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Mini-Review

An Approach to The Safety Assessment of Nanoparticles and Nanomaterials

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Abstract

Nanotechnology is a fast-developing discipline, which is why we consider it of interest to look for and summarize information in order to know the benefits, challenges and risks which its use will bring to light, and which will need to be addressed. As is often the case, the regulations that govern this field within the sciences are one step behind the applications being discovered. After posing the questions that we consider can and should currently be answered, in this paper we will attempt to provide answers based on existing sources.

Finally, we will lay out our conclusions and opinions so that such a promising discipline may develop with maximum benefits and minimum risks.

Keywords: Nanomaterials, Nanoparticles, Assessment, Safety, Cell Cultures, Cytotoxicity

Introduction

Nanotechnology is a fast-growing scientific field which is used in numerous aspects of human life, animal life, and the environment. However, the use of nanomaterials (NMs) or nanoparticles (NPs) in medical devices (MDs) or nanodrugs (NDs) which implement any of these technologies have not been properly regulated yet, and its adverse effects are not fully known. There are many research teams working on these issues and developing new experimental models in order to carry out toxicity evaluations and gather information on the risks which using these materials entails [1]. Work teams from international agencies, which have the task of regulating their use, are trying to produce appropriate Technical Reports and Regulations, such as ISO STR 10993-22: 2017 Biological evaluation of medical devices- Part 22: Guidance on nanomaterials, in order to assess their safety and effectiveness since there is an ever-growing assortment of products in the health market that have in one way, or another adopted these technologies.

Problem Statement

NMs, NPs, MDs, and NDs are suitable candidates for the application of nanotechnology in the health field. Devices containing any of those materials demand an advanced characterization which, oftentimes, is not available before the MD is launched to the market. We consider this issue needs to be addressed in order to develop possible solutions.

Objective of This Minireview

Due to the wide scope and difficulty of the subject, in this minireview we aim to show the current possibilities at hand, so that manufacturers may have tools to start assessing nanotoxicity according to Technical Guidelines or Reports in order to reduce the potential risks of using these products and keep up with this fast-developing discipline.

Focus Questions

How to proceed when no regulations have been established yet

Some of the most relevant works that discuss and comment on the different voluntary ways to control nanotechnology, known as a whole as "soft regulation", are reviewed. Information is sought in different agencies related to the standardization of nanotechnology, paying special attention to the work of the Technical Committee in Nanotechnology of the International Organization for Standardization, ISO/TC-229.

In exceptional circumstances, when a Technical Committee has gathered data of a different kind from that which is usually published as an International Standard (for example, state of the art), by a simple majority of votes of its participating members, it may decide to publish a Technical Report. This Technical Report is meant to be used in all countries, whether or not they have legal or regulatory frameworks in place that approach manufactured nanomaterials, and it is purely informative. It is aimed mainly at organizations that manufacture or process NMs or manufacture, process and distribute products containing NMs. Nevertheless, government authorities, professionals, and members of the public may also find this information useful [2].

Which are the Potential Effects of Nanoparticles to Human Health and The Environment

The exposure of living beings to NPs and NMs becomes unavoidable when considering their fast-expanding application in all areas of human activity, including health. At the same time, the studies devoted to the characterization of their effects after exposure, and the corresponding Application Rules are lagging behind.

Is it possible for the advantages of NMs to outweigh their disadvantages? Are we capable of assessing the environmental and health impact that an extended and/or unlimited use of the NMs may cause in the different biological systems? If that should be the case, which are the best models to assess it? In the interest of the ongoing development of nanotechnology and nanomedicine, it is necessary to understand the risks derived from NPs, NMs, and NDs, because those risks are closely related to the safety and efficiency when they are being used. We need to be able to assess them.

Findings

What we currently know

a) The safety of materials at a nanoscale is a growing concern.

b) When NPs enter the body via inhalation, ingestion or contact with the skin, they reach organs such as the lungs, kidneys, liver or the brain, and interact with molecules potentially causing adverse effects.

c) Most methods used were "*in vitro*" cell tests.

d) Reducing and replacing animal usage in lab tests is a global demand.

The progress made in molecular biology offers the possibility of testing the toxicity of different NPs using single cells, cell population, reconstructed tissue, and whole organisms. These developments will allow us to carry out increasingly accurate assays to evaluate nanotoxicity.

Nanotoxicity is Related to Cytotoxicity and Cell Stress

Cytotoxicity happens when there are disturbances to the metabolism, the integrity of the plasma membrane, adhesion properties, the cytoskeleton, replication or other cellular functions. Oxidative stress occurs when in the cell there appear compounds such as free radicals, hydrogen peroxide, and there is a lack of antioxidants to neutralize them. When in excess, it has negative consequences because it alters some cell functions.

Origin and Reasons Behind the Toxicological Effects

They are related to certain properties of the NPs:

a. Size

b. Surface area

c. Shape

d. Charge

e. Physicochemical properties

f. Layers of protein corona

Exposure pathways, as we mentioned before, are also important. The lungs and heart are the organs most frequently affected by NPs which are easily airborne and widely distributed in the lung regions, resulting in systemic effects. When NPs interact with biologic components, they may influence their functioning and result in adverse outcomes.

Due to their small size and large surface area, NPs, NMs, and NDs interact with biological systems differently from the bulk substance, Contrary to the beneficial biological effects, most often they result in adverse effects.

Common Mechanisms Involved

To rule out the toxic effects of any NMs or NPs, it is essential to understand the mechanisms associated with nanotoxicity.

According to the previously published literature, these are some NMs-induced initial toxic effects:

a) Activation of the immune response.

b) Inflammatory stimuli.

c) Inflammatory cytokines overproduction.

d) Increased reactive oxygen and nitrogen species production (RONS).

Initial effects will lead to any of the following outcomes:

a) Apoptosis.

b) Necrosis.

c) Necroptosis.

d) Autophagy-mediated cell death.

If we already know that NMs cause cell death, later studies should analyze the kind of cell death which is induced by the

NM, and determine whether necrosis, apoptosis, necroptosis or autophagy has occurred. The information obtained from assays will allow to define the biological and molecular mechanism of toxicity and deduce beforehand whether the NM could have a potential genotoxic or inflammatory effect on a specific biological system.

Conventional Methods for Assessing Nanotoxicity

***In vitro* assays:**

Advantages

a) Compliance with the 3Rs rule

b) Quick and inexpensive

c) Endpoints: microscopic, colorimetric, fluorescent, luminescent, etc. observations

Disadvantages

a) Possible interference of the chemical reagents with NPs may lead to misinterpretations of the results and concerns which warrant more advanced and specific techniques for assessing the toxicity of NPs.

b) As in all instances, the matter of the most convenient model for each case also arises here: *in vivo* vs *in vitro*.

c) Culture model selection.

We Shall Bear in Mind the Cellular Uptake Mechanisms:

a) Endocytosis (after plasma membrane vesicle formation)

b) Ion channels

c) Pores in the membrane

d) Passive uptake in the cells: by diffusion and interaction between nano-object surfaces and cell surfaces without vesicle formation.

And also

a. Presence of certain receptors.

b. Cell sensitivity to the toxic substance.

By way of example, we may summarize/refer to the work of Handule Lee [3], where Statens Seruminstitut Rabbit Cornea (SIRC) cells were used in an *in vitro* cytotoxicity assay to study the damage to the eye that may be caused by exposure to Zn, Ag, Ce, Si, and TI NPs with concentrations of 1-100 μg/mL. The Statens Seruminstitut Rabbit Cornea (SIRC) cell line was used in this study: the obtained cells were cultured in culture plates for 24 hrs. in established conditions (ISO 10993-5) and were later exposed to concentrations of 10 to 100 μg/mL of the different NPs in order to assess the cytotoxicity though the MTT quantitative assay laid out in ISO 10993-5. At the tested concentrations, cell viability only decreased in the group treated with ZnO NPs.

The other NPs did not show cytotoxicity in these concentrations. AG NPs seem to show low cytotoxicity at higher concentrations.

In that same study, also the effect of those nanoparticles was analyzed in the production of ROS through fluorescence. Those results were compared to the cytotoxicity results, and they proved to be coincident, only the SIRC cells treated with ZnO NPs showed fluorescence images, and these cells were gradually detached from the bottom of the plate. Since the possibility of eye exposure exists for workers employed in the production of nanoparticles and for users of medical products or personal protection equipment that contain them, carrying out this assay would be an improvement for guidance. It is important to remember that in the assay the proper controls need to be in place.

Interaction of NPs in in-vitro cultures

a) Nano-objects reach the cell through sedimentation aggregates NPs (added NPs) or through diffusion (individual NPs).

b) This will be especially dependent on their size.

c) The addition of NPs is also influenced by the culture medium and physicochemical properties.

What happens when the nano-object has entered the cell [4]?

1. NP distribution in different organs is dependent on the physicochemical characteristics, including electrical charge, shape, and composition of the NP core and shell.

2. It may interact with biological components and alter cell function.

3. Intracellular location will depend on its physicochemical properties, size, and dosage.

4. In vivo, nano-objects usually end up in cells from the macrophage system (MPS).

5. Carrying out the *in vitro* cytotoxicity assay in phagocytic and non-phagocytic cells should be considered.

Interference to be Considered

It has been proven that NPs may interfere in MTT, XTT, LDH, and DCF colorimetric assays for cytotoxicity.

Some NPs may absorb or scatter light and, thereby, interfere with the measurements. Separating nano-objects though centrifugation before reading the assay may reduce the variations in the data obtained.

Cytotoxicity Induced by Oxidative Stress

In cases where toxicity is induced by Reactive Oxygen Species

(ROS), the nanomaterial does not necessarily enter the cell to produce cytotoxicity. A similar situation happens when cytotoxicity is induced by the release of ions, as it happens with ZnO and Ag nanomaterials.

Methods for the assessment of nanotoxicity in 3D cell models (cell spheroids)

Many important characteristics of cells that grow in monolayers differ substantially from those of the same cells in live tissue. In several publications, experimental 3D models have been used to analyze NP toxicity [5].

There are currently *in vitro* Reconstructed Human Epidermis (RHE) 3D models from different organs that were previously tested in nanotechnology assays. The results showed a good correlation with the data obtained *in vivo*.

The following may serve as examples:

EpiDerm was used to assess corrosion and irritation of the skin, and potential nanotoxicity caused by titanium dioxide particles [6]. On the other hand, Epi Alveolar was used to predict the formation of pulmonary fibrosis due to extended exposure to carbon nanotubes [7]. These *in vitro* organotypic models are promising as to their application in nanotoxicology. Cellular spheroids are yet another *in vitro* biological model used in toxicology. Because they have a greater average life, they are suitable for studies of chronic exposure to certain toxic agents [8].

What are cell spheroids?

Structures that naturally form a multicellular sphere, organized in cell layers and showing properties that differ from those of cells growing in a 2D monolayer.

Their main characteristics compared to 2D cultures are:

a) Higher cell viability

b) Stable morphology

c) Polarization ability

d) Cell–cell and cell–extracellular matrix interactions

e) Increased metabolic activity

Additionally, it has been observed that 3D behavior in cells arranged into spheroids is very similar to the behavior cells have *in vivo* in a multicellular organism [9,10].

Advantages of Using Cell Spheroids

Some of the advantages of using spheroids as opposed to their counterpart in 2D, is that they enable the study of cell–cell and cell–matrix extracellular interactions. Research carried out in 3D cultures has proliferated given the need to produce human cell *in vitro* models, which will allow an in-depth study of molecule and cell responses derived from these cell types. An example can be found in the case of hepatitis C, where clinical assessments of the response to speciesspecific pathogens and hepatotoxicity have been evaluated in 3D cultures, enabling the creation of an appropriate predictive model in order to prevent clinical assessments from failing [11].

In the case of NM evaluations in 3D systems, it has been reported their binding and internalization may differ from the monolayer response due to the complex physiology of spheroid cultures. The cytoskeleton plays a decisive role in NP internalization in 3D cultures, but not so in 2D cultures, since macropinocytosis is the main mechanism through which NPs are internalized in 3D cultures. These *in vitro* cell culture models attempt to imitate as much as possible the *in vivo* cellular microenvironment; additionally, they are a relatively simple model, and much more cost effective than animal models. At present, spheroids have been recognized as the intermediary cell model between *in vitro* tests and *in vivo* studies [12].

Are there examples of spheroids used for the assessment of NMs?

Most reported studies on the use of spheroids for the cytotoxic assessment of NPs re based on the comparison of their cytotoxic effects, internalization, and diffusion ability in 2D monolayers and 3D spheroids cell cultures.

Metal NP and oxide NP assessments have been described in these 3D systems. The cytotoxic effect and internalization ability of titanium dioxide NPs (TiO2 NPs) have been studied in cell cultures of human osteoblasts in order to analyze the responses to cell–cell interaction in bone tissue. After a 72-hour exposure to TiO2 NPs, cell viability was not affected. In addition, high concentrations of TiO2 NPs induced the production of proinflammatory cytokine and growth factors involved in bone homeostasis and osteolysis [13].

Other oxide NPs that have been assessed in 2D and spheroid cultures are zinc oxide NPs (ZnO NPs). Chia & Co. showed in 2015 the ability of ZnO NPs with a 25 nm diameter to induce a cytotoxic response of death cell depending on the type of dimensional arrangement of the colon cells. In addition, these authors suggested the idea of cell turnover dynamics in spheroids, which behave as "onion layers," and whose cells become detached as a result of the damage inflicted and the inflammatory processes caused by exposure to NPs. This allows for there to be a lower layer of viable cells which responds differently to the effects of the NPs depending on their dimensional arrangement [14]. The cytotoxicity of some luminescent nanomaterials has also been assessed using spheroids, for example, gadolinium loaded mesoporous silica [15].

What We Learnt from Using Nanomaterials in Medical Applications

a. NMs showed their effectiveness in the treatment of both simple and complex diseases.

b. They have transformed some traditional treatments: a new approach to bone tissue regeneration, for example, consists of culturing, on a graphene scaffold, mouse bone marrow stem cells which tend to transform into bone generating cells, called osteoblasts. The cells multiplied and spread within the network of pores, a positive sign which also demonstrated the scaffold biocompatibility, and the ability of the cells to colonize them [16].

Vaccine production [17]

a. The development and deployment of first generation COVID-19 vaccines, which include nucleic acids or viral vectors, has progressed at a phenomenal pace.

b. Nanoparticles may imitate the structural characteristics of the natural virus.

c. Vaccines containing NPs provide ways to promote strong responses because they are capable of stimulating the immune system, generating robust solutions, but also adverse reactions in some subjects.

d. They use a lipid nanoparticle (LNP) nano delivery to protect the mRNA so that it may reach the ribosome intact and produce antigenic proteins.

Surface Or Capsular Antigens

Nanoparticle vaccines can be divided into two groups:

a) Nanoparticles which contain *encapsulated* viral proteins or nucleic acids encoded for these proteins.

b) Nanoparticles *with vaccine antigens on their surface*.

Differences Between both Groups

a) Encapsulating nanoparticle-based vaccines protect the antigens during intracellular transport and release them in a controlled manner.

b) Nanoparticles that show vaccine antigens on their surface may involve antigen presenting cells (APCs) and/or efficiently promote the B cell receptor (BCR), leading to a strong immunogenicity.

Conclusion

As we stated in the introduction, nanotechnology is evolving fastly, and its applications are expanding to every aspect of human life, animal life, and the ecosystem of the whole world. In view of this, all research related to possible toxic effects of nanomaterials, nanodrugs, and nanoparticles in general become of paramount importance. It is necessary to study in greater depth the range of different toxic effects any NM or NP may cause by way of intentional use or unnoticed exposure. Any toxicology study of a new nanomaterial demands an *in vitro* test in order to assess biocompatibility using different types of cells in several cell culture medium formulations and suspensions of nanomaterials with the same physical and chemical properties assessed simultaneously. Conventional protocols for toxicology assessments have had to be adapted, and sometimes improving them is complicated due to the physicochemical characteristics of NMs. Consequently, the assessments of cytotoxic effects and cell proliferation of NMs have been carried out traditionally in two-dimensional cultures (2D). The three-dimensional arrangement of cell cultures is being increasingly used due to conclusive evidence about the susceptibility difference between 2D cultures and spheroid or 3D cultures.

An additional source of concern for future endeavors is the standardization of conditions and procedures; therefore, different laboratories need to carry out studies, and then compare the obtained data. We believe that, although assessments of these products with assays which are not normalized may lead to conclusions not entirely accurate, it is necessary to start carrying them out as rigorously as possible, especially regarding controls, because we will always get valuable insights which will prevent serious damage.

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