CHAPTER 4

Nanocytotoxicity

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4.1 INTRODUCTION

Widespread applications of nanoparticles in today's medicine, industry, and ordinary life have increased the importance of studying nanotoxicity. However, despite the continuous rise in types and applications of nanoparticles, a relatively small number of studies have been conducted to investigate their effects upon exposure and to assess their potential toxicity. Diagnostic and therapeutic applications of nanoparticles have shown great potential to improve the understanding, detection, and treatment of human diseases. Such applications involve intentional contact or administration. This demonstrates the importance of studying the properties and the effects of nanoparticles on the body prior to clinical applications.

Unfortunate events such as the "nanoscare" incident in 2006 in Germany, related to the aerosol glass protective Magic Nano [1], or the sunscreen controversy triggered by the United States Environmental Protection Agency findings regarding the possibility of brain damage in mice resulting from titanium dioxide nanoparticles found in sunscreens [2] clearly demonstrated the urgent need for thorough investigations about the safety of nanoparticles.

When used in drug delivery, biosensors, or medical imaging, nanoparticle application involves deliberate and direct ingestion or injection into the body. The toxicity of nanoparticles is a critical factor to consider for biomedical purposes, especially for in vivo applications. When used in imaging and drug delivery, bioconjugate coatings of nanoparticles such as DNA, proteins, and monoclonal antibodies can facilitate targeting of specific cell types. Therefore, it is particularly important to certify that these enhancements do not cause any adverse effects. More significantly, the occurrence and the effect of biodegradation of naked or coated nanoparticles in the cellular environment should be confirmed. Biodegraded nanoparticles can cause

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intracellular changes such as the disruption of organelle integrity or genetic changes when they accumulate within cells. Because in vitro applications do not involve administration of the nanoparticles to the body, they offer more freedom for toxicological characterizations compared to in vivo applications. Researchers are increasingly investigating in vitro cytotoxicity of nanoparticles using different cell lines and a number of colorimetric assays.

4.2 CYTOTOXICITY

Cell-culture studies are usually the first step to understand the behavior of exogenous agents in the body. Cellular testing is less ethically ambiguous and easier to control and reproduce. Regarding cytotoxicity, it should be emphasized that cell cultures are sensitive to environmental changes such as pH, fluctuations in temperature, nutrients, and waste concentration and concentration of the potentially toxic agents under investigation. Therefore, maintaining stringent control over the experimental conditions is crucial to certify that the measured cell death is occurring as a result of the toxicity of the added nanoparticles rather than unstable culturing conditions. Moreover, usage of appropriate cytotoxicity assays is also crucially important as nanoparticles can adsorb dyes and be redox active. Finally, conducting multiple different tests allows for greater confidence in the validity of the conclusions.

Using bright-field microscopy to visually inspect the changes in cellular or nuclear morphology represents a simple test of cytotoxicity. Fiorito et al. used this method to assess the cytotoxicity of single-walled carbon nanotubes (SWNTs) [3]. However, measuring cell death via colorimetric methods is the most popular approach among cytotoxicity assays. Two major categories of colorimetric methods are tests that measure the integrity of the plasma membrane or mitochondrial activity.

The cell membrane may be damaged via exposure to certain cytotoxic agents, which often leads to the leakage of cellular contents. Viability tests that make use of this phenomenon are neutral red and trypan blue assays. Neutral red, or toluylene red, is a weak cationic dye capable of diffusively crossing the plasma membrane and accumulates in lysosomes within the cell. Alteration of the cell membrane decreases the uptake of neutral red and results in its leakage, allowing for the differentiation of live and dead cells. Studying the uptake of neutral red under different exposure conditions via spectrophotonic measurements can provide quantitative information about cytotoxicity [4]. Flahaut et al. and Monterio-Riviere et al. utilized the

neutral red assay to examine the cytotoxicity of carbon nanotubes [5,6]. Trypan blue, a diazo dye, can only penetrate cells with compromised membranes; therefore, it does not stain the live cells, and they remain colorless, while dead cells are stained blue. Light microscopy can be used to determine the number of dead cells [7]. Bottini et al. and Goodman et al. utilized the trypan blue assay to investigate the cytotoxicity of SWNTs and gold nanoparticles [8,9].

Another method to measure the number of dead cells is the LIVE/DEAD viability test. This assay utilizes two chemicals, calcein acetoxymethyl (calcein AM) and ethidium homodimer, and has been used to evaluate fullerenes and gold nanoshells [10,11]. Calcein AM, an electrically neutral, esterified molecule, can easily enter cells by diffusion. After entering the cells, calcein AM converts to calcein, a green fluorescent molecule, via intracellular esterases. On the contrary, ethidium homodimer can only penetrate compromised membranes. Once bound to nucleic acid, ethidium homodimer becomes red fluorescent. Using a 495 nm excitation, calcein AM and ethidium homodimer produce separable fluorescent peaks at 515 and 635 nm, respectively [12].

Monitoring the release of lactate dehydrogenase (LDH) is another assay that has been generally used in cytotoxicity assessment of carbon nanotubes [10,13,14]. In this assay, LDH that is released from damaged cells oxidizes lactate to pyruvate. Pyruvate, in turn, enhances the conversion of tetrazolium salt INT to formazan. Formazan is a water-soluble molecule that absorbs light at 490 nm. The number of damaged or lysed cells can then be assessed by measuring the amount of LDH released [15].

Other colorimetric cytotoxicity assays also utilize the mechanism of induced cell death by observing the amount of cells with damaged plasma membranes. Tetrazolium salts can be used to evaluate mitochondrial activity, since mitochondrial dehydrogenase enzymes cleave the tetrazolium ring. This reaction can only happen in living cells, because mitochondrial dehydrogenase enzymes are only present in active mitochondria [16]. One of the most common tests is the MTT viability assay [5,6,10,17–22]. Although MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, exhibits a pale-yellow color in solution, it produces a dark-blue formazan product within living cells. Many researchers have also used cell titer 96 aqueous one solution cell proliferation assay distributed by Promega, which is a variation of the MTT [23–25].

Malich et al. used phenazine ethosulfate, MTS, and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-

2H-tetrazolium for in vitro cytotoxicity evaluation. Here, similar to the MTT assay, the absorbance at 492 nm is measured to quantify the formation of formazan and determine the number of living cells [26]. The WST assay is another cytotoxicity evaluation assay based on tetrazolium [27]. The formazan product formed from WST-1 or WST-8 has a yellow-orange color and quantifying its absorbance at 450 nm can be used for determining its concentration [28]. Cytotoxicity evaluation methods using resazurin (aka alamar blue) have also been reported [29,30]. Alamar blue is nonfluorescent, but cell metabolic activity reduces it to a pink fluorescent dye. This occurs because during oxygen consumption alamar blue acts as an electron acceptor for enzymes such as NADP and FADH [31].

Other than causing deficiency in membrane or metabolic functions, nanoparticles might have other adverse effects; therefore, more extensive cytotoxicity studies have focused on the sublethal effects of nanoparticles. A glutathione assay can be used to detect oxidative stress. Glutathione (GSH) is a common antioxidant compound that can form glutathione disulfide (GSSG) when it is oxidized in the presence of reactive oxygen species (ROS). To be able to protect against oxidative stress, GSH/GSSG ratio should be high, which is sustained by the enzyme glutathione reductase. The levels of glutathione are detected using Ellman's reagent, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), that forms a yellow-colored product, 5-thio-2-nitrobenzoic acid (TNB), upon reaction with the sulfhydryl group of GSH. GSH is recycled from the GSH-TNB complex by glutathione reductase, leading to further TNB production. The level of GSH can be investigated by determining the absorbance of TNB at 405 or 412 nm given that TNB production rate is directly proportional to the GSH concentration in the sample [32].

Various methods including GSH and thiobarbituric acid (TBA) assay can detect lipid peroxidation of the plasma membrane. In the TBA assay, when malondialdehyde (MDA), which is a toxic by-product of lipid peroxidation, is heated at an environment with an acidic pH, it forms a fluorescent pink chromogen via reaction with 2-thiobarbituric acid. Upon excitation at 532 nm, pink fluorescence is produced, and the intensity can be measured colorimetrically. Halliwell et al. have provided an extensive list of other methods of lipid peroxidation [33].

Nanoparticles can also cause inflammation. Commonplace pro-inflammatory cytokines of inflammatory response include IL-1b, IL-6, and TNF-a and chemokine IL-8 [34,35]. Using enzyme-linked immunosorbent assay (ELISA), these cytokines can be quantified when the absorbance of

either alkaline phosphatase or streptavidin-horseradish-peroxidase-labeled antibodies is measured at 405 or 620 nm, respectively [36]. Genotoxic potential of nanoparticles has been investigated in extensive cytotoxicity studies by studying the level of DNA damage. Flow cytometry is a common method for investigating the effect of carbon nanoparticles [37–40]. Here, cells are differentiated based on their size and density using a laser beam. Using DNA-intercalating dyes, the proportion of cells undergoing apoptosis is evaluated by studying the cellular DNA content. For example, propidium iodide, a membrane-impermeable red dye, is commonly used in this way. As the number of damaged cells increases, so does the intensity of the fluorescent dye, being that an increase in membrane permeability increases the binding of the dye to nucleic acids [41]. DNA damage in individual cells has also been studied by the comet assay using gel electrophoresis. Cells with damaged DNA form "comets"; healthy DNA maintains the head portion, while damaged DNA pieces migrate away and form the tail. The gel is read using a DNA-specific dye such as propidium iodide, and DNA damage is evaluated based on the amount of gel found in the tail [42]. Furthermore, preliminary DNA microarray studies have focused on determining which specific genes are up- or downregulated due to nanoparticle exposure [43–46]. Interactions with plasma membrane, organelles, or macromolecules are the major effects of nanoparticles. Each nanoparticle might cause specific biological responses; therefore, the cytotoxicity of each particular nanoparticle should be studied separately.

4.3 SUPERPARAMAGNETIC IRON OXIDE NANOPARTICLES

Although bare iron oxide nanoparticles demonstrate some toxic effects, coated superparamagnetic iron oxide nanoparticles (SPIONs) are relatively nontoxic. Gupta et al. demonstrated the biocompatibility of PEG-coated nanoparticles showing that cells exposed to such nanoparticles remained more than 99% viable relative to control at an upper concentration of 1 mg mL^{-1} [47]. In contrast, a 25%–50% loss in fibroblast viability at 250 mg mL^{-1} was observed upon exposure to bare iron oxide nanoparticles. Gupta et al. have also shown the dose dependence of SPION cytotoxicity, with 20% cell viability reduction at 0.05 mg mL^{-1} and 60% cell viability reduction at 2.0 mg mL^{-1} concentration [48]. In contrast, using a different PEGylation approach, Yu et al. showed relative nontoxicity for PMAO-PEG-coated SPIONs, illustrated in Fig. 4.1. Only 9% cell viability decrease was observed when tested at 400 nm exposure [49].

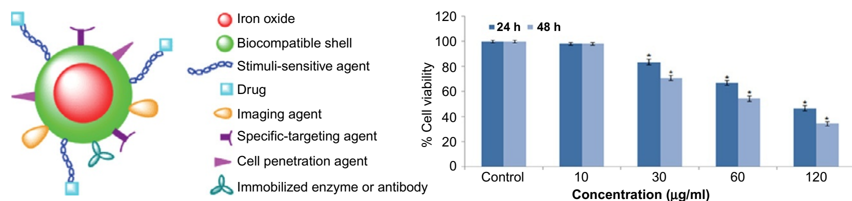

Fig. 4.1 Example iron oxide nanoparticle structure and cell viability data of human breast cancer cells treated with iron oxide nanoparticles. *(From L. Zhou, J. Yuan, Y. Wei, Core–shell structural iron oxide hybrid nanoparticles: from controlled synthesis to biomedical applications, J. Mater. Chem. 21 (2011) 2823, doi:10.1039/C0JM02172E; S. Alarifi, D. Ali, S. Alkahtani, et al., Iron oxide nanoparticles induce oxidative stress, DNA damage, and caspase activation in the human breast cancer cell line, Biol. Trace Elem. Res. 159 (2014) 416. https://doi.org/10.1007/s12011-014-9972-0transfer.)*

On the other hand, some groups have considered bare iron oxide nanoparticles biocompatible. Although Hussain et al. observed 30% decrease in cell viability at 250 mg mL^{-1}, they reported it as little to no toxicity [50]. Cheng et al. evaluated a higher concentration of 23.05 mM of nanoparticles and did not observe any considerable differences between the exposed cells and the control. However, the relatively short exposure time of 4 h might have played a key role in this observation [51].

Cytotoxicity of iron oxide particles with other surface coatings other than PEG has also been studied, and little cytotoxicity has been reported. Pullulan (Pn)-coated SPIONs were studied by Gupta et al., and no cytotoxic effects were observed, and at 2.0 mg mL^{-1}, cell viability was only reduced by 8% [48]. It was hypothesized that the pullulan coating prevented the iron oxide core from interacting with cells and led to the low toxicity of Pn-SPIONs. Petri-Fink et al. showed that amino-SPIONs with all polymer/iron ratios exhibit no cytotoxicity in melanoma after 2 h of exposure [52]. However, high polymer concentrations showed cytotoxicity after 24 h exposure. Cengelli et al. studied a SPION with similar coating and found that N11 microglial cells only took up aminoPVA-coated SPIONs, and no nitric oxide was produced, hence suggesting no toxicity [53]. Wan et al. evaluated the cytotoxicity of SPIONs with three different coatings and observed minimal cytotoxicity for MPEG-Asp3-NH_2-coated iron oxide nanoparticles at the studied concentrations [54]. In contrast, MPEG-PAA- and PAA-coated iron oxide nanoparticles caused an 84% reduction in cell viability at 400 mg mL^{-1} concentration of iron. Because bare iron oxide nanoparticles are adsorbed to the surface of the cells, it was not possible to perform MTS analysis. Cell counts after incubation,

on the other hand, revealed that uncoated iron oxide nanoparticles reduced cell viability to a large extent.

Cellular uptake and ROS production are the suggested mechanisms for SPION cytotoxicity (if present). Hu et al. observed greater than 93% cell viability for cells exposed to PACHTUNGTRENUNG(PEGMA)-immobilized nanoparticles [55]. Cells exposed to pristine iron oxide nanoparticles showed 70% viability after 2 days, while the viability increased to 90% after 5 days. It was hypothesized that the increase in viability after 5 days stems from the reduction in nanoparticle concentration, as a result of dilution by increasing cell numbers after mitosis. It was noticed that the cell uptake of particles was 154 pg $cell^{-1}$ on the first day and 58 pg $cell^{-1}$ on the fifth day. On the other hand, cell uptake of PACHTUNGTRENUNG(PEGMA) nanoparticles was 2 pg $cell^{-1}$, suggesting a link between their lower toxicity and the lack of cellular uptake. A cell response to bare iron oxide nanoparticle exposure was revealed by Brunner et al. [56]. In the presence of 30 ppm iron oxide, 3T3 cells maintained proliferation, while only 3.75 ppm iron oxide reduced the viability of human mesothelioma cells. Production of iron-induced free radicals by the Fenton or Haber-Weiss reactions and internalization of the iron oxide particles were suggested to be the cause for the observed toxicity. Pisanic et al. demonstrated that rat pheochromocytoma cells can easily endocytose anionic dimercaptosuccinic acid (DMSA)-coated iron oxide nanoparticles. Such nanoparticles are found in the cytoplasm, inside endosomes, or can accumulate in the perinuclear region within the cells [57]. The first 48 h following the exposure is when the majority of cell death occurs. Moreover, cytotoxicity and cell detachment of such nanoparticles have shown dose dependency.

A relatively consistent 20%–30% reduction in neutral red uptake in monocyte-macrophages with 10 mg mL^{-1} ferumoxtran-10 was observed by Muller et al. when evaluated with a range of incubation times [58], and the MTT assay yielded similar results. Ferumoxtran-10-pretreated cells and control cells maintained similar viability after 2 weeks of exposure. It is suggested that the observed cytotoxicity stems from ROS production via the Fenton reaction. This can lead to lipid peroxidation, DNA damage, and protein oxidation. No increase in cytokines IL-1b, TNF-a, IL-6 or IL-12, or superoxide anion production was observed when the inflammatory responses were evaluated for exposure to ferumoxtran-10. Therefore, it was concluded that the nanoparticles did not activate the monocyte-macrophages.

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