, 10 μM, and 100 μM). It is worth noting that 100 μM of doxorubicin approximately corresponds to a therapeutic dose of 3 mg/kg for in vivo conditions.[194]

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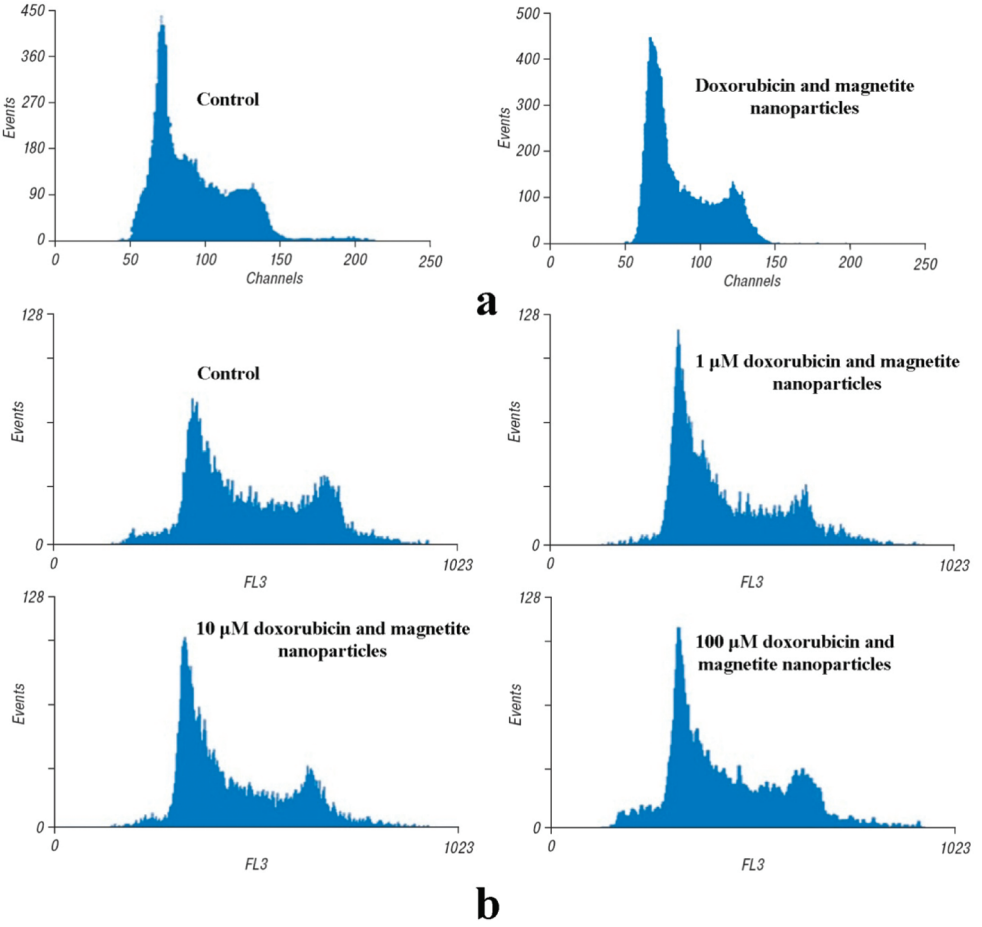

Figure 9. (a) In vivo and (b) in vitro cell cycle analysis of both control and treated cells (i.e., with a combination of doxorubicin and magnetite nanoparticles). Reprinted with permission from ref 146. Copyright 2002 Saunders.

The results of in vivo tests confirmed the significant decrease of the cell population in S phase of the cells treated with a combination of doxorubicin and magnetite nanoparticles, as compared to control groups. Further, the treated cells showed higher rates of apoptosis in comparison to the control cells (see Figure 9). Considering the in vitro results, the Ehrlich ascitic tumor cells treated with a combination of doxorubicin and magnetite nanoparticles for 48 h showed a decreased amount of the cell population in S phase together with increasing number of cells in sub G_0/G_1 phase (see Figure 9). It should be noted that the cell distribution in the cell life cycle was changed; however, the variations were not proportional to the drug concentration, which suggests that the cell cycle was mainly influenced by the magnetite nanoparticles.

4.4.2. Alteration of Organelle and Mitochondrial Function. Mitochondria produce energy via the citric acid cycle and are critically dependent upon redox reactions from the respiration chain. Electrons that leak from this process have been shown to be a constant source of superoxide and hydrogen peroxide[196] and are free to react with iron via Fenton chemistry. Nanoparticles have been implicated to be in direct contact with, and to produce damage within, mitochondria.[107] Given this proximity to the mitochondria, it is highly likely that the redox active surface of SPIONs could significantly affect electron flow and act to alter mitochondrial functionality.

An assay employed for testing cell viability is the MTT (and MTS) assay, which indirectly measures cell viability by testing for the presence of active reductase enzymes within the mitochondria of living cells, ultimately causing a color change in the MTT reagent upon its reduction. Although commonly used as a viability assay, the MTT assay more specifically represents a measure of mitochondrial function rather than of cell viability and can in principle be used to measure alteration of mitochondrial function in cells. Several studies have utilized the MTT assay to assess mitochondrial function; however, other studies typically have assumed nonviability in cells exhibiting reduced reductive activin.[197,198]

Another potential intracellular target for SPIONs-associated toxicity is the plasma membrane and proteins. In addition to the induction of cell signaling pathways, SPIONs-induced redox reactions can activate and upregulate plasma membrane proteins such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[199] This activation is known to occur within phagocytic cells such as the previously mentioned macrophages, which are known to reliably take up SPIONs in vitro and within the body.[129,130,137,200−205]

4.4.3. Long-Term Nanotoxicity of SPIONs - General and Neurological Toxicity. Because of the relatively recent advent of SPIONs engineered for in vivo use, there are currently no studies that have investigated the long-term effects that exposure to them might have. There is, however, knowledge that may be extrapolated from analogous studies in PM toxicology that might indicate potential long-term implications. For example, the well-studied

oxidative stress paradigm is known to be involved in the development of numerous disorders, such as pulmonary injury and morbidity,[206] atherosclerosis,[207] cardiovascular disease,[208] diabetes mellitus,[209] reproductive dysfunction,[210] cancer,[211] and others.[212,213]

It has become well-established that iron and iron accumulation is associated with multiple neurodegenerative diseases including Parkinson's and Alzheimer's disease.[214] Since Kirschvink et al.[215] discovered iron oxide particles in the brains of humans 15 years ago, investigators have repeatedly reported the presence of high levels of accumulated iron in the brains of patients suffering from neurodegenerative disorders. Iron has traditionally been thought to cross the blood−brain barrier primarily through a transferrin-mediated route through the capillary endothelium; however, there are multiple other routes including passage through olfactory nerves or circumventricular organs.[216] Additionally, iron has also been shown to be transported via ferritin,[217] and this may represent a method of transport of dissolved iron derived from SPIONs to the brain. Alekseenko et al.[192] demonstrated that the iron nanoparticles within ferritin proteins could generate ROS within brain synapses, as well as interfere with the transmission of neuronal signals. Furthermore, several studies on the biodistribution of SPIONs have shown particle migration to the brains of exposed subjects.[140,142,218] This, coupled with the fact that nanoparticles have also been shown to be selectively toxic to neuronal type cells,[132,219,220] gives rise to concern regarding the potential effects that exposure to SPIONs may have; even when deliberate, as in the case of MRI applications.

The brain is especially susceptible to the effects of oxidative stress, as it is highly reliant upon iron for its high respiratory activity, as well as for myelinogenesis and the production of numerous neurotransmitters. This high oxidative metabolism and the resulting generation of ROS make the brain particularly susceptible to imbalances in the oxidative equilibrium that are thought to be related to, or cause, neurodegeneration and associated disorders. While excess iron can directly produce toxic effects on neurons, according to the classical oxidative stress paradigm, it is thought that one of the prime mediators and culprits of ROS-mediated neurotoxicity are microglia.[221] Microglia are the professional phagocytic cells of the central nervous system and serve many of the same functions as macrophages do for the rest of the body, including phagocytosis, antigen presentation, and cytokine release. Exposure to metal oxides has been shown to specifically activate these cells in vitro and result in the generation of ROS that can damage and kill dopaminergic neurons.[220,222] But what is particularly troublesome is that damaged or dead neurons, particularly those from an instigating stimulus such as might be derived from SPION exposure, can also further activate microglia and induce a condition known as reactive microgliosis that results in a vicious and destructive cycle progressing to neurodegenerative disease.[221]

5. EFFECT OF NONMAGNETIC NANOPARTICLES ON THE CELL CYCLE

5.1. Metallic Nanoparticles

5.1.1. Gold. Gold is one of the most widely studied nanostructures. It has a number of unique physicochemical properties at the nanoscale, and the manipulation and synthesis of these properties have been studied in great depth.[223] Given the ubiquity of gold nanoparticles, their compatibility with the cell cycle is imperative. Despite the inert nature of gold, some gold nanostructures can lead to DNA damage secondary to an oxidative stress response, which is also thought to be dependent on the size of the particle.[224] Li et al.[224] noticed that, regardless of the underlying genetic damage and transcriptional changes observed in the cells treated with gold nanoparticles, no ultimate cytotoxicity was observed in the study. The total internal dose of gold nanoparticles has been shown to be influenced by a number of factors including the size and shape of the particles.[225,226] Such properties must be taken into account when one considers the possibility of gold nanoparticles impacting the cell cycle. Moreover, as gold nanoparticles are being engineered to interface with other materials and conjugated to a variety of chemicals, the subsequent effect on the cell cycle will have to be accounted for.

5.1.2. Silver. Silver is also an extremely well studied metal given its unique nanoscale properties and potential applications in biotechnology. This material is particularly popular for study and application due to the novel bactericidal properties.[227,228] Nevertheless, the volume of silver nanoparticle applications has led to concern over possible toxicity and in particular damage to cellular components such as DNA.

One study manipulated particles to compare the effect of surface coated silver nanoparticles with uncoated ones using mouse embryonic stem cells and embryonic fibroblasts.[229] The authors demonstrated that coated silver nanoparticles exhibited a greater degree of DNA damage response, as shown by increased expression of repair proteins and H2AX phosphorylation, than uncoated particles. It was postulated that there was a more efficient distribution of coated particles, as compared to the observed agglomeration of the uncoated silver particles, thereby enhancing the total surface area of the particle dose and thus exposing the cellular contents to more particulate properties. It was noted that both types of nanoparticles (coated and uncoated) increased p53 expression and p53 phosphorylation, upregulated the DNA damage repair protein Rad51, and also increased the phosphorylated form of H2AX, which shows that at some exposure levels genetic alteration may occur.

It is also known that particulate metals can be a source of oxidative stress, which is linked to perturbations in the cell cycle and DNA anomalies.[17,230] With respect to silver nanoparticles, Carlson et al. have show a dose-dependent response in ROS generation.[231] Further study is required to determine specific impacts on the cell cycle as a result of pathways activated from oxidative stress.

Starch coated silver nanoparticles at concentrations of 25 and 80 mg/mL (around 0.2−3.2 mM) have shown cell life arrest in the G_2/M phase, evidenced by the increasing cell population in this phase compared to control, on the human glioblastoma cells (U251) and IMR-90 normal human lung fibroblast cells.[232] It is worth noting that the massive enhancement in the G_2/M phase was observed by increasing the concentration of silver nanoparticles; the massive enhancement was observed in the G_2/M phase (see Figure 10). It is hypothesized that the oxidative stress in cells treated with silver nanoparticles possibly caused DNA damage and the early effect is evidenced in cell cycle progression. It is well recognized[233] that irreversible damage of the cells could cause apoptosis phenomena which would be tracked by the accumulation of cells in the sub G_0/G_1 phase. Since in this study there was no significant difference in the cell population in sub G_0/G_1 phases between treated and control cells, it was concluded that no significant apoptosis had occurred (see Figure 10).

As discussed above, metals can play an important role in the generation of oxidative stress in nanoparticle toxicity. Their close

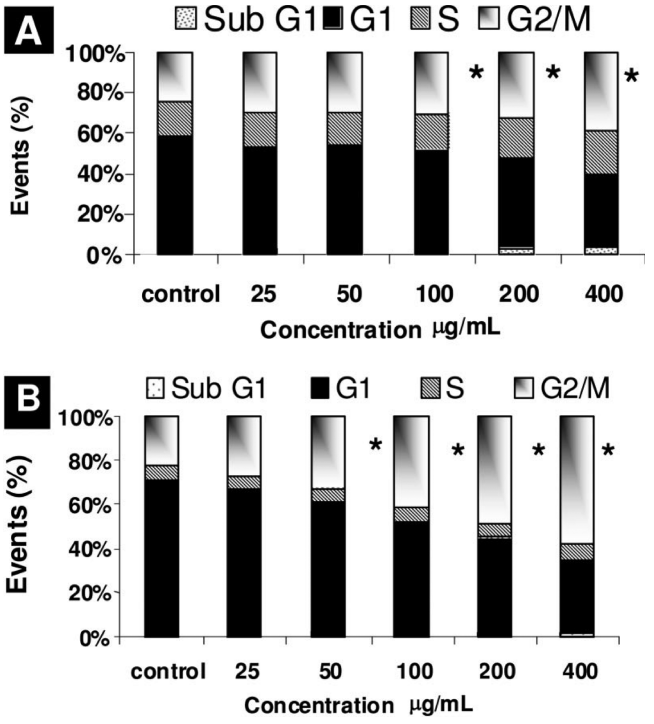

Figure 10. Ag nanoparticle treated U251 cells (A) showed a slow increase in the S/G_2 population, and IMR-90 cells (B) showed a concentration-dependent G_2/M arrest. Markers were set at regions of interest (sub G_0, G_1, S, and G_2/M); * represents $P < 0.05$. Reprinted with permission from ref 232. Copyright 2009 American Chemical Society.

cousins include ceramic metal oxides and they have also been shown to cause oxidative stress and cytotoxicity under certain conditions.[234,235]

5.2. Ceramic Nanoparticles

5.2.1. Titanium Dioxide. Given that oxidative stress and inflammation are associated with cell cycle disruption via genotoxicity secondary to reactive oxygen species adducts, metal oxides are a possible source of cell cycle toxicity. For example, several studies have shown it is possible that nanosized TiO_2 may indirectly result in DNA changes. TiO_2 is a commonly studied material in its nanoscale format and is also abundant industrially. Exposure to nanoscale TiO_2 has been shown to result in chromosomal damage in Syrian hamster embryo (SHE) fibroblasts, blood lymphocytes, and in WIL2-NS (a human B-cell lymphoblastoid cell line) using the micronucleus assay.[236−238] Therefore, a number of different species including human cells lines have been studied. To better understand the mechanistic outcome of possible genetic damage, Rahman et al. showed an increase in the micronucleus frequency at a concentration range of 0.5−5.0 mg/cm^2 in the hamster embryo fibroblast model.[238] An interesting observation, however, was the acuity with which the observations occurred. Specifically, the authors observed an increase over time in micronuclei formation at 12−24 h, but this increase did not continue when evaluated at 48 and 72 h time points. Using other techniques to determine the origins of the micronuclei increase, they concluded that the nanosized TiO_2 caused clastogenicity, which is mostly associated with ROS-related DNA injury. This mechanism is in accordance with prior studies on known oxidative injury to cells. Early nanotoxicity studies suffered from inadequate characterization of the content of nanoparticle batches leading to incorrect conclusions about nanostructure toxicity. In the above study, particle batch characterization of the TiO_2 particles also was incomplete.

A number of other studies have shown DNA damage which is known to set into action pathways linked to cell cycle arrest and cell death.[236,237,239] Two of these studies demonstrated DNA strand breakage using the Comet assay in epithelial cells exposed to TiO2.[237,239] Karlsson et al. studied an array of particle types, including CuO, TiO_2, ZnO, $CuZnFe_2O_4$, Fe_3O_4, Fe_2O_3, carbon black, and multiwalled carbon nanotubes, to test their effects on DNA in an A549 lung cell line. They concluded that ROS were linked to nanoparticle toxicity. Kang et al.[237] showed relationships to the cell cycle by reporting the accumulation of p53 protein in combination with DNA damage checkpoint kinases in nanosized TiO_2 treated lymphocytes from a 33-year-old female. It was noted, however, that TiO_2 nanoparticles do not stimulate transactivational activity of p53. These observations are in line with oxygen species causing DNA lesions which lead to p53 activation and thereby triggering p53-mediated cell cycle checkpoint signaling.

Prior to these studies Wang et al.[236] reported an increased micronucleus frequency, at a concentration of 65 mg/mL, and noted that nanosized TiO_2 can lead to DNA strand breaks and point mutations. An additional study by Xu et al.[240] also showed that nanoscale TiO_2 can cause genotoxic and thus DNA-harmful effects. The authors did further work to show that reactive nitrogen species such as peroxynitrite anions may be the underlying mechanism for the DNA perturbation. Their data supported their conclusion by showing that the genotoxic response could be inhibited by cycloxygenase-2 and nitric oxide synthase inhibitors. As the reader can recognize, a recurrent theme in metal and metal oxide nanoparticle toxicity is the potential to induce oxidative stress which can lead to intracellular changes causing cell cycle arrest. TiO_2, however, is under some debate in the literature at present. For example, Theogaraj et al.[241] used a chromosomal aberration test in Chinese Hamster Ovary cells and did not observe gene toxicity or an increase in the frequency of DNA damage with or without the photoactivation stimulus of UV light. These results are important given the use of metal oxide components in cosmetics and sunscreens.[242]

Nanoscale TiO_2 is one of the earlier particle types to demonstrate size-specific features related to toxicity. For example, nanoscale TiO_2 anatase less than 20 nm can cause chromosomal damage at a culture concentration of 10 mg/mL as evidenced by micronucleus frequency. Interestingly, a particle size of 200 nm in either the rutile or anatase form did not cause the same effects.[243] As nanotoxicity studies on cell cycle disruption are in their infancy, many of these data need to be scrutinized and revisited as we learn more about in vitro dosimetry and experimental design. As noted before, batch variation, purity, and exposure scenarios may all affect cell cycle signal pathways. Indeed, dosage can affect the type of genes activated, associated with either necrosis or apoptosis, as demonstrated by Ding et al. using multiwalled carbon nanotubes and nano-onions.[244]

5.2.2. Zinc Oxide. ZnO nanoparticles are particularly common and were one of the original foci of study in sunscreens as nanotechnology evolved. These particles are generally assumed to show very little or no toxicity, but, under certain conditions of stress, they have received more attention from toxicologists.[245] Nanoscale ZnO has been shown to induce cytotoxicity and inflammatory changes.[246,247] Dufour et al. examined the possibility of DNA damage in ZnO exposed cells.[248] Specifically, they

Table 3. Effect of Various Nanoparticles on the Cell Life Cycle

nanoparticle	coating	size (nm)	cell type	exposure concentration	exposure time (h)	phase arrest	phase enhanced	remark	ref
SPIONs	polyvinyl alcohol	48	mouse tissue connective	80 mM	72	none	G_2/M	Surface passivated nanoparticles were used.	10
SPIONs	none	4.5	mouse tissue connective	80 mM	72	none	sub G_0/G_1 and G_2/M	Surface passivated nanoparticles were used.	10
SPIONs	polyvinyl alcohol	12	mouse tissue connective	200−400 mM	72	none	none	Surface active nanoparticles were used.	88
SPIONs	none	4	mouse tissue connective	200−400 mM	72	G_0G_1	sub G_0G_1	Surface Active nanoparticles were used.	11, 88
SPIONs	carboxy dextran	45−60	human mesenchymal stem cells	300 μg/mL	1	none	S and G_2/M	SPION-promoted cell growth is due to its ability to diminish intracellular H_2O_2 through intrinsic peroxidase-like activity.	187
silver	starch	6−20	human lung fibroblast and human glioblastoma	25 μg/mL	48	G_2/M	sub G_0/G_1	A possible mechanism of toxicity is proposed which involves disruption of the mitochondrial respiratory chain by Ag-np leading to production of ROS and interruption of ATP synthesis, which in turn cause DNA damage.	232
prodrug		140−180	breast cancer	100 μg/mL	6	G_2/M		Prodrug consists of a succinylated-heparin carrier conjugated to paclitaxel via a single amino acid spacer, either valine, leucine, or phenylalanine.	259
nanoparticulate carbon black	none	14	adenocarcinomic, human alveolar, basal epithelial	100 μg/mL	3, 6, and 24	S	G_2/M	Model of the carbonaceous nanoparticles in urban dust. The specific sample has been shown to elicit oxidative stress in various biological cell systems.	260
nanoparticulate carbon black and benzo(a)pyrene	none	14	adenocarcinomic, human alveolar, basal epithelial	100 μg/mL	3, 6, and 24	none	G_2/M	Model of nanoparticles with organic component. Has been used in lung carcinogenicity studies in rats.	260
gold	polyethylene glycol	30	human oral squamous cell carcinoma	0.4 nM	4	cytokinesis	Sub G_0/G_1	The nanoparticles were bioconjugated with an arginine-glycine-aspartic acid peptide (RGD) and a nuclear localization signal (NLS) peptide; hence, the nucleus of the cells was targeted by gold nanoparticles.	261
gold	bound to fluoro -6-deoxy-D -glucose	10.8	human prostate carcinoma and human diploid fibroblast	15 nM	1, 2, 4, 6, 18, and 24	sub G_0/G_1	G_2/M	Taking into consideration the time dependence of the cell cycle changes induced by GNPs, optimized radiation sensitivity was obtained.	262
poly (D,L-lactide-co-glycolide)	none	226	breast cancer cell	100 μM	48	G_0/G_1 and S	G_2/M and sub G_0/G_1	Doxorubicin drug has been encapsulated in nanoparticles with an efficacy of 46%.	263
						none	none	There is no drug encapsulated in nanoparticles.	
europium oxide	none	60	human cervical carcinoma	200 μM	72	none	none		264

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Table 3. Continued

nanoparticle	coating	size (nm)	cell type	exposure concentration	exposure time (h)	phase arrest	phase enhanced	remark	ref
chitosan	none	46−83 (average of 65)	human gastric carcinoma	25, 50, and 75 μM	24	none	none	All concentrations induced apoptosis.	265
				100 μM		G_0/G_1	S and G_2/M		
polyethylene glycol) -poly(D,L-lactic-co-glycolicacid)-monomethoxy	none	70−180	Chinese hamster ovary	5 mg/mL	4	none	none	Nanoparticles were prepared using a water-in-oil-in-water (w/o/w) emulsion solvent extraction/evaporation technique	266
silica	Berberine	50	human cervical carcinoma, human hepatocellular liver carcinoma, and human embryonic kidney	IC50 concentrations of berberine for the respective cell line	24	G_0/G_1	sub G_0/G_1	Berberine is a plant alkaloid known to have antiproliferative activity.	267
Silica	none	20 and 50	Human embryonic kidney	25, 50, and 100 μg/mL	24	G_2/M	sub G_0/G_1	The G_0/G_1 arrest was observed for nanoparticles with diameter of 20 nm and concentration of 100 μg/mL.	250
linoleic acid	none	<25	breast cancer	100−200 μM		G_0/G_1	sub G_0/G_1	pro-apoptotic proteins such as Bax were up-regulated, whereas antiapoptotic proteins, such as Bcl-2, were down-regulated by treatment with nanoparticles.	268
linoleic acid	polyethylene glycol	25	breast cancer	100−200 μM		G_0/G_1	sub G_0/G_1	The tumor suppressor gene p53 was significantly up-regulated by nano-particles treatment with increasing concentrations, suggesting that nanoparticle-induced apoptosis is regulated by a p53-mediated signaling pathway.	268
titanium oxide	none	25	human lymphocytes	100 μg/mL	24	none	sub G_0/G_1	Nanoparticles increased the proportion of Sub G_0/G_1 cells, activated caspase-9 and caspase-3, and induced caspase-3-mediated PARP cleavage.	269
peptide	none	260 ± 50	fibroblasts and human cervical carcinoma	100−500 nM		G_2/M	none		270
cobalt ferrite	silica	50	human mesenchymal stem cells	0.2 mg/mL	24, 48, and 72	none	none	The nanoparticles do not shown arrest on the cell cycle while they can be taken up by stem cells.	271

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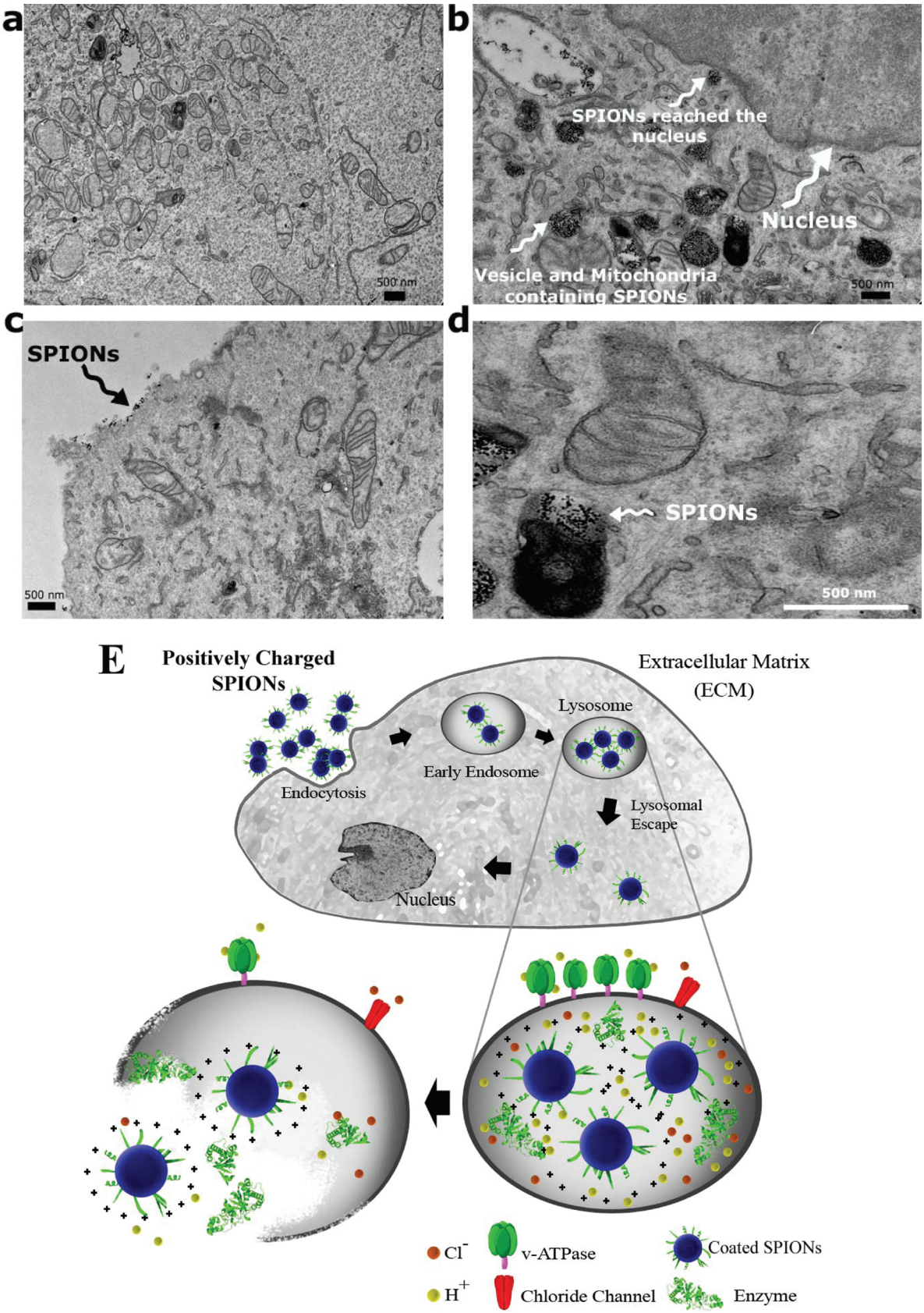

Figure 11. Bio-TEM images of HeLa cells: control (A) and exposed to positively (B) and negatively (C) charged SPIONs, and the internalization of magnetite nanoparticles inside mitochondria (D). Reprinted with permission from ref 8. Copyright 2011 Elsevier. (E) Schematic representation of the endosomal escape of positively charged nanoparticles.

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were looking for possible genomic toxicity in Chinese hamster ovary cells by using the chromosome aberration technique and they factored in the variable of UV light stimulation, which is central to the cosmetic and sunscreen industries' applications of oxide nanoparticles. The authors reported that chromosomal aberrations caused by nanosized ZnO with an average diameter of 100 nm were enhanced by UV light, with increased clastogenicity under preirradiation and simultaneous irradiation conditions as compared to no light stimulation. The dosages used in the study are considerably high and poor batch description of the particles was reported. For example, in preirradiated and simultaneously irradiated cultures an increase in DNA injury was observed at 54 mg/mL as compared to 105 mg/mL when exposure was performed with no light stimulation. This dose demonstrates a proof of principle effect; however, the levels of ZnO exposure were significantly higher than current nanotoxicity studies. Given the current and future likelihood of ZnO in consumer products, it is imperative that realistic exposure conditions be studied.

5.2.3. Silica. SiO_2 has also received tremendous attention given its long industrial history as an occupational carcinogen. Thus, its nanoscale format has been the focus of many studies as it could have significant implications for the cell cycle.[249−251] In fact, even the traditional form has been used as a positive control particle in many toxicity studies. Specifically, nanoscale SiO_2 can disrupt nuclear integrity by forming intranuclear protein aggregates that can lead to inhibition of replication, transcription, and cell proliferation, as reported by Chen et al.[252] These authors also found that SiO_2 nanoparticles reduced replication activity down to 67% and 60% after 6 and 24 h, respectively, as well as transcriptional activity down to 82% after 4 h. They concluded that there was less proliferation after 24 and 48 h postexposure. It was also noted that nanoparticle size may be a significant determinant in their observations, as particles larger than 200 nm did not enter the nucleus, alter nuclear structure and function, or alter gene expression.

Silica is another nanoscale particle that is incorporated into many new nanostructures; thus, more research is required to elucidate its mechanism of action in cells. Furthermore, realistic exposure scenarios will need to be developed to provide accurate data for occupational and consumer disease prevention.

5.3. Polymeric Nanoparticles

Polymeric nanoparticles represent a vast and rapidly expanding area of nanomaterials. There is an enormous body of literature on specific types of nanoparticles and, thus, a brief overview of the most important points concerning these nanoparticle types will be given here. Early polymeric nanoparticles reported by Gref et al. were composed of poly(lactic acid)/poly(lactic-co-glycolic acid) (PLA/PLGA) and PEG block copolymers and were noted to be "long-circulating nanoparticles" due to their stealth properties.[253] Such properties have enabled various drugs to evade the reticuloendothelial system for enhanced drug delivery in cancer therapy and to change the pharmacokinetics of various anticancer agents.

With respect to nanomedicine, polymer−drug conjugates are relatively preliminary nanocarrier systems; however, significant research and development is underway to create novel formulations with value-added physicochemical properties. For example, small changes in the polymer−drug conjugation efficiency may significantly modify the pharmacokinetic parameters and tissue biodistribution.[254]

The prime directive of cancer drugs is to interrupt the rapidly expanding cell-cycle in malignant tissues. As polymer nanoparticles work to target the delivery of such agents to tumors, it is hoped that significant impacts on the tumor cell cycle with systemic toxicity will be observed in chemotherapy. Given that few studies have looked at the specific cytotoxic effect of polymeric nanoparticles without a pharmacological cargo, it is difficult to discern at this time whether polymeric particles alone have an impact on the cell cycle in either a positive or a negative manner.

Biodegradable high-molecular-weight poly (lactic-co-glycolic acid) (PLGA) was used as an encapsulating vehicle to obtain contrast agents composed of Gd-DTPA and fluorocarbon-filled microbubbles. The efficacy of the contrast agent for ultrasound and magnetic resonance imaging was evaluated by in vivo imaging of hepatic vessels and the liver parenchyma of rabbit following injection of the contrast agent. Additionally, the liver and kidney functions were examined before and at 7, 14, 30 days after injection. Neither marked changes in the liver and kidney functions nor other severe complications were detected during the follow-up period.[255]

Dendritic polymers have been found to be a suitable carrier for a variety of drugs with capacity to improve their solubility and their bioavailability. The use of dendrimers in a biological system is constrained because of inherent toxicity associated with them.[256] This toxicity is attributed to the interaction of surface cationic charge of dendrimers with negatively charged biological membranes in vivo.[257] This interaction results in membrane disruption via nanohole formation, membrane thinning and erosion. Dendrimer toxicity in biological system is generally characterized by hemolytic toxicity, cytotoxicity, and hematological toxicity. To minimize this toxicity, two strategies have been utilized; first, designing and synthesis of biocompatible dendrimers; and second, masking of peripheral charge of dendrimers by surface engineering. Conjugated polymer nanoparticles are highly versatile nanostructured materials that can potentially find applications in various areas such as bioimaging, biosensing, or nanomedicine. Their synthesis with desired sizes and properties, biocompatibility, and nontoxicity make these materials highly attractive for different applications.[258]

6. CONCLUSION AND FUTURE PERSPECTIVES

The effects of various nanoparticles on the cell life cycle are presented in Table 3. A widespread deep understanding of the various interactions of nanoparticles with the biomolecules in both in vitro and in vivo systems could have significant effects on the cell life cycle. One of the effects, for instance, could be changes in the surface charge of nanoparticles such as with SPIONs. Specifically, the surface charge can determine the direct or indirect interactions of nanoparticles with the DNA, which can define the induced type of problem to the cell life phases. For example, the positively charged SPIONs (coated with PVA-NH_2^+) are capable of entering the nucleus of the cells by escaping from lysosomes. The precise mechanism of the lysosomal escape is likely particle specific but may be related to the acidic pH of the lysosomal milieu. The reason for the specific nuclear location of the particles is unknown, but other nanoparticles have also been found at various organelles in the cell. In the above study, this nuclear localization persisted for only 72 h.[8] In contrast, negatively and neutrally charged SPIONs (e.g., with PVA-COO^- and PVA coatings, respectively) can be either attached to cell membranes or be endocytosed by the cells without the chance of escaping from the lysosomes (see Figure 11).

Hence, the various effects on the cell cycle may depend on the intracellular location of the nanoparticles.

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Sophie Laurent was born in 1967. Her studies were performed at the University of Mons-Hainaut (Belgium) where she received her Ph.D. in Chemistry in 1993. She then joined Prof. R. N. Muller's team and was involved in the development (synthesis and physicochemical characterization) of paramagnetic Gd complexes and super paramagnetic iron oxide nanoparticles as contrast agents for MRI. She is currently working on the vectorization of contrast agents for molecular imaging. She is lecturer and coauthor of about 90 publications and more than 180 communications in international meetings.

REFERENCES

(1) Bensaude-Vincent, B.; Hessenbruch, A. *Nat. Mater.* **2004**, *3*, 345.
(2) Joachim, C. *Nat. Mater.* **2005**, *4*, 107.
(3) Smaglik, P. *Nature* **2006**, *439*, 506.
(4) Venema, L. *Nature* **2006**, *442*, 994.
(5) de la Fuente, M.; Ravia, M.; Paolicelli, P.; Sanchez, A.; Seijo, B.; Alonso, M. J. *Adv. Drug Delivery Rev.* **2010**, *62*, 100.
(6) Jones, C. F.; Grainger, D. W. *Adv. Drug Delivery Rev.* **2009**, *61*, 438.
(7) Whitesides, G. M. *Small* **2005**, *1*, 172.
(8) Mahmoudi, M.; Sant, S.; Wang, B.; Laurent, S.; Sen, T. *Adv. Drug Delivery Rev.* **2011**, in press; DOI: 10.1016/j.addr.2010.05.006.
(9) Mahmoudi, M.; Simchi, A.; Milani, A. S.; Stroeve, P. *J. Colloidal Interface Sci.* **2009**, *336*, 510.
(10) Mahmoudi, M.; Simchi, A.; Vali, H.; Imani, M.; Shokrgozar, M. A.; Azadmanesh, K.; Azari, F. *Adv. Eng. Mater.* **2009**, *11*, B243.
(11) Mahmoudi, M.; Shokrgozar, M. A.; Simchi, A.; Imani, M.; Milani, A. S.; Stroeve, P.; Vali, H.; Hafeli, U. O.; Bonakdar, S. *J. Phys. Chem. C* **2009**, *113*, 2322.
(12) Helmus, M. N. *Nat. Nanotechnol.* **2006**, *1*, 157.
(13) Helmus, M. N. *Nat. Nanotechnol.* **2007**, *2*, 130.
(14) Jones, R. *Nat. Nanotech.* **2009**, *4*, 75.
(15) Mazzola, L. *Nat. Biotechnol.* **2003**, *21*, 1137.
(16) Rouch, D. A.; Lee, B. T. O.; Morby, A. P. *J. Indust. Microbiol. Biotechnol.* **1995**, *14*, 132.
(17) Xia, T.; Kovochich, M.; Brant, J.; Hotze, M.; Sempf, J.; Oberley, T.; Sioutas, C.; Yeh, J. I.; Wiesner, M. R.; Nel, A. E. *Nano Lett.* **2006**, *6*, 1794.
(18) Nel, A.; Xia, T.; Madler, L.; Li, N. *Science* **2006**, *311*, 622.
(19) Van Houten, B.; Woshner, V.; Santos, J. H. *DNA Repair (Amst)* **2006**, *5*, 145.
(20) Murphy, M. P. *J. Biochem.* **2009**, *417*, 1.
(21) Smith, J. A.; Martin, L. *Proc. Natl. Acad. Sci. U. S. A.* **1973**, *70*, 1263.
(22) Morgan, D. O. *The Cell Cycle: Principles of Contro*; New Science Press in association with Oxford University Press: London, 2007.
(23) Lewin, B., Cassimeris, L., Lingappa, V. R. *Cell*; Jones & Bartlett Publishers: London, 2007, 371.
(24) Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. *Mol. Biol. Cell*, 5th ed.; Garland Science: New York, 2008.
(25) Alberts, B., Bray, D., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. *Essential Cell Biology: An Introduction to the Molecular Biology of the Cell*; Garland Publishing: New York, 1997.
(26) Nigg, E. A. *Bioessays* **1995**, *17*, 471.
(27) Robbins, C., Kumar, A. *Pathological Basis of Disease*; Elsevier: Philadelphia, 2004.
(28) Norbury, C. Hardie, D. G., Hanks, S., Eds.; In *Protein Kinase factsBook*; Academic Press: Boston, 1995.
(29) Barbour, L.; Ball, L. G.; Zhang, K.; Xiao, W. *Genetics* **2006**, *174*, 1789.
(30) Callegari, A. J.; Clark, E.; Pneuman, A.; Kelly, T. J. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 8219.
(31) Malumbres, M.; Barbacid, M. *Nat. Rev. Cancer* **2009**, *9*, 153.
(32) Simic, M. G.; Hunter, E. P. L. *J. Free Radicals Biol. Med.* **1986**, *2*, 227.
(33) Jones, C. F.; Grainger, D. W. *Adv. Drug Delivery Rev.* **2009**, *61*, 438.
(34) Chaudhuri, P.; Paraskar, A.; Soni, S.; Mashelkar, R. A.; Sengupta, S. *ACS Nano* **2009**, *3*, 2505.
(35) Chekhun, V. F.; Todor, I. N.; Lukyanova, N. Y.; Shpyleva, S. I.; Naleskina, L. A.; Khaetsky, I. K.; Kulik, G. I. *Exp. Oncol.* **2009**, *31*, 163.
(36) Cooper, S. *Cell. Mol. Life Sci.* **2003**, *60*, 1099.
(37) Prindull, G. *Exp. Hematol.* **2008**, *36*, 1403.
(38) Roa, W.; Zhang, X.; Guo, L.; Shaw, A.; Hu, X.; Xiong, Y.; Gulavita, S.; Patel, S.; Sun, X.; Chen, J.; Moore, R.; Xing, J. Z. *Nanotechnology* **2009**, *20*, 375101.
(39) AshaRani, P. V.; Low Kah Mun, G.; Hande, M. P.; Valiyaveettil, S. *ACS Nano* **2009**, *3*, 279.
(40) Asharani, P. V.; Xinyi, N.; Hande, M. P.; Valiyaveettil, S. *Nanomedicine (Lond)*, *5*, 51.
(41) Reddy, L. H.; Dubernet, C.; Mouelhi, S. L.; Marque, P. E.; Desmaele, D.; Couvreur, P. *J. Controlled Release* **2007**, *124*, 20.
(42) Moreno, D.; Zalba, S.; Navarro, I.; Tros de Ilarduya, C.; Garrido, M. J. *Eur. J. Pharm. Biopharm. 74*, 265.
(43) Huang, D. M.; Hsiao, J. K.; Chen, Y. C.; Chien, L. Y.; Yao, M.; Chen, Y. K.; Ko, B. S.; Hsu, S. C.; Tai, L. A.; Cheng, H. Y.; Wang, S. W.; Yang, C. S. *Biomaterials* **2009**, *30*, 3645.
(44) Kushwaha, S.; Tripathi, D. N.; Vikram, A.; Ramarao, P.; Jena, G. B. *Reg. Toxicol. Pharmacol. 58*, 145.
(45) Shapiro, H. M. *Cytometry A* **2004**, *58*, 13.
(46) Poot, M. *Current Protocols in Cytometry*; John Wiley & Sons: New York, 2004.
(47) Darzynkiewicz, Z.; Juan, G.; Bedner, E. *Current Protocols in Cell Biology*; John Wiley & Sons: New York, 2001.
(48) Darzynkiewicz, Z.; Crissman, H.; Jacobberger, J. W. *Cytometry A* **2004**, *58*, 21.
(49) Wersto, R. P.; Chrest, F. J.; Leary, J. F.; Morris, C.; Stetler-Stevenson, M. A.; Gabrielson, E. *Cytometry* **2001**, *46*, 296.
(50) Nunez, R. *Curr. Issues Mol. Biol.* **2001**, *3*, 67.
(51) Buchegger, F.; Dupertuis, Y. M.; Perillo-Adamer, F. *Cancer. Res.* **2007**, *67*, 5576.
(52) Lupi, M.; Cappella, P.; Matera, G.; Natoli, C.; Ubezio, P. *Cancer Chemother. Pharmacol.* **2006**, *57*, 443.
(53) Asmuth, D. M.; Wang, N.; Lu, Y.; Li, X. D.; Reece, L.; Terry, N. H.; Pollard, R. B.; Nokta, M.; Leary, J. F.; White, R. A. *Cytometry A* **2005**, *66*, 41.
(54) Huang, S.; Chueh, P. J.; Lin, Y. W.; Shih, T. S.; Chuang, S. M. *Toxicol. Appl. Pharmacol.* **2009**, *241*, 182.

(55) Shedden, K.; Cooper, S. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 4379.
(56) Darzynkiewicz, Z.; Juan, G. *Current Protocols in Cytometry*; John Wiley & Sons: New York, 2001.
(57) Dolbeare, F. *Histochem. J.* **1995**, *27*, 339.
(58) Terry, N. H.; White, R. A. *Nat. Protoc.* **2006**, *1*, 859.
(59) White, R. A.; Meistrich, M. L.; Pollack, A.; Terry, N. H. *Cytometry* **2000**, *41*, 1.
(60) Torricelli, A.; Bisiach, M.; Spinelli, L.; Ubezio, P. *Cytometry* **1997**, *29*, 222.
(61) Terry, N. H. A.; White, R. A. *Nat. Protoc.* **2006**, *1*, 859.
(62) Buck, S. B.; Bradford, J.; Gee, K. R.; Agnew, B. J.; Clarke, S. T.; Salic, A. *Biotechniques* **2008**, *44*, 927.
(63) Salic, A.; Mitchison, T. J. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 2415.
(64) Wallace, P. K.; Muirhead, K. A. *Immunol. Investigations* **2007**, *36*, 527.
(65) Quah, B. J. C.; Warren, H. S.; Parish, C. R. *Nat. Protoc.* **2007**, *2*, 2049.
(66) Oostendorp, R. A. J.; Audet, J.; Eaves, C. J. *Blood* **2000**, *95*, 855.
(67) Lyons, A. B.; Doherty, K. V. *Current Protocols in Cytometry*; John Wiley & Sons: New York, 2004.
(68) Lyons, A. B. *J. Immunol. Methods* **2000**, *243*, 147.
(69) Parish, C. R.; Glidden, M. H.; Quah, B. J. C.; Warren, H. S. *Curr. Protoc. Immunol.* **2009**, *4*, 1.
(70) Park, M. S.; Koff, A. *Curr. Protoc. Cell Biol.* 2001; Chapter 8, Unit 8 1.
(71) Satyanarayana, A.; Kaldis, P. *Oncogene* **2009**, *28*, 2925.
(72) Juan, G.; Darzynkiewicz, Z. *Current Protocols in Cytometry*; John Wiley & Sons: New York, 2001.
(73) Gong, J.; Traganos, F.; Darzynkiewicz, Z. *Cancer Res.* **1993**, *53*, 5096.
(74) Larsen, J. K.; Landberg, G.; Roos, G. *Methods Cell Biol.* **2001**, *63*, 419.
(75) Gerdes, J.; Lemke, H.; Baisch, H.; Wacker, H. H.; Schwab, U.; Stein, H. *J. Immunol.* **1984**, *133*, 1710.
(76) Darzynkiewicz, Z.; Juan, G.; Bedner, E. *Current Protocols in Cell Biology*, John Wiley & Sons: New York, 2004.
(77) Toba, K.; Kishi, K.; Koike, T.; Winton, E. F.; Takahashi, H.; Nagai, K.; Maruyama, S.; Furukawa, T.; Hashimoto, S.; Masuko, M.; Uesugi, Y.; Kuroha, T.; Tsukada, N.; Shibata, A. *Exp. Hematol.* **1996**, *24*, 894.
(78) Kamentsky, L. A.; Kamentsky, L. D. *Cytometry* **1991**, *12*, 381.
(79) Darzynkiewicz, Z.; Bedner, E.; Li, X.; Gorczyca, W.; Melamed, M. R. *Exp. Cell Res.* **1999**, *249*, 1.
(80) Krysko, D. V.; Vanden Berghe, T.; D'Herde, K.; Vandenabeele, P. *Methods* **2008**, *44*, 205.
(81) Holme, A. L.; Yadav, S. K.; Pervaiz, S. *Cytometry Part A* **2007**, *71*, 80.
(82) Galbavy, S.; Kuliffay, P. *Bratislava Med. J.* **2008**, *109*, 3.
(83) Aillon, K. L.; Xie, Y.; El-Gendy, N.; Berkland, C. J.; Forrest, M. L. *Adv. Drug Delivery Rev.* **2009**, *61*, 457.
(84) Laurent, S.; Forge, D.; Port, M.; Roch, A.; Robic, C.; Vander Elst, L.; Muller, R. N. *Chem. Rev.* **2008**, *108*, 2064.
(85) Mahmoudi, M.; Hosseinkhani, H.; Hosseinkhani, Boutry, S.; M.; Simchi, A.; Journeay, W. S.; Subramani, K.; Laurent, S. *Chem. Rev.* **2011**, *111*, 253.
(86) Mahmoudi, M.; Milani, A. S.; Stroeve, P. *Int. J. Biomed. Nanosci. Nanotechnol.* **2010**, *1*, 164.
(87) Mahmoudi, M.; Simchi, A.; Imani, M. *J. Iranian Chem. Soc.* **2010**, *7*, S1.
(88) Mahmoudi, M.; Simchi, A.; Imani, M. *J. Phys. Chem. C* **2009**, *113*, 9573.
(89) Mahmoudi, M.; Simchi, A.; Imani, M.; Hafeli, U. O. *J. Phys. Chem. C* **2009**, *113*, 8124.
(90) Mahmoudi, M.; Simchi, A.; Imani, M.; Milani, A. S.; Stroeve, P. *J. Phys. Chem. B* **2008**, *112*, 14470.
(91) Mahmoudi, M.; Simchi, A.; Imani, M.; Milani, A. S.; Stroeve, P. *Nanotechnology* **2009**, *20*, 225104.
(92) Mahmoudi, M.; Simchi, A.; Imani, M.; Shokrgozar, M. A.; Milani, A. S.; Hafeli, U.; Stroeve, P. *Colloids Surf. B* **2010**, *75*, 300.
(93) Mahmoudi, M.; Simchi, A.; Imani, M.; Stroeve, P.; Sohrabi, A. *Thin Solid Films* **2010**, *518*, 4281.
(94) Fischer, H. C.; Chan, W. C. W. *Curr. Opin. Biotechnol.* **2007**, *18*, 565.
(95) Cedervall, T.; Lynch, I.; Foy, M.; Berggard, T.; Donnelly, S. C.; Cagney, G.; Linse, S.; Dawson, K. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 5754.
(96) Cedervall, T.; Lynch, I.; Lindman, S.; Berggard, T.; Thulin, E.; Nilsson, H.; Dawson, K. A.; Linse, S. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 2050.
(97) Riyahi-Alam, N.; Behrouzkia, Z.; Seifalian, A.; Jahromi, S. H. *Biol. Trace Elem. Res.* **2010**, *137*, 324.
(98) Lynch, I.; Cedervall, T.; Lundqvist, M.; Cabaleiro-Lago, C.; Linse, S.; Dawson, K. A. *Adv. Colloid Interface Sci.* **2007**, *134*−*135*, 167.
(99) Lynch, I.; Dawson, K. A.; Linse, S. *Science* **2006**, *327*, 14.
(100) Nel, A.; Xia, T.; Madler, L.; Li, N. *Science* **2006**, *311*, 622.
(101) Nel, A. E.; Madler, L.; Velegol, D.; Xia, T.; Hoek, E. M. V.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. *Nat. Mater.* **2009**, *8*, 543.
(102) Oberdörster, G.; Oberdörster, E.; Oberdörster, J. *Environ. Health Perspect.* **2005**, *113*, 823.
(103) Oberdörster, G.; Maynard, A.; Donaldson, K.; Castranova, V.; Fitzpatrick, J.; Ausman, K.; Carter, J.; Karn, B.; Kreyling, W.; Lai, D.; Olin, S.; Monteiro-Riviere, N.; Warheit, D.; Yang, H. *Part. Fibre Toxicol.* **2005**, *2*, 8.
(104) Hoet, P.; Bruske-Holfeld, I.; Salata, O. *J. Nanobiotechnology* **2004**, *2*, 12.
(105) Ryman-Rasmussen, J.; Riviere, J.; Monteiro-Riviere, N. *Toxicol. Sci.* **2006**, *91*, 159.
(106) Tinkle, S.; Antonini, J.; Roberts, J.; Salmen, R.; DePree, K.; Adkins, E. *Environ. Health Perspect.* **2003**, *111*, 1202.
(107) Li, N.; Sioutas, C.; Cho, A.; Schmitz, D.; Misra, C.; Sempf, J.; Wang, M.; Oberley, T.; Froines, J.; Nel, A. *Environ. Health Perspect.s* **2003**, *111*, 455.
(108) Savic, R.; Luo, L.; Eisenberg, A.; Maysinger, D. *Science* **2003**, *300*, 615.
(109) Geiser, M.; Rothen-Rutlshauser, B.; Knapp, N.; Schurch, S.; Kreyling, W.; Schulz, H.; Semmler, M.; Im, H.; Heyder, J.; Gehr, P. *Environ. Health Perspect.* **2005**, *113*, 1555.
(110) Porter, A.; Gass, M.; Muller, K.; Skepper, J.; Midgley, P.; Welland, M. *Environ. Health Perspect.* **2007**, *41*, 3012.
(111) Magrez, A.; Kasa, S.; Salicio, V.; Pasquier, N.; Won Seo, J.; Celio, M.; Catsicas, S.; Schwaller, B.; Forro, L. *Nano Lett.* **2006**, *6*, 1121.
(112) Weissleder, R.; Stark, D. D.; Engelstad, B. L.; Bacon, B. R.; Compton, C. C.; White, D. L.; Jacobs, P.; Lewis, J. *Am. J. Roentgenol.* **1989**, *152*, 167.
(113) Lacava, Z. G. M.; Azevedo, R. B.; Lacava, L. M.; Martins, E. V.; Garcia, V. A. P.; Rebula, C. A.; Lemos, A. P. C.; Sousa, M. H.; Tourinho, F. A.; Morais, P. C.; Da Silva, M. F. *J. Magn. Magn. Mater.* **1999**, *194*, 90.
(114) Lacava, Z. G. M.; Azevedo, R. B.; Martins, E. V.; Lacava, L. M.; Freitas, M. L. L.; Garcia, V. A. P.; Rebula, C. A.; Lemos, A. P. C.; Sousa, M. H.; Tourinho, F. A.; Da Silva, M. F.; Morais, P. C. *J. Magn. Magn. Mater.* **1999**, *201*, 431.
(115) Lewin, M.; Carlesso, N.; Ihng, C. H.; Tang, X. W.; Cory, D.; Scadden, D. T.; Weissleder, R. *Nat. Biotechnol.* **2000**, *18*, 410.
(116) Bulte, J. W.; Douglas, T.; Witwer, B.; Zhang, S. C.; Strable, E.; Lewis, B. K.; Zywicke, H.; Miller, B.; van Gelderen, P.; Moskowitz, B. M.; Duncan, I. D.; Frank, J. A. *Nat. Biotechnol.* **2001**, *19*, 1141.
(117) Moghimi, S. M.; Hunter, A. C.; Murray, J. C. *Pharmacol. Rev.* **2001**, *53*, 283.
(118) Schulze, E.; Ferrucci, J. T.; Poss, K.; Lapointe, L.; Bogdanova, A.; Weissleder, R. *Invest. Radiol.* **1995**, *30*, 604.
(119) Mueller, R. H.; Maassen, S.; Weyhers, H.; Specht, F.; Lucks, J. S. *Int. J. Pharm. (Amsterdam)* **1996**, *138*, 85.
(120) Hilger, I.; Fruhauf, S.; Linss, W.; Hiergeist, R.; Andra, W.; Hergt, R.; Kaiser, W. A. *J. Magn. Magn. Mater.* **2003**, *261*, 7.

(121) Berry, C. C.; Wells, S.; Charles, S.; Curtis, A. S. G. *Biomaterials* **2003**, *24*, 4551.
(122) Berry, C. C.; Wells, S.; Charles, S.; Aitchison, G.; Curtis, A. S. G. *Biomaterials* **2004**, *25*, 5405.
(123) Gupta, A. K.; Curtis, A. S. G. *Biomaterials* **2004**, *25*, 3029.
(124) Gupta, A. K.; Gupta, M. *Biomaterials* **2005**, *26*, 1565.
(125) Gupta, A. K.; Berry, C.; Gupta, M.; Curtis, A. *IEEE Transact. Nanobiosci.* **2003**, *2*, 255.
(126) van den Bos, E. J.; Wagner, A.; Mahrholdt, H.; Thompson, R. B.; Morimoto, Y.; Sutton, B. S.; Judd, R. M.; Taylor, D. A. *Cell Transplant.* **2003**, *12*, 743.
(127) Stroh, A.; Zimmer, C.; Gutzeit, C.; Jakstadt, M.; Marschinke, F.; Jung, T.; Pilgrimm, H.; Grune, T. *Free Radic. Biol. Med.* **2004**, *36*, 976.
(128) Brunner, T. J.; Wick, P.; Manser, P.; Spohn, P.; Grass, R. N.; Limbach, L. K.; Bruinink, A.; Stark, W. J. *Environ. Health Perspect.* **2006**, *40*, 4374.
(129) Soto, K. F.; Carrasco, A.; Powell, T. G.; Garza, K. M.; Murr, L. E. *J. Nanoparticle Res.* **2005**, *7*, 145.
(130) Soto, K.; Garza, K. M.; Murr, L. E. *Acta Biomater.* **2007**, *3*, 351.
(131) Jeng, H. A.; Swanson, J. *J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng.* **2006**, *41*, 2699.
(132) Pisanic, T. R.; Blackwell, J. D.; Shubayev, V. I.; Finones, R. R.; Jin, S. *Biomaterials* **2007**, *28*, 2572.
(133) Auffan, M.; Decome, L.; Rose, J.; Orsiere, T.; De Meo, M.; Briois, V.; Chaneac, C.; Olivi, L.; Berge-Lefranc, J. l.; Botta, A.; Wiesner, M. R.; Bottero, J. Y. *Environ. Sci. Technol.* **2006**, *40*, 4367.
(134) de Freitas, E. R. L.; Soares, P. R. O.; Santos, R. D.; dos Santos, D. L.; da Silva, J. R.; Porfirio, E. P.; Bao, S. N.; Lima, E. C. D.; Morais, P. C.; Guillo, L. A. *J. Nanosci. Nanotechnol.* **2008**, *8*, 2385.
(135) de la Fuente, J. M.; Alcantara, D.; Penades, S. *IEEE Transact. Nanobiosci.* **2007**, *6*, 275.
(136) Lewinski, N.; Colvin, V.; Drezek, R. *Small* **2008**, *4*, 26.
(137) Raynal, I.; Prigent, P.; Peyramaure, S.; Najid, A.; Rebuzzi, C.; Corot, C. *Invest. Radiol.* **2004**, *39*, 56.
(138) Doiron, A. L.; Chu, K.; Ali, A.; Brannon-Peppas, L. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 17232.
(139) Garcia, M. P.; Veloso, V. N.; Lacava, Z. G. M.; Morais, P. C.; Azevedo, R. B. *J. Magn. Magn. Mater.* **2005**, *293*, 277.
(140) Kim, J. S.; Yoon, T. J.; Yu, K. N.; Kim, B. G.; Park, S. J.; Kim, H. W.; Lee, K. H.; Park, S. B.; Lee, J. K.; Cho, M. H. *Toxicol. Sci.* **2006**, *89*, 338.
(141) Gamarra, L. F.; Pontuschka, W. M.; Amaro, E., Jr; Costa-Filho, A. J.; Brito, G. E. S.; Vieira, E. D.; Carneiro, S. M.; Escriba, D. M.; Falleiros, A. M. F.; Salvador, V. L. *Mater. Sci. Eng., C* **2008**, *28*, 519.
(142) Jain, T. K.; Reddy, M. K.; Morales, M. A.; Leslie-Pelecky, D. L.; Labhasetwar, V. *Mol. Pharmaceutics* **2008**, *5*, 316.
(143) Bourrinet, P.; Bengele, H. H.; Bonnemain, B.; Dencausse, A.; Idee, J. M.; Jacobs, P. M.; Lewis, J. M. *Invest. Radiol.* **2006**, *41*, 313.
(144) Corot, C.; Robert, P.; Idle, J. M.; Port, M. *Adv. Drug Delivery Rev.* **2006**, *58*, 1471.
(145) Sun, C.; Lee, J. S. H.; Zhang, M. *Adv. Drug Delivery Rev.* **2008**, *60*, 1252.
(146) Pollard, T. D., Earnshaw, W. C. *Cell Biology*; Saunders: New York, 2002.
(147) Knepper, M. A.; Pisitkun, T. *Kidney Int.* **2007**, *72*, 1043.
(148) Korn, E. D.; Weisman, R. A. *J. Cell Biol.* **1967**, *34*, 219.
(149) Swanson, J. A.; Watts, C. *Trends Cell Biol.* **1995**, *5*, 424.
(150) Mukherjee, S.; Ghosh, R. N.; Maxfield, F. R. *Physiol. Rev.* **1997**, *77*, 759.
(151) Banks, W. A.; Freed, E. O.; Wolf, K. M.; Robinson, S. M.; Franko, M.; Kumar, V. B. *J. Virol.* **2001**, *75*, 4681.
(152) Drin, G.; Rousselle, C.; Scherrmann, J. M.; Rees, A. R.; Temsamani, J. *AAPS Pharm. Sci.* **2002**, *4*, 61.
(153) Kaloyanides, G. J. *Nephrol. Dial. Transplant.* **1994**, *9*, 130.
(154) Wilhelm, C.; Billotey, C.; Roger, J.; Pons, J. N.; Bacri, J. C.; Gazeau, F. *Biomaterials* **2003**, *24*, 1001.
(155) Ghinae, N.; Simionescu, N. *J. Cell Biol.* **1985**, *100*, 606.
(156) Bulte, J. W. M. *J. Magn. Magn. Mater.* **2005**, *289*, 423.
(157) Bulte, J. W. M.; Douglas, T.; Witwer, B.; Zhang, S. C.; Strable, E.; Lewis, B. K.; Zywicke, H.; Miller, B.; Van Gelderen, P.; Moskowitz, B. M.; Duncan, I. D.; Frank, J. A. *Nat. Biotechnol.* **2001**, *19*, 1141.
(158) Prasad, P. D.; Mahesh, V. B.; Leibach, F. H.; Ganapathy, V. *Biochim. Biophys. Acta - Mol. Cell Res.* **1994**, *1222*, 309.
(159) Takizawa, F.; Tsuji, S.; Nagasawa, S. *FEBS Lett.* **1996**, *397*, 269.
(160) Palecanda, A.; Paulauskis, J.; Al-Mutairi, E.; Imrich, A.; Qin, G.; Suzuki, H.; Kodama, T.; Tryggvason, K.; Koziel, H.; Kobzik, L. *J. Exp. Med.* **1999**, *189*, 1497.
(161) Peiser, L.; Mukhopadhyay, S.; Gordon, S. *Curr. Opin. Immunol.* **2002**, *14*, 123.
(162) Ofek, I.; Goldhar, J.; Keisari, Y.; Sharon, N. *Annu. Rev. Microbiol.* **1995**, *49*, 239.
(163) Fedele, G.; Stefanelli, P.; Spensieri, F.; Fazio, C.; Mastrantonio, P.; Ausiello, C. M. *Infect. Immun.* **2005**, *73*, 1590.
(164) Mobberley-Schuman, P. S.; Weiss, A. A. *Infect. Immun.* **2005**, *73*, 7317.
(165) von zur Muhlen, C.; von Elverfeldt, D.; Bassler, N.; Neudorfer, I.; Steitz, B.; Petri-Fink, A.; Hofmann, H.; Bode, C.; Peter, K. *Atherosclerosis* **2007**, *193*, 102.
(166) Rogers, W. J.; Basu, P. *Atherosclerosis* **2005**, *178*, 67.
(167) Thorek, D. L. J.; Tsourkas, A. *Biomaterials* **2008**, *29*, 3583.
(168) Zhang, Y.; Kohler, N.; Zhang, M. *Biomaterials* **2002**, *23*, 1553.
(169) Huth, S.; Lausier, J.; Gersting, S. W.; Rudolph, C.; Plank, C.; Welsch, U.; Rosenecker, J. *J. Gene Med.* **2004**, *6*, 923.
(170) Pan, X.; Guan, J.; Yoo, J. W.; Epstein, A. J.; Lee, L. J.; Lee, R. J. *Int. J. Pharm.* **2008**, *358*, 263.
(171) Harush-Frenkel, O.; Debotton, N.; Benita, S.; Altschuler, Y. *Biochem. Biophys. Res. Commun.* **2007**, *353*, 26.
(172) Berry, C. C.; Charles, S.; Wells, S.; Dalby, M. J.; Curtis, A. S. G. *Int. J. Pharm.* **2004**, *269*, 211.
(173) Kohler, N.; Sun, C.; Wang, J.; Zhang, M. *Langmuir* **2005**, *21*, 8858.
(174) Lu, C. W.; Hung, Y.; Hsiao, J. K.; Yao, M.; Chung, T. H.; Lin, Y. S.; Wu, S. H.; Hsu, S. C.; Liu, H. M.; Mou, C. Y.; Yang, C. S.; Huang, D. M.; Chen, Y. C. *Nano Lett.* **2007**, *7*, 149.
(175) Zhao, M.; Kircher, M. F.; Josephson, L.; Weissleder, R. *Bioconjugate Chem.* **2002**, *13*, 840.
(176) Gump, J. M.; Dowdy, S. F. *Trends Mol. Med.* **2007**, *13*, 443.
(177) Wilhelm, C.; Gazeau, F. *Biomaterials* **2008**, *29*, 3161.
(178) Oberdarster, G.; Stone, V.; Donaldson, K. *Nanotoxicology* **2007**, *1*, 2.
(179) Panyala, N. R.; Pena-Mendez, E. M.; Havel, J. *J. Appl. Biomed.* **2009**, *7*, 75.
(180) Atanasijevic, T.; Shusteff, M.; Fam, P.; Jasanoff, A. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 14707.
(181) Unfried, K.; Albrecht, C.; Klotz, L. O.; Von Mikecz, A.; Grether-Beck, S.; Schins, R. P. F. *Nanotoxicology* **2007**, *1*, 52.
(182) Risom, L.; Muller, P.; Loft, S. *Mutat. Res. - Fundam. Mol. Mech. Mutagenesis* **2005**, *592*, 119.
(183) Soenen, S. J. H.; De Cuyper, M. *Contrast Media Mol. Imaging* **2009**, *4*, 207.
(184) Soenen, S. J. H.; Brisson, A. R.; De Cuyper, M. *Biomaterials* **2009**, *30*, 3691.
(185) Tang, M.; Wang, M.; Xing, T.; Zeng, J.; Wang, H.; Ruan, D. Y. *Biomaterials* **2008**, *29*, 4383.
(186) Gao, L.; Zhuang, J.; Nie, L.; Zhang, J.; Zhang, Y.; Gu, N.; Wang, T.; Feng, J.; Yang, D.; Perrett, S.; Yan, X. *Nat. Nanotechnol.* **2007**, *2*, 577.
(187) Huang, D. M.; Hsiao, J. K.; Chen, Y. C.; Chien, L. Y.; Yao, M.; Chen, Y. K.; Ko, B. S.; Hsu, S. C.; Tai, L. A.; Cheng, H. Y.; Wang, S. W.; Yang, C. S. *Biomaterials* **2009**, *30*, 3645.
(188) Walczak, P.; Kedziorek, D. A.; Gilad, A. A.; Barnett, B. P.; Bulte, J. W. M. *Magn. Res. Med.* **2007**, *58*, 261.
(189) Pernodet, N.; Fang, X.; Sun, Y.; Bakhtina, A.; Ramakrishnan, A.; Sokolov, J.; Ulman, A.; Rafailovich, M. *Small* **2006**, *2*, 766.
(190) Lipski, A. M.; Pino, C. J.; Haselton, F. R.; Chen, I. W.; Shastri, V. P. *Biomaterials* **2008**, *29*, 3836.

(191) Kostura, L.; Kraitchman, D. L.; Mackay, A. M.; Pittenger, M. F.; Bulte, J. W. M. *NMR in Biomedicine* **2004**, *17*, 513.
(192) Alekseenko, A. V.; Waseem, T. V.; Fedorovich, S. V. *Brain Res.* **2008**, *1241*, 193.
(193) Theil, E. C.; Matzapetakis, M.; Liu, X. *J. Biol. Inorg. Chem.* **2006**, *11*, 803.
(194) Chekhun, V. F.; Todor, I. N.; Lukyanova, N. Y.; Shpyleva, S. I.; Naleskina, L. A.; Khaetsky, I. K.; Kulik, G. I. *Exp. Oncol.* **2009**, *31*, 163.
(195) Potter, A. J.; Gollahon, K. A.; Palanca, B. J. A. *Carcinogenesis* **2002**, *23*, 389.
(196) Cadenas, E. *Mol. Aspects Med.* **2004**, *25*, 17–26.
(197) Hussain, S. M.; Hess, K. L.; Gearhart, J. M.; Geiss, K. T.; Schlager, J. J. *Toxicol. in Vitro* **2005**, *19*, 975.
(198) Au, C.; Mutkus, L.; Dobson, A.; Riffle, J.; Lalli, J.; Aschner, M. *Biol. Trace Elem. Res.* **2007**, *120*, 248.
(199) Amara, N.; Bachoual, R.; Desmard, M.; Golda, S.; Guichard, C.; Lanone, S.; Aubier, M.; Ogier-Denis, E.; Boczkowski, J. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **2007**, *293*, L170.
(200) Storm, G.; Belliot, S. O.; Daemen, T.; Lasic, D. D. *Adv. Drug Delivery Rev.* **1995**, *17*, 31.
(201) Moore, A.; Weissleder, R.; Bogdanov, A., Jr. *J. Magn. Res. Imaging* **1997**, *7*, 1140.
(202) Kool, M. E.; Cappendijk, V. C.; Cleutjens, K. B. J. M.; Kessels, A. G. H.; Kitslaar, P. J. E. H. M.; Borgers, M.; Frederik, P. M.; Daemen, M. J. A. P.; Van Engelshoven, J. M. A. *Circulation* **2003**, *107*, 2453.
(203) Ruehm, S. G.; Corot, C.; Vogt, P.; Kolb, S.; Debatin, J. F. *Circulation* **2001**, *103*, 415.
(204) Muller, K.; Skepper, J. N.; Posfai, M.; Trivedi, R.; Howarth, S.; Corot, C.; Lancelot, E.; Thompson, P. W.; Brown, A. P.; Gillard, J. H. *Biomaterials* **2007**, *28*, 1629.
(205) Muller, K.; Skepper, J. N.; Tang, T. Y.; Graves, M. J.; Patterson, A. J.; Corot, C.; Lancelot, E.; Thompson, P. W.; Brown, A. P.; Gillard, J. H. *Biomaterials* **2008**, *29*, 2656.
(206) Li, N.; Xia, T.; Nel, A. E. *Free Radic. Biol. Med.* **2008**, *44*, 1689.
(207) Bonomini, F.; Tengattini, S.; Fabiano, A.; Bianchi, R.; Rezzani, R. *Histol. Histopathol.* **2008**, *23*, 381.
(208) Dhalla, N. S.; Temsah, R. M.; Netticadan, T. *Hypertension* **2000**, *18*, 655.
(209) Stefanovia, A.; Kotur-Stevuljevia, J.; Spasia, S.; Vekia, J.; Zeljkovia, A.; Spasojevia-Kalimanovska, V.; Jelia-Ivanovia, Z. *Clin. Biochem.* **2010**, *43*, 1230.
(210) Turner, T. T.; Lysiak, J. L. *J. Andrology* **2008**, *29*, 488.
(211) Gago-Dominguez, M.; Castelao, J. E. *Crit. Rev. Oncol./Hematol.* **2008**, *68*, 107.
(212) Cachofeiro, V.; Goicochea, M.; De Vinuesa, S. G.; Ouba, P.; Lahera, V.; Lo, J. *Kidney Int.* **2008**, *74*, S4.
(213) Chrissobolis, S.; Faraci, F. M. *Trends Mol. Med.* **2008**, *14*, 495.
(214) Barnham, K. J.; Bush, A. I. *Curr. Opin. Chem. Biol.* **2008**, *12*, 222.
(215) Kirschvink, J. L.; Kobayashi-Kirschvink, A.; Diaz-Ricci, J. C.; Kirschvink, S. J. *Bioelectromagnetics* **1992**, No. Suppl 1, 101.
(216) Begley, D. J. *Pharmacol. Ther.* **2004**, *104*, 29.
(217) Fisher, J.; Devraj, K.; Ingram, J.; Slagle-Webb, B.; Madhankumar, A. B.; Liu, X.; Klinger, M.; Simpson, I. A.; Connor, J. R. *Am. J. Physiol. - Cell Physiol.* **2007**, *293*, C641.
(218) Kwon, J. T.; Hwang, S. K.; Jin, H.; Kim, D. S.; Minai-Tehrani, A.; Yoon, H. J.; Choi, M.; Yoon, T. J.; Han, D. Y.; Kang, Y. W.; Yoon, B. I.; Lee, J. K.; Cho, M. H. *J. Occup. Health* **2008**, *50*, 1.
(219) Block, M. L.; Wu, X.; Pei, Z.; Li, G.; Wang, T.; Qin, L.; Wilson, B.; Yang, J.; Hong, J. S.; Veronesi, B. *FASEB J.* **2004**, *18*, 1618.
(220) Ali, S.; Hussain, S. *Toxicol. Lett.* **2008**, *18*, S21.
(221) Block, M. L.; Zecca, L.; Hong, J. S. *Nat. Rev. Neurosci.* **2007**, *8*, 57.
(222) Long, T. C.; Saleh, N.; Tilton, R. D.; Lowry, G. V.; Veronesi, B. *Environ. Sci. Technol.* **2006**, *40*, 4346.
(223) Daniel, M. C.; Astruc, D. *Chem. Rev.* **2004**, *104*, 293.
(224) Li, J. J.; Zou, L.; Hartono, D.; Ong, C.-N.; Bay, B.-H.; Lanry, Y.-Y. *Adv. Mater.* **2008**, *20*, 138.
(225) Chithrani, B. D.; Ghazani, A. A.; Chan, W. C. W. *Nano Lett.* **2006**, *6*, 662.
(226) Chithrani, B. D.; Chan, W. C. W. *Nano Lett.* **2007**, *7*, 1542.
(227) Fong, J.; Wood, F. *Int. J. Nanomed.* **2006**, *1*, 441.
(228) Morones, J. R.; Elechiguerra, J. L.; Camacho, A.; Holt, K.; Kouri, J. B.; Ramirez, J. T.; Yacaman, M. J. *Nanotechnology* **2005**, *16*, 2346.
(229) Ahamed, M.; Karns, M.; Goodson, M.; Rowe, J.; Hussain, S. M.; Schlager, J. J.; Hong, Y. *Toxicol. Appl. Pharmacol.* **2008**, *233*, 404.
(230) Ghio, A. J.; Stonehuerner, J.; Dailey, L. A.; Carter, J. D. *Inhalation Toxicol.* **1999**, *11*, 37.
(231) Carlson, C.; Hussain, S. M.; Schrand, A. M.; Braydich-Stolle, L. K.; Hess, K. L.; Jones, R. L.; Schlager, J. J. *J. Phys. Chem. B* **2008**, *112*, 13608.
(232) AshaRani, P. V.; Mun, G. L. K.; Hande, M. P.; Valiyaveettil, S. *ACS Nano* **2009**, *3*, 279.
(233) Ishikawa, K.; Ishii, H.; Saito, T. *DNA Cell Biol.* **2006**, *25*, 406.
(234) Ivask, A.; Bondarenko, O.; Jepihhina, N.; Kahru, A. *Anal. Bioanal. Chem.* **2010**, *398*, 701.
(235) Ding, M.; Kisin, E. R.; Zhao, J.; Bowman, L.; Lu, Y.; Jiang, B.; Leonard, S.; Vallyathan, V.; Castranova, V.; Murray, A. R.; Fadeel, B.; Shvedova, A. A. *Toxicol. Appl. Pharmacol.* **2009**, *241*, 260.
(236) Wang, J. J.; Sanderson, B. J.; Wang, H. *Mutat. Res.* **2007**, *628*, 99.
(237) Kang, S. J.; Kim, B. M.; Lee, Y. J.; Chung, H. W. *Environ. Molec. Mutagenesis* **2008**, *49*, 399.
(238) Rahman, Q.; Lohani, M.; Dopp, E.; Pemsel, H.; Jonas, L.; Weiss, D. G.; Schiffmann, D. *Environ. Health Perspect.* **2002**, *110*, 797.
(239) Karlsson, H. L.; Cronholm, P.; Gustafsson, J.; Moller, L. *Chem. Res. Toxicol.* **2008**, *21*, 1726.
(240) Xu, A.; Chai, Y.; Hei, T. K. *Part. Fibre Toxicol.* **2009**, *6*, 3.
(241) Theogaraj, E.; Riley, S.; Hughes, L.; Maier, M.; Kirkland, D. *Mutat. Res.* **2007**, *634*, 205.
(242) Nohynek, G. J.; Lademann, J.; Ribaud, C.; Roberts, M. S. *Crit. Rev. Toxicol.* **2007**, *37*, 251.
(243) Gurr, J. R.; Wang, A. S.; Chen, C. H.; Jan, K. Y. *Toxicology* **2005**, *213*, 66.
(244) Ding, L.; Stilwell, J.; Zhang, T.; Elboudware, O.; Jiang, H.; Selegue, J. P.; Cooke, P. A.; Gray, J. W.; Chen, F. F. *Nano Lett.* **2005**, *5*, 2448.
(245) Qi, Q.; Zhang, T.; Yu, Q.; Wang, R.; Zeng, Y.; Liu, L.; Yang, H. *Sens. Actuators, B* **2008**, *133*, 633.
(246) Jeng, H. A.; Swanson, J. *J. Environ. Sci. Health A Toxicol. Hazard Subst. Environ. Eng.* **2006**, *41*, 2699.
(247) Sayes, C. M.; Reed, K. L.; Warheit, D. B. *Toxicol. Sci.* **2007**, *97*, 163.
(248) Dufour, E. K.; Kumaravel, T.; Nohynek, G. J.; Kirkland, D.; Toutain, H. *Mutat. Res.* **2006**, *607*, 215.
(249) Huang, X.; Zhuang, J.; Teng, X.; Li, L.; Chen, D.; Yan, X.; Tang, F. *Biomaterials* **2010**, *31*, 6142.
(250) Wang, F.; Gao, F.; Lan, M.; Yuan, H.; Huang, Y.; Liu, J. *Toxicol. in Vitro* **2009**, *23*, 808.
(251) He, X.; Liu, F.; Wang, K.; Ge, J.; Qin, D.; Gong, P.; Tan, W. *Chin. Sci. Bull.* **2006**, *51*, 1939.
(252) Chen, M.; von Mikecz, A. *Exp. Cell Res.* **2005**, *305*, 51.
(253) Gref, R.; Minamitake, Y.; Peracchia, M. T.; Trubetskoy, V.; Torchilin, V.; Langer, R. *Science* **1994**, *263*, 1600.
(254) Alexis, F.; Pridgen, E. M.; Langer, R.; Farokhzad, O. C. *Exp. Pharmacol.* **2010**, *197*, 55.
(255) Ao, M.; Wang, Z.; Ran, H.; Guo, D.; Yu, J.; Li, A.; Chen, W.; Wu, W.; Zheng, Y. *J. Biomed. Mater. Res. B Appl. Biomater.* **2010**, *93*, 551.
(256) McNerny, D. Q.; Leroueil, P. R.; Baker, J. R. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2010**, *2*, 249.
(257) Jain, K.; Kesharwani, P.; Gupta, U.; Jain, N. K. *Int. J. Pharm.* **2010**, *394*, 122.
(258) Tuncel, D.; Demir, H. V. *Nanoscale* **2010**, *2*, 484.
(259) Wang, Y.; Xin, D.; Liu, K.; Zhu, M.; Xiang, J. *Bioconjugate Chem.* **2009**, *20*, 2214.
(260) Mroz, R. M.; Schins, R. P. F.; Li, H.; Jimenez, L. A.; Drost, E. M.; Holownia, A.; MacNee, W.; Donaldson, K. *Eur. Respir. J.* **2008**, *31*, 241.

(261) Kang, B.; Mackey, M. A.; El-Sayed, M. A. *J. Am. Chem. Soc.* **2010**, *132*, 1517.
(262) Roa, W.; Zhang, X.; Guo, L.; Shaw, A.; Hu, X.; Xiong, Y.; Gulavita, S.; Patel, S.; Sun, X.; Chen, J.; Moore, R.; Xing, J. Z. *Nanotechnology* **2009**, *20*, 375101.
(263) Misra, R.; Sahoo, S. K. *Eur. J. Pharm. Sci.* **2010**, *39*, 152.
(264) Li, Y.; Li, Z.; Zhou, X.; Yang, P. *Nano Biomed. Eng.* **2010**, *2*, 32.
(265) Qi, L. F.; Xu, Z. R.; Li, Y.; Jiang, X.; Han, X. Y. *World J. Gastroenterol.* **2005**, *11*, 5136.
(266) He, L.; Yang, L.; Zhang, Z. R.; Gong, T.; Deng, L.; Gu, Z.; Sun, X. *Nanotechnology* **2009**, *20*, 455102.
(267) Halimani, M.; Chandran, S. P.; Kashyap, S.; Jadhav, V. M.; Prasad, B. L. V.; Hotha, S.; Maiti, S. *Langmuir* **2009**, *25*, 2339.
(268) Seo, J. H.; Moon, H. S.; Kim, I. Y.; Guo, D. D.; Lee, H. G.; Choi, Y. J.; Cho, C. S. *Eur. J. Pharm. Biopharm.* **2008**, *70*, 621.
(269) Kang, S. J.; Kim, B. M.; Lee, Y. J.; Hong, S. H.; Chung, H. W. *Biochem. Biophys. Res. Commun.* **2009**, *386*, 682.
(270) Crombez, L.; Morris, M. C.; Dufort, S.; Aldrian-Herrada, G.; Nguyen, Q.; Mc Master, G.; Coll, J. L.; Heitz, F.; Divita, G. *Nucleic Acids Res.* **2009**, *37*, 4559.
(271) Park, K. S.; Tae, J.; Choi, B.; Kim, Y. S.; Moon, C.; Kim, S. H.; Lee, H. S.; Kim, J.; Park, J.; Lee, J. H.; Lee, J. E.; Joh, J. W.; Kim, S. *Nanomedicine: Nanotechnol. Biol. Med.* **2010**, *6*, 263.