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Chapter 2

Evaluation Strategies of Nanomaterials Toxicity

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Additional information is available at the end of the chapter

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Abstract

The revolutionary development of nanoscience during the last years has increased the number of studies in the field to evaluate the toxicity and risk levels. The design of different nanomaterials together with biological components has implemented the advances in biomedicine. Specifically, nanoparticles seem to be a promising platform due to their features, including nanoscale dimensions and physical and chemical characteristics than can be modified in function of the final application. Herein, we review the main studies realized with nanoparticles in order to understand and characterize the cellular uptake mechanisms involved in biocompatibility, toxicity, and how they alter the biological processes to avoid disease progression.

Keywords: Nanoparticle, toxicity, nanomaterial, cellular uptake, immunogenicity

1. Introduction

The nanoscience revolution that sprouted throughout the 1990s is becoming part of our daily life in the form of cosmetics, food packaging, drug delivery systems, therapeutics, or biosensors, among others [1]. It has been estimated that the production of nanomaterials would increase in 2020 by 25 times what it is today.

This is due to the wide range of applications that they have in numerous fields, ranging from commercial products such as electronic components, cosmetics, household appliances, semiconductor devices, energy sources, food color additives, surface coatings, and medical

products such as biological sensors, drug carriers, biological probes, implants, and medical imaging. Despite this future dependence on nanomaterials, studies regarding their safe incorporation in our lives are very limited [2].

Recently, several studies suggested that nanoparticles (NPs) could easily enter into the human body [3]. This is mainly because their nanoscale dimensions are of a similar size to typical cellular components. Moreover, proteins–NPs may bypass natural mechanical barriers, possibly leading to adverse tissue reactions. As a result, the particles might be taken up into cells. Generally, NPs of different physical and chemical properties may enter the cells by different mechanisms, such as phagocytosis, macropinocytosis, endocytosis, or directly by "adhesive interactions" [4].

In order to understand the exact cellular influences of NPs, a thorough characterization of individual nanoparticles is necessary. Nanoparticles can get into the human body through various ways, such as skin penetration, inhalation, or injection, and due to their small size and diffusion abilities; they have the potential to interact with cells and organs. In addition to involuntary exposure to NPs by means of contacting nanomaterials-based products, there are cases where nanoparticles would interact with the human body for biomedical purposes.

In case of using nanoparticles for targeted-drug delivery, NPs are required to traverse the cell membranes and interact with specific components. Hence, the success rate of drug delivery is based on the biocompatibility of NPs. Research has shown that different physicochemical properties of NPs result in different cellular uptake. Currently, it has been described that several factors play a critical role in toxicity (Fig. 1); such as (i) size and surface, very important for liposomes, silicon microparticles, quantum dots, polymeric NPs, or gold NPs; (ii) concentration, crystallinity, and mechanical strength, toxicity is directly related to these parameters [2]; (iii) chemical attributes, the development of hydrophilic polymer functionalization (i.e. polyethylene glycol, polycarboxybetaine) at the surface of NPs enhances the systemic circulation; however, the response of the immune system is also related with this hydrophilic coating.

The discovery of Enhanced Permeation and Retention (EPR) effect and its combination with hydrophilic polymers is related to the accumulation of NP-based carrier systems in tumor tissues followed by the release of the drug either in the proximity to the tissue. However, EPR effect is commonly inconsistent due to the heterogeneity associated with the tumor tissue. For this reason, novel nanomedicines are being designed and developed in order to target only a particular cell, tissue, and organ by linking an affinity reagent to the NP, which is targeting a specific biomolecule differentially expressed at the tissue or cells of interest.

Although some concerns have been raised about poor systemic circulation, enhanced clearance by the mononuclear phagocyte system and limited tissue penetration has been shown to improve the cellular uptake and efficacy of their payload in comparison with passively targeted counterparts. This improvement in cellular uptake is a key point because mostly of the targets present intracellular location. Bearing this in mind, the characterization of endocytosis pathways plays a critical role in designing efficient intracellular trafficking, subcellular

targeting, and nanomedicines with ideal features (biocompatibility, low-toxicity, and low-immune response) [4].

Herein, we present a comprehensive review on recent developments and outline future strategies of nanotechnology-based medicines. Specifically, the trials *in vivo*/*in vitro*, requested by The National Cancer Institute, that evaluate NP toxicity for nanomedicines are detailed below. They can be sorted in two large groups: biocompatibility and immunological studies.

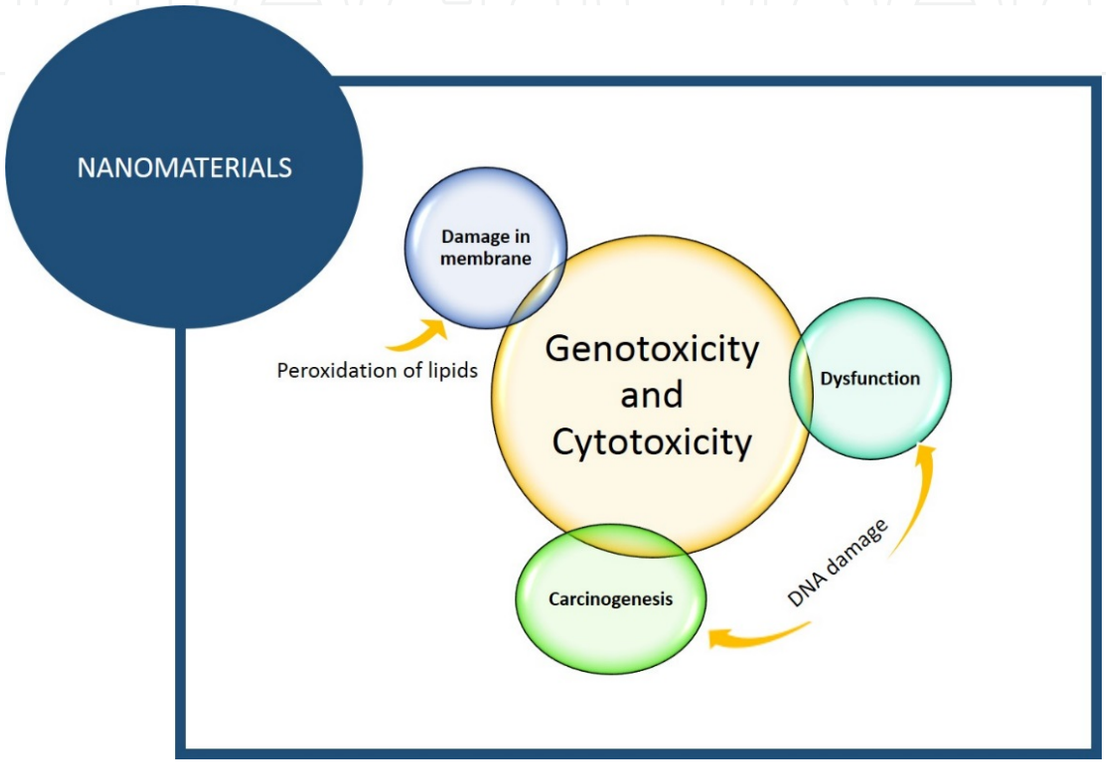

Figure 1. Effects of nanomaterials on cells.

2. Biocompatibility studies

Once the NPs are in biological environment, it is expected that their interaction with biomolecules, such as proteins, lipids, nucleic acids, and even metabolites, is to a large extent because of their high surface-to-mass ratio. Bearing in mind that proteins are one of the majority components in biological fluids, formation of a protein corona at the surface of NPs is expected. This protein corona may substantially influence the biological response [5].

2.1. Relation of biomolecular corona and nanoparticles toxicity

Herein, we briefly describe how this biomolecular corona influences mainly in cellular uptake, toxicity, and biodistribution and targeting ability to a lesser extent.

2.1.1. Effect of physicochemical properties

2.1.1.1. Size

The size of nanomaterials has a direct and significant impact on the physiological activity. In fact, the NP size may be expanded by the biomolecular corona. Then, the NP size plays a critical role in cellular uptake, efficiency of particle processing in the endocytic pathway, and physiological response of cells to NPs. Kim and collaborators [6] thoroughly studied the size-dependent cellular toxicity of Ag NPs using different characteristic sizes against several cell lines, including MC3T3-E1 and PC12. They demonstrated that NP toxicity was precisely size- and dose-dependent in terms of cell viability, intracellular reactive oxygen species generations, LDH release, and ultra-structural changes in the cell.

In general, biodegradable NPs are less cytotoxic than non-biodegradable ones [7]. Apart from the nature of NP coating, particle size can also affect the degradation of the polymer matrix. With the decrease of particle size, the surface area-to-volume ratio increases greatly, leading to an easier penetration and release of the polymer degradation products. Even though it can be assumed that the smaller the NP size, the more likely it can enter into cells and cause potential damages, the mechanisms of toxicity are very complicated, so the size factor cannot be viewed as the only influence parameters.

Yuan and collaborators studied the effect of size of hydroxyapatite NPs on the antitumor activity and apoptotic signaling proteins. They studied the effect of particle size on cell apoptosis, the Hep62 cells (incubated with and without hydroxyapaptite NPs), presented morphological changes related to apoptosis which were related to the size of the NPs [8].

2.1.1.2. Nanomaterial and shape

The structure and shape influence in the toxicity of nanomaterials (Fig. 2). Commonly, nanomaterials have different shapes and structures such as tubes, fibers, spheres, and planes. For instance, several studies compared cytotoxicity of multi-wall carbon nanotubes *vs.* single-wall carbon nanotubes or graphene [9, 10], obtaining results that suggest a strong influence of the shape and toxicity. Furthermore, other authors have evaluated the toxicity of nanocarbon materials *vs.* NPs [11].

2.1.1.3. Concentration of nanomaterial

In 2013, a research was carried out to inspect the cytotoxicity of a cisplatin derivative, known as PtU2. Minor toxicity was detected when this compound was conjugated with 20 nm gold NPs (Au-NPs). Cisplatin is one of the most used anticancer agents and its conjugation with Au-NPs gives it benefits thanks to Au characteristics: biocompatibility, inactivity, non-toxicity, and stability. In this way, the compound becomes a powerful tool for the treatment of solid tumors. In the present trial, osteosarcoma cell line (MG-63) was treated with different concentrations of AuNPs, PtU2 and a combination of both, PtU2-AuNPs. Firstly, one of the aims was the determination of the carrier activity. In order to achieve this, the metal content (gold and platinum) was measured in cells and supernatants separately. The results showed that metal

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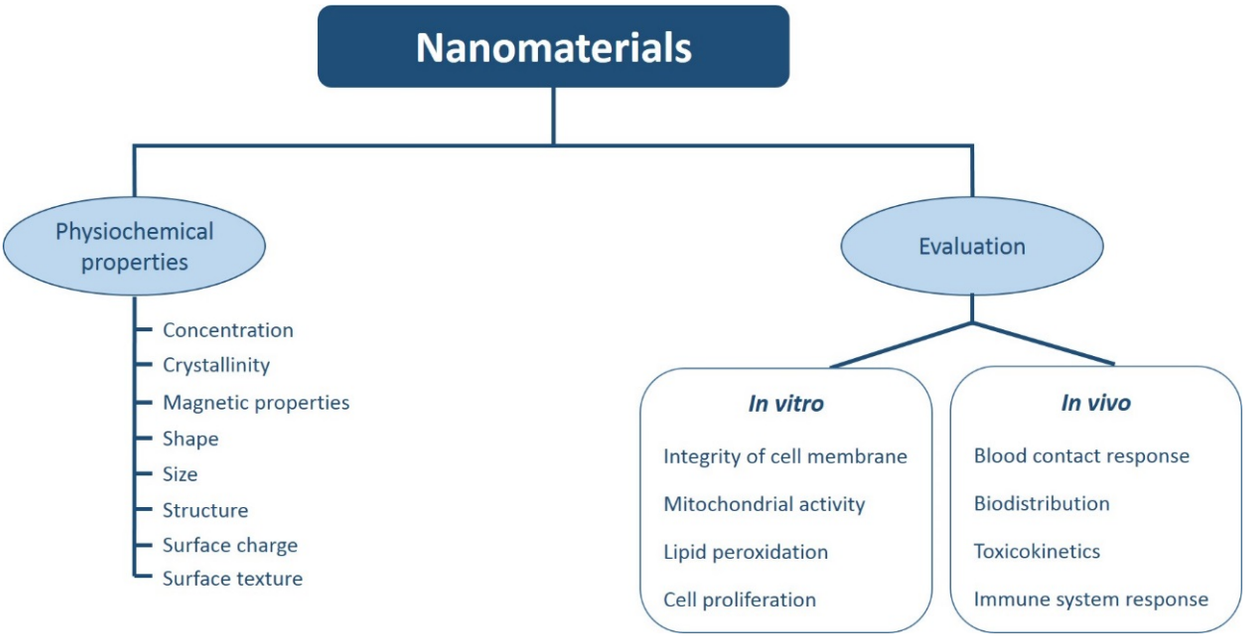

Figure 2. Characteristics and studies about the nanomaterials effect. Adapted from Li X. et al. [2].

uptake capacity from cells is the same for AuNPs or AuNPs conjugated with PtU2. Then, the cytotoxicity was evaluated by Annexin V-FITC assay by flow cytometry. As a result of MG-63 incubation with the two compounds, higher cytotoxicity was detectable after 48 hours of culture in cells treated with PtU2-AuNPs. To sum up, PtU2-AuNPs are more effective inciting cellular toxicity on the same culture conditions [12].

2.2. Relation of biomolecular corona and cellular uptake

Due to protein nature of the biomolecular corona, it is important to distinguish between specific and nonspecific cellular uptake. Specific uptake is regulated by membrane receptors that are internalized by interaction with specific ligands. In turn, nonspecific uptake is considered a random process without control by the cell [5].

Overall, nonspecific uptake seems to be decreased in the presence of a corona whereas specific uptake seems to be promoted by protein corona because a misfolding of corona proteins triggers NPs uptake by specific cells that otherwise would not have done so or because there is a protein in the corona able to target a specific receptor expressed in the cell line used. So far, all the performed studies suggest how important cell line specificity is for this protein corona effect. However, a more extensive revision of literatures is recommended because in many occasions some inconsistencies of cellular uptake of NPs have been found, particularly regarding incubation conditions or fluidic For example, several studies for cellular uptake of differential macrophage-like cell line (dTHP1) have different outcomes. In such a way, Yan and colleagues [13] did not observe any changes in effective association and internalization in the presence of serum. However, these cells present phagocytosis activity when unfolded BSA is presented in the protein corona; in this case, phagocytosis is mediated by Scavenger receptor subclass A.

2.3. Effect of protein corona on biodistribution

Despite the knowledge about the influence of NP PEGylation on biodistribution, the characterization and consequences of a biomolecular corona formed *in vivo* has not been investigated yet.

Hence, it has been described that, independently of the nature of the NPs, pre-coating with proteins, such as serum albumin, or apolipoprotein E, increases the blood circulation time and reduces the clearance speed. This effect is explained by a reduction in opsonization and phagocytosis; meanwhile, liver is the main organ for NP accumulation and the protein used for pre-coating seems to be distributed in other organs (i.e. albumin targeting and apolipoprotein E target lungs and brain, respectively) [14].

2.4. Different assays for evaluation of cytotoxicity/biocompatibility

In general, the mechanisms of toxicity are very complicated. Several studies have been developed for biological characterization of nanomaterials which are vital to guarantee the safety of the material that will be in contact with food or humans. Here, a brief description of the most conventional assays to evaluate cytotoxicity/biocompatibility is reported.

2.4.1. Cytotoxicity analysis

In order to determine the viability of cells exposed to NPs, toxicity tests *in vitro* are very useful to understand the toxic mechanisms [2]. Some of these tests are listed: Alamar Blue Assay, MTT, LDH leakage assay [2, 15], and quick cell [16]. First and second approaches constitute an index of intrinsic cytotoxicity.

On the one hand, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay (mitochondrial toxicity, MTT, assay) is based on the transformation of tetrazolium salt by mithocondrial succinate dehydrogenases in metabolically active cells generating purple formazan crystals [17]. This oxidation–reduction reaction can be only produced in presence of dehydrogenase enzymes, so it is a good way to determine the activity of mitochondria [2]. Thus, the number of living cells is proportional to the amount of formazan produced.

Cells with culture medium and NPs are seeded in 96-well plates and then 20 μL/well of MTT, with a final concentration of 5 mg/mL added to each well. This compound must be incubated for 4 h at 37ºC and 5% CO_2. After the incubation, the solution is removed and the crystals that have been formed are dissolved by DMSO. Finally, the optical density is measured at 595 nm expressing the percent cell viability [3, 17]. This method also allows the measurement of cell survival and proliferation.

Although MTT is the most accepted assay method [2], there is another test to evaluate the nanotoxicity, the resazurin assay (Alamar Blue, AB, assay). This study is based on the reduction of blue, nonfluorescent resazurin to pink, fluorescent resorufin by living cells [18]. This reduction is mediated by mitochondrial enzymes located in the mitochondria, cytosol, and microsomal fractions [17, 18]. The decrease in the magnitude of resazurin reduction indicates loss of cell viability.

The AB assay is commonly used with a final concentration of 10% (w /v). Then, plates are exposed to an excitation wavelength of 530 nm and emission at 590 nm to determine if any of the dyes interact with the compound. Lastly, the fluorescence is read 5 hours later and the percent viability is calculated [17]. Moreover, AB assay has many advantages: it is a simple, rapid and versatile test and reveals a high correlation with other methods to evaluate nanotoxicity [18].

Sometimes, problems with interference between NPs and this type of assay arise [17], so care must be taken with the dyes used. The confidence degree of toxicity studies significantly depends on this interaction. Few researchers have found that carbon nanotubes can interact with dyes such as AB and neutral red [2].

According to the analysis carried out by Hamid R and collaborators, AB assay and MTT are advisable to identify the cytotoxic compound. However, the AB assay is homogenous and presents more sensitivity that can detect densities as low as 200 cells per well [17].

In turn, cell death is also determined by evaluating the activity of the enzyme lactate dehydrogenase (LDH). LDH is an enzyme generally located in the cytosol, but it is quickly released when cellular damage is produced. In this way, the LDH release assay allows the assessment of the membrane integrity of cells by measuring this enzyme in the extracellular medium. This method, like MTT, uses the measure of a color compound absorbance to determine the cell viability that can be affected by the uptake of NPs [19].

The Quick Cell Proliferation Colorimetric Assay Kit works in a similar way. This is based on the cleavage of the tetrazolium salt to formazan by mitochondrial dehydrogenases. An increase in the activity of these enzymes is connected with cellular proliferation. The formazan dye produced by viable cells can be quantified by a spectrophotometer by measuring the absorbance of the solution at 440 nm. Moreover, the Quick Cell Assay has several advantages in face of MTT because it is a new simple method, requiring no washing, no harvesting, and no solubilization steps, and it is more sensitive and faster too [20].

2.4.2. Assays for studying cell death by effects of nanomaterials

The cytotoxicity analysis can be complemented by other studies. Here, we present different methods to determine cell death or apoptosis, including trypan blue (TB) and propidium iodide (PI) protocols.

TB exclusion test marks which cells are viable. This is because live cells have intact cell membranes and certain dyes, such as TB or PI, cannot entry into them [21]. In dead cells, the membrane is ruptured and the dye is able to cross it and stain the cytoplasm of blue. In 2014, Mendes and colleagues [22] published a work where their aim was to investigate different diameters of iron oxide NPs. Four cell lines were incubated with NPs to assess the material toxicity and the possible size dependence. Cell viability was measured using the MTT and TB tests. For the dye exclusion assay, the cells were seeded in 6-well flat-bottom plates and incubated for 12 or 48 h with a NP suspension at 10 μg/mL concentration. Then, 20 μL of each suspension was mixed with 0.4% TB to count the number of living and dead cells. This method was used because with the MTT it was not clear if NPs caused cell death or whether they only

reduced the cellular metabolic activity. The results showed that cells incubated with the carbon-coated iron oxide NPs tend to decrease their mitochondrial activity (indicated by MTT test) rather than die (indicated by the dye exclusion test). In conclusion, cytotoxicity analysis showed no apparent difference between the diameters studied, whereas there are clear differences in particle uptake.

On the other hand, Alshatwi and collaborators [23] have published a work where the toxicity of platinum NPs is evaluated. The objective in this project is to investigate the effects of platinum NPs on cell viability, nuclear morphology, and cell cycle distribution on SiHa cells (a cervical cancer cell line). To study the nuclear morphology, SiHa cells were incubated with platinum NPs for 24 hours. Then, cell nucleus were stained by 1mg/mL PI and examined under a fluorescence inverted microscope. In treated cells, nuclear fragmentation, chromatin condensation, and nuclear swelling were observed. The nuclear fragmentation is a hallmark of late apoptosis.

In the same way, PI was also used to determine the cell cycle stage of treated cells by a flow cytometer. The results showed that these NPs induced a G2/M phase cell cycle arrest due to DNA damage. Briefly, this investigation suggests that platinum NPs inhibit cell proliferation because they induce cell death via apoptosis. Moreover, the NPs also have effect by reducing cell viability and causing DNA fragmentation and G2/M cell cycle arrest. That is why; they can be a potential therapy agent in the cervical cancer treatment.

Secondly, we describe two different ways to evaluate the cell death induced by apoptosis. Apoptosis or program cell death occurs in the normal physiology during development and aging to keep a balance between proliferation and cell death [24, 25]. It is also a defense mechanism and it is important for removing damaged cells and decreasing the damage on neighbor cells.

This process is carried out by loss of the mitochondrial transmembrane potential and activation of caspases (cysteine proteases). These proteins can be categorized into initiators (caspases 2, 8, 9, 10), effectors (caspases 3, 6, 7), and inflammatory caspases (caspases 1, 4, 5) [24].

Frequently, cell apoptosis is usually evaluated using a caspase-3 activation assay. For instance, Xun et al. put into effect a work where they tried to study the effect of silica NP size (7, 20, and 50 nm) on cytotoxicity. The cell line HepG2, a human hepatoma model, was selected for the study. HepG2 cells were treated with silica NPs of 20 nm (SNP20) at concentrations of 160 μg/mL and 320 μg/mL for 24 and 48 hours, respectively. Then, caspase-3 assay buffer and caspase-3 lysis buffer were added into the cell culture. After reaction, the fluorescence intensity was detected under a fluorescence plate reader. Caspase-3 is an essential molecule in the final phase of apoptosis induced by diverse stimulus. Results obtained in this analysis showed an increase of caspase-3 activity about 2–3 fold higher in cells treated with SNP20 than that of controls after 24 hours of incubation and about 3–5 fold after 48 hours. About this evidence, silica NPs could activate caspase-3 and downregulate procaspase-9, indicating an activation of caspase-9 in HepG2 cells. That is, these NPs can change apoptotic protein expression and greatly increase apoptosis in mitochondria-dependent pathways in hepatoma cells. In

addition, Annexin V-FITC/PI assay was used in this study to quantify cell apoptosis. This test allows distinguishing between normal, apoptotic, and necrotic cells.

HepG2 cells and normal L-02 hepatic cell lines exposed to SNP20, at the same two concentration used before, were stained using Annexin V-FITC and PI and analyzed by flow cytometry. Apoptotic cells undergo changes in the distribution of their membrane lipids. Phosphatidylserine is a phospholipid commonly presented inside the membrane, whereas during apoptosis, processes are expressed on the cellular surface.

In this way, Annexin V, which has a high affinity for phosphatidylserine, is used as a marker of early apoptosis. However, PI is used to distinguish necrotic cells from apoptotic cells. This is an agent which is intercalated in the DNA of dead cells when losing the membrane integrity. As a result, almost no apoptotic cells were detected in controls and treated L-02 cells and in control HepG2 cells. On the contrary, many apoptotic cells were found in HepG2 treated with SNP20, indicating that apoptosis induced by NPs is dose-dependent [25].

Annexin V is a method commonly used for assessing cellular apoptosis. For example, Ashokkumar and collaborators as well as Grudzinski et al. employed this procedure in their studies. In the first one, the aim was to evaluate whether gold NPs are able to induce apoptosis in cancer cells. HepG2 cell line was used for the investigation and these were treated for 24 hours with gold NPs. After that, cells were stained with Annexin V and the level of apoptosis was quantified as a percentage of Annexin V positive cells. Finally, the results showed that HepG2 treated with gold NPs undergo cell apoptosis whereas untreated cells did not show it [26]. In the second investigation, they tried to study the cytotoxicity of carbon-encapsulated iron nanoparticles (CEIN) in murine glioma cells (GL261). These cells were divided into two groups: one was treated at two different concentrations during 24 hours, whereas the other group was the control group (untreated cells). Then, both groups were stained with Annexin V and the analysis was performed by flow cytometer. The results indicated that the samples treated with the higher concentration of CEIN induced some pro-apoptotic and necrotic events in the glioma cell line. As a summary, this work have supposed a huge progress because it is the first report which clearly displays that CEINs with surface modifications with acidic groups cause murine glioma cell-specific cytotoxicity [27].

3. Immunological studies

Besides the fact that NPs play an important role in medicine area and their properties can be used to improve traditional treatments and diagnostic agents [28], there are many biocompatibility studies about size, shape, charge, solubility, and surface modification of NPs. However, the interphase related to interactions between NPs and immune system is still not well understood.

According to literature, NPs can activate and/or suppress immune response and the compatibility with this system is determined by its surface chemistry. Therefore, NPs can be designed to avoid immunotoxicity and reach desirable immunomodulation [29, 30].

Preclinical data shows that NPs are not more immunotoxic than conventional drugs, so NPs employed like drug carriers can provide advantages, such as the reduction of systemic immunotoxicity. For instance, NPs can release the drug in a specific tissue in order to not alter safe tissues and they may keep drugs away from blood cells. Moreover, NPs can also decrease drug immunotoxicity by raising their solubility. However, NPs are generally picked up by phagocytic cells of the immune system, such as macrophages. This incident can produce immunostimulation or immunosuppression, which may promote inflammatory or autoimmune disorders. For example, granuloma formation was observed in the lungs and skin of animals treated with carbon nanotubes [29].

Next, we briefly describe immunostimulation and immunosuppression linked to NPs uptake.

3.1. Immunosuppression

There are not many studies about this area for NPs because most of the researches focused on the inflammatory properties of NPs [29].

One of the studies about immunosuppression has revealed that inhalation of carbon nanotube (CNT) results in a reduction of immune system in mice. This is produced through a mechanism that involves the release of TFG-β1 from lungs. Then, TFG-β1 goes into circulation and increases the expression of two molecules whose function is to inhibit T-cell proliferation [31].

Other NPs that can produce immunosuppression are zinc oxide (ZnO) particles. They are able to induce immunosuppression *in vitro* and *in vivo* in function of the different size and charge. ZnO NPs suppress innate immunity such as natural killer cell activity. Moreover, the $CD4^+/CD8^+$ ratio, a marker for matured T-cells, serum levels of T helper-1 cytokines (interferon-γ and IL-12p70) and pro/anti-inflammatory were slightly reduced. In the opposite sense, no significant changes were detected in T- and B-cell proliferation [32].

3.2. Immunostimulation

Biological therapeutics, where NPs are included, are able to activate the immune system. In other words, nanomaterials are identified by this system and innate or adaptive immune responses are produced. We briefly describe several effects of NPs on cytokine secretion, immunogenicity and the mechanism through which nanoparticles are recognized by the immune system.

On the one hand, many immunostimulatory reactions, driven by NPs, are mediated by the release of inflammatory cytokines. Cytokines are signaling molecules inducted by different types of nanomaterials: gold, dendrimers, or lipid nanoparticles, among others. Moreover, NP size is an important factor for determining whether antigens loaded into NPs stimulate type I (interferon-γ) or II (IL-4) [29, 30]. For example, a study carried out from peripheral blood mononuclear cells of non-atopic women showed that palladium NPs improved the release of IFN-γ [33]. In other study about THP1-macrophages, the results showed that chitosan-DNA nanoparticles did not produce pro-inflammatory cytokines, whereas the secretion of metalloproteinase 9 and 2 was increased in cell supernatants [34].

This kind of analysis is often evaluated by enzyme-linked immunosorbent assay (ELISA). Antibodies and an enzyme-mediated color change are used to determine the presence and relative concentrations of particular cytokines present in the tissue or cell culture media. [35, 36]. ELISA is based on the concept of an antigen binding to its specific antibody, which allows identification of small quantities of molecules such as cytokines [36].

In turn, NPs induce antibody response (immunogenicity). NPs raise a special interest in this area because immunogenicity is improved by stimulating the production of antibodies [30]. Plasma B cells are responsible for making antibodies, specialized proteins, in response to an antigen [29].

A recent *in vivo* study about a novel dengue nanovaccine (DNV) has demonstrated that the vaccine can stimulate humoral and cell-mediated immune responses. This vaccine is composed by the dengue virus type 2 inactivated. Moreover, the adjuvant chitosan together with NPs including cell wall components from *Mycobacterium bovis* were used to improve the action of the DNV. Mice treated with this compound showed an increase of cytokine levels and a strong anti-dengue IgM and IgG antibody response. The release of IFN-γ produced by $CD4^+$ and $CD8^+$ T cell was also incremented. In conclusion, these results demonstrated that the DNV can be an important vaccine candidate for treatment of dengue disease [37].

Finally, we briefly mention the mechanism through which NPs are phagocyted into the cells. Macrophages are responsible for the first line of defense in the organism. They detect and uptake foreign molecules and synthesize mediators which warn the immune system about infection. Raw 264.7, a mouse leukemic monocyte macrophages cell line, is the model line used for the phagocytosis assays. For instance, Raw 264.7 was utilized in a new *in vitro* research about the effect of silica and gold NPs in macrophages. The results showed that silica and gold NPs decreased the ability of phagocytosis in 50%, while surface markers and cytokine secretion were not disturbed due to the particles [38]. To evaluate this analysis, different methods can be used depending on the composition of the nanomaterial. These procedures include confocal microscopy, optical and fluorescence microscopy, transmission electron microscopy (TEM), or scanning electron microscopy (SEM) [38, 39].

4. Conclusions and perspectives

Bearing in mind the importance and relevancy of the NPs in biomedical field, a better understanding of their effects on the human body is therefore required. According to the points described above, the physicochemical properties of nanomaterials play a critical role in toxicity. Thus, the alteration of these properties could be used to modify the toxicity and/or biocompatibility of these materials. On the other side, it is also necessary to obtain the maximum amount information about the interaction of biological interactions of NPs with cells, tissues, and proteins. In fact, this could be a critical parameter for the future application of nanomaterials in the biomedical area. In this review, special attention has been paid to the protein corona because it plays a critical role in toxicity and biocompatibility. Many studies have been performed; however, further studies are needed to know how to exploit the benefits

of the corona *in vivo*; mainly, because it seems quite complicated to predict the composition of proteins corona and its biological consequences.

Despite immense progress on the evaluation of toxicity and biocompatibility of nanomaterials, from this comprehensive review it is pointed out that further experimentation is still ongoing in this field to obtain a better and optimal understanding of the interaction between nanomaterials and the human body.

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