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The investigation of the parameters affecting the ZnO nanoparticles cytotoxicity behaviour: a tutorial review

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In the last 30 years the research about the zinc oxide nanoparticles (ZnO NPs) and related toxicity had a boom. ZnO NPs show cytotoxicity for both prokaryotic and eukaryotic cells and many studies demonstrated a selective toxicity towards cancer cells. However, with the increasing number of publications, we assisted to an increase in the discrepancies between the various results obtained. Soon the scientific community understood that the ZnO NCs toxicity behaviour is affected by many factors, related not only to the ZnO NPs themselves, but also to the experimental conditions used. Many recent reviews discuss about these parameters reporting experimental evidences and try to assess general statements about the ZnO NPs cytotoxicity. These informations are extremely useful for the evaluation of which type of ZnO NPs is more or less suitable for a specific study or application. However, despite that, a deep comprehension of the ZnO NPs behaviour in relation to the different experimental conditions is still lacking. Actually, a full understanding of the reasons behind the NPs behaviour is essential to better assess their biological activity and in particular their therapeutic application, avoiding undesired effects both in the experimental and clinical contexts.

This tutorial review aims to be an experimental and practical guide for scientists that face with the use of ZnO NPs for biomedical applications and, in particular, for their therapeutic purposes. The driving idea is to not simply resume the results obtained in the literature, but to provide the instruments for a deep comprehension of the mechanisms affecting the ZnO NPs cytotoxicity and behavior. This review also aims to point out the attention on the critical experimental parameters to be considered when working with these NPs, underlying the main related risks and limitations that the scientists have to face.

Introduction

The role of nanomaterials, i.e. those materials having by definition at least one size lower than 100 n, has gained increased interest in the last decades. Nanomaterials can be categorized based on their chemical composition, structure, size, morphology or origin. In this context, zinc oxide nanoparticles (ZnO NPs) are metal oxide particles made of ZnO with a size < 100 nm. The ZnO is a semiconductor material of group II–VI, with both a covalent and ionic behaviour. At ambient conditions it is normally found in a crystalline structure named wurtzite, made by tetrahedral units of zinc ions coordinated with four oxygen atoms and oriented in the space in one direction to produce the characteristic hexagonal symmetry. This particular crystal structure is the responsible of the high attractiveness of this material, because it confers it many useful properties. The wide band gap of of 3.37 eV and the large excitation binding of 60 meV are responsible for the semiconducting and optical properties, like the ability to adsorb the UV radiation. The lack of a center of symmetry in the crystal structure, combined with the anisotropic crystalline framework, confer to this material both piezoelectric and pyroelectric properties. In addition, the high synthesis versatility of ZnO could give rise to a myriad of micro and nano-structures, obtained through different easy and low cost kinds of synthesis (1).

Due to these special properties, ZnO NPs have found large applications in many different fields and, surprisingly, they are constituents of many common and daily used products. For instance, they are present in sunscreen and cosmetics due to their ability to adsorb the UV radiation, in baby lotion and dental paste for their antibacterial properties and even in the breakfast cereals as food addictive, because primary source of zinc (2,3).

Actually, this wide application of ZnO NPs was supported by their safety perception. In fact, the ZnO is listed as “generally recognized as safe” (GRAS) by the Food and Drug Administration, that considers its use not dangerous for the human health (4). Moreover, the consideration that the zinc is an essential trace element in the human body, also involved in many physiological functions (5), supported the idea of biocompatibility attributed to these NPs (6).

In the last recent years ZnO NPs have emerged as promising candidates in nanomedicine, which is the branch of medicine that utilizes nanomaterials for medical purposes (7). Actually, the reduction in size to the nanometric form, confers to this material new properties particularly useful in the biomedical field. As other NPs, ZnO NPs display a high surface to volume ratio that increases their reactivity, facilitates the interaction with the similar sized biological molecules and confers them the

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ability to spontaneously target and accumulate in the tumor tissues (7). These properties, added to the special ones intrinsic of the ZnO, promoted, in the last years, extensive studies about this material in the biomedical field, for the development of both diagnostic and therapeutic agents (6). However, from the biological results, it soon emerged that the ZnO NPs were able to kill both prokaryotic and eukaryotic cells (8)(9), namely, they have the intrinsic ability called "cytotoxicity". Considering the wide diffusion of these NPs, this discovery forced the scientific community to perform a re-consideration of the ZnO NPs toxicity, to evaluate the possible risks for the human health connected with the use of these NPs. The purposes of these analyses were to consider the risks for the final users of the NPs-based products, the ones connected with the occupational exposure and finally, the toxicological impact of the ZnO NPs environment pollution (10–12). From the numerous in vitro and in vivo studies it then emerged an unexpected ZnO NPs property, namely the ability of these NPs to selectively kill the cancer cells (13). This discovery profoundly changed the image of these NPs and produced a real boom in the ZnO NPs research, aimed to the use of NPs as anticancer agents.

However, the numerous results obtained about the ZnO NPs cytotoxicity are also highly jeopardized and the scientific community faces with many difficulties when trying to compare the results obtained by the different laboratories to provide general statements about the ZnO NPs toxicity. Indeed, the cytotoxicity studies show a high experimental variability and seem to be strongly influenced by the context in which they were performed. Therefore, these differences moved the scientists to investigate more in depth the NPs toxicity, trying to identify which parameters are crucial for determining their cytotoxicity behaviour.

Comparing different NPs formulations, it clearly emerged that more than one NPs characteristics is involved in the determination of the cytotoxic behaviour, like the NPs size and surface charge obtained at the end of the synthesis route. In addition, scientists discovered that ZnO NPs are highly reactive entities, changing their physico-chemical properties in response to the different experimental settings. They demonstrated that the NPs cytotoxicity behaviour depends not only from the intrinsic NPs characteristics, but also by the experimental conditions, and remarked that both these parameters must be carefully taken in consideration in the cytotoxicity studies in order to reduce, as most as possible, the experimental variability. For instance, the choice of the synthesis procedures must be aimed at obtaining the most homogenous NPs formulation, while the NPs cytotoxic behaviour must be checked in different experimental conditions, to determine the most reproducible ones (14). In addition, scientists discovered that, due to their cytotoxicity mechanisms, ZnO NPs show a different cytotoxicity in the different cell lines, that was demonstrated to be highly influenced by cell-related parameters, like the cell culture conditions or the kind and state of the cell lines used (15). Differences in the NPs behaviour were detected also in the different in vivo settings, in which NPs acquired new physico-chemical properties in response to the encountered conditions (16).

Therefore, considering this complex situation, this tutorial review has two main aims:

first, to provide useful information about the ZnO NPs cytotoxicity, for both scientists that are actually working and also those who are approaching the studies on ZnO NPs. Actually, this tutorial review tries to summarize the present knowledge about the ZnO NPs cytotoxicity, bringing out the general statements obtained by the scientific community, but also pointing out the differences between the published works, as powerful starting points for the comprehension of the NPs toxicity. Whenever possible, the results are accompanied with explanations of the mechanisms behind the different cytotoxic effects, to provide the instruments for a deep comprehension for the ZnO NPs cytotoxicity mechanisms, fundamental for the correct interpretation of the experimental results.

Secondly, this review aims to be an experimental guide for the scientists that face with the nano-world, making warnings and advices to avoid recurrent mistakes that often occurred in the NPs cytotoxicity studies. For this reason, it is configured not only like a resume of the published results, but as a concrete help for the scientists to follow the right path in the ZnO NPs' related research.

ZnO NPs characteristics

Many reports in the literature try to relate some of ZnO NPs characteristics with their potential cytotoxic behaviour.

The two mainly investigated characteristics of ZnO NPs are their size and surface charge.

Considering them together, it seems that the majority of the literature agrees with the statement, that the size of the ZnO NPs inversely correlates with the NPs cytotoxicity and that positively charged ZnO NPs are more cytotoxic that their negatively charged counterparts. Baek et al. reported that the smallest (20 nm) and positive ZnO NPs exert the highest cytotoxic effect, inhibiting the cell proliferation (WST-1 assay) and damaging the cell membrane (LDH assay) (Fig1a,b). In addition, Punnose et al. demonstrated that it is possible tailoring the cytotoxicity just modifying the surface charge of the NPs, producing most positive and cytotoxic ZnO NPs (Fig1e,f). Indeed, as discussed later, the positive charge enhances the NPs interaction with the cell membrane (Fig1c). Studies with the same kind of NPs that differ only in dimension or charge demonstrated that the smallest and most positive ZnO NPs produce the highest cytotoxic effects. In particular, the cytotoxic potential is explored detecting the cell proliferation/viability (WST-1, MTT, Alamar blue assays), the damage to cellular structures, like the cell membrane, the mitochondria and the DNA (LDH assay, Mitochondrial Membrane Potential (MMP) check, comet assay, respectively), or the formation of Reactive Oxygen Species (ROS) and Zinc ions

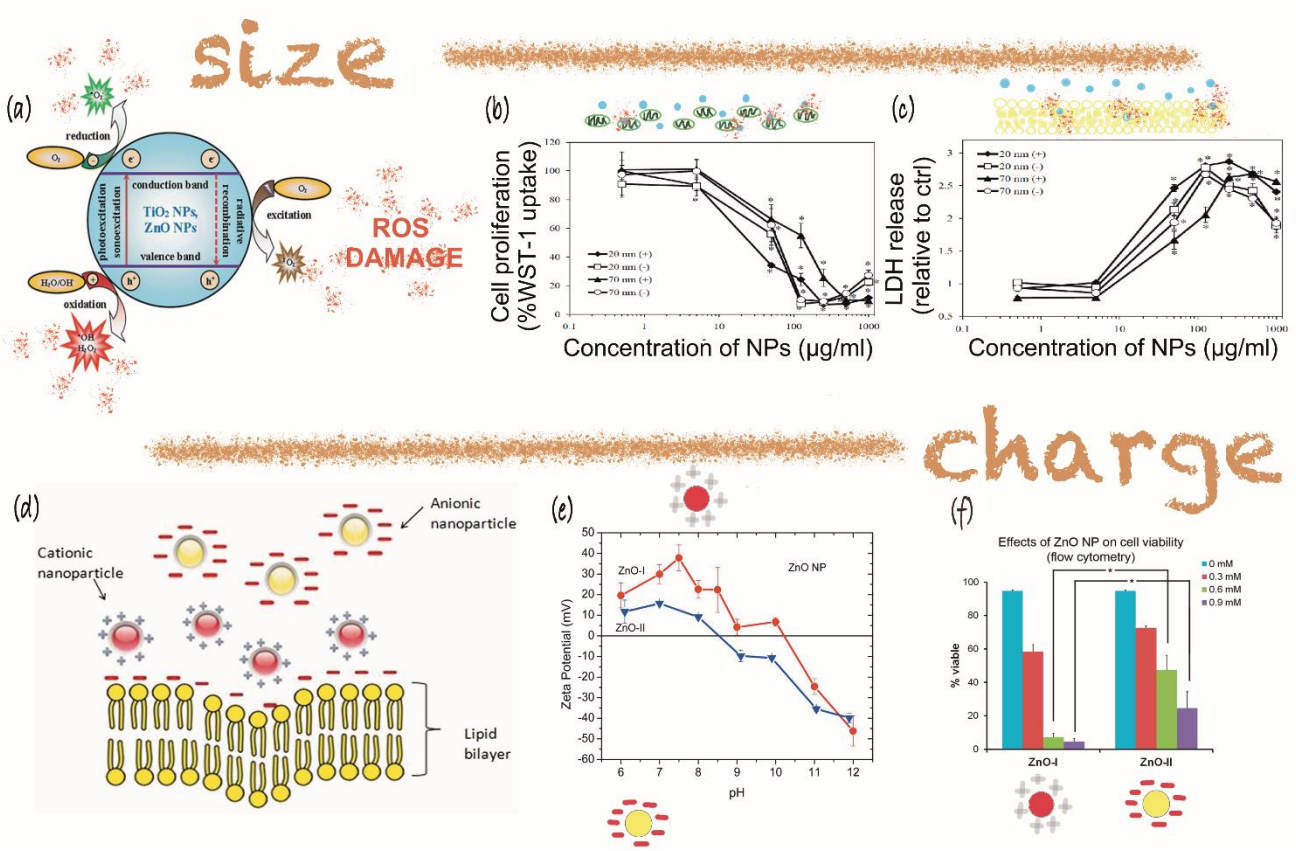

Figure 1 The ZnO NPs small size and positive charge inversely correlate with the NPs cytotoxicity. (**a**) Scheme of the possible mechanisms in metal oxide nanoparticles to generate reactive oxygen species (ROS). Adapted under a Creative Common Licence CC-BY 4.0. Co yright 2020. Bogdan et al. from Nanoscale Res. Letters 12 (2017) 225); Effects of different sized ZnO NPs on cell proliferation (**b**) and LDH leakage (**c**) of A549 cells after 48 h. Reprinted under a Creative Common LIcence CC-BY 4.0. Copyright 2020. From (23); (**d**) The effect of surface charge on nanoparticle-cell interactions. Reprinted by permission of the publisher Taylor and Francis LTD, http://www.tandofonline.com from "Biocompatibility and nanostructured materials: applications in nanomedicine" Mahdi Adabi, Majid Naghibzadeh et al., May 19,2017, Taylor and Francis; (**e**) Zeta potentials measurements as a function of pH of two kinds of ZnO NPs (ZnO-I and ZnO-II) different in surface chemical structure because synthesized using the same precursors but different reaction solvents; (**f**) Viability of Hut-78 cancer cells after treatment with ZnO-I and ZnO-II NPs for 24 hours. Reprinted under permission of ACS https://pubs.acs.org/doi/full/10.1021/sc500140x.

(Dichlorofluorescein (DCF) test, Zinq probes), associated by the literature to the ZnO NPs cytotoxicity mechanisms (17,18).

The reasons behind the effects of these two parameters on the NPs cytotoxicity have been hypothesized. First, it must be taken into account that the reduction of ZnO to the nanometric size confers to this material new structural, physicochemical and optical properties that could dramatically affect its behaviour and interaction with the biological systems. In fact, the reduction in size increases the surface area to volume ratio, leading to a major percentage of atoms able to interact and potential damage cellular structures. Furthermore, the nanometric dimension of the nanoparticles resembles those of many naturally occurring biomolecules, making their interaction easier and direct than their larger counterparts (19). Therefore, the smallest NPs harbor a high surface reactivity and enhanced interactions with the cellular structures, in particular with the plasma membrane that is the main barrier that separates the cell from the surrounding environment. It seems that this interaction is enhanced also by the surface charge. Actually, mammalian cell membranes are mainly formed by negatively charged domains, therefore the adsorption of the positively charged ZnO NPs could be favored by the electrostatic interactions. This adsorption is not only able to cause membrane damage, but also to drive the penetration of the NPs inside the cells. Therefore, the contact with the cell membrane, driven by the electrostatic force and enhanced by the high surface area, makes easier the cell internalization processes that are the same used for the biomolecules, due to the similar size range (19). In this context the smallest NPs, other than be able to cross the membrane through passive diffusion, could be internalized by the cell through the endocytosis process. As reported by Chen et al., the endocytic process is an energy-consuming process. Therefore, for the same concentration, smallest ZnO NPs consume less energy compared to the biggest ones, resulting in a highest extent of internalization (20). However, Shang et al. analyzed the internalization behaviour of different sized NPs and assessed that there is an optimal size for the internalization process. This size is around 30-50 nm, independently from the nanoparticle composition. They reported that the smallest nanoparticles have the highest probability to be internalized by passive diffusion, while the biggest ones bring on their surface the largest number of ligands able to interact with cell surface receptors and trigger endocytosis (19).

The relationship between ZnO NP characteristics and their cellular uptake is of great importance because the NPs internalization was often associated in the literature to their cytotoxic effect (21). Indeed, the internalized ZnO NPs can

directly interact and damage many different cellular structures, leading to two main detrimental effects, namely the release of zinc ions and the ROS production. Both these events are almost always associated to the ZnO NPs cytotoxicity.

Concerning the zinc ion release, the NPs internalization in the lysosomal compartment, used by the cells to degrade foreign objects and characterized by low pH, elicits the dissolution of the ZnO NPs, inducing the so called "lysosome-enhanced Trojan horse effect" (22) and further cytotoxic effects.

About the ROS production, Baek et al., suggested that the surface charge is the most critical parameter and that, independently by the size, the most positive ZnO NPs generate the highest ROS concentrations (23).

In view to a ZnO NPs therapeutic application, it is important to consider how the NPs characteristics could affect their *in vivo* fate. A possible strategy relies in the study of how these characteristics could modulate the NPs absorption and excretion, determining the long NPs circulation time and tissues accumulation *in vivo*. The group of Paek et al. deeply investigated these aspects. They suggested that the pharmacokinetic of the ZnO NPs is independent by the size, but mainly affected by the surface charge: they demonstrated that negatively charged ZnO NPs are better adsorbed by the systemic circulation than their positive counterparts. In addition, they showed that this parameter does not affect the NPs biodistribution, while the particle size is the main determining parameter for the excretion, promoting the elimination of the smallest ZnO NPs (24). This data confirmed a high correlation between the NPs characteristics and their *in vivo* pharmacokinetics, even if the reasons and mechanisms behind this behaviour still remain unclear.

Despite all these considerations and as already pointed out in the introduction, it is too simplistic considering only the two parameters size and surface charge to evaluate the ZnO NPs behaviour and toxic effects: the experimental setting must be actually taken into account. Indeed, the NPs hydrodynamic diameter and the surface charge are dramatically affected by the interaction with the surrounding environment, thus producing different biological responses. For instance, Zhang et al. reported no correlation between the ZnO NPs cytotoxic effect and particle size, suggesting that their quick agglomeration, in both water and cell culture media, flattens the dimensional differences (25). In addition, the characteristics extrinsic to the NPs, like the kind of cell line used, could prevail on these intrinsic ones, as suggested by Deng et al. reporting that the ZnO NPs size is an indifferent parameter in the viability evaluation of neural stem cells (26).

Considering all of these data, it becomes of great importance to obtain the correct NPs formulation (by own lab synthesis or from commercially purchased NPs) with the most homogeneous physicochemical characteristics. Actually, the uniformity of the preparation could improve the experimental reproducibility and avoid adding another layer of complexity to the already uncontrolled experimental variability. This consideration has been recently stressed by Garino et al. who demonstrated how different synthesis routes could produce ZnO NPs with differences in size and shape, that dramatically affect their biological response (14).

Table 1 Resume of the lessons about ZnO NPs characteristics and related warnings.

LESSONS FROM THE LITERATURE:
• **In general, positively charged and small-sized NPs exert the most cytotoxic effect** (23,27)**.** • **These NPs are highly reactive and favorably interact with the cell membrane** (7,28) **.** • **The high interaction could result in a high intracellular internalization that enhances the cytotoxic effect** (21)**.**
WARNINGS:
• **The above general statements must be customized to the specific experimental setting.** • **Carefully consider the *in vivo* fate, thus adopt biocompatible and stealth surface coating for the NPs.** • **To minimize the variability among experiments, work with the most possible homogenous NPs' formulation is strongly advised.**

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Cell characteristics

As discussed in the previous paragraph, the NPs toxic behaviour is not only dependent on the intrinsic NPs characteristics but also influenced by external parameters, like the cell lines used in the cytotoxicity experiments.

As underlined already in the Introduction, ZnO NPs are able to kill both prokaryotic and eukaryotic cell lines and despite the numerous cytotoxicity mechanisms reported in the literature, it seems that the ZnO NPs cytotoxicity could be ascribed to two main factors: the NPs dissolution with the release of zinc ions (Zn^{2+}) and the formation of Reactive Oxygen Species (ROS) (7, 29). Indeed, even if zinc is an essential element for the human life, its homeostasis in the human body is strictly controlled and excessive levels of free zinc ions in the cytoplasm could produce many deleterious effects, as the dysregulation of the cellular signalling and the collapse of the mithocondrial membrane potential (30). In addition, the interaction of the ZnO NPs with the cellular structures, especially the mithocondria, could give rise to different kind of ROS, which can produce several cellular damages and eventually lead to cell death (31).

Despite these mechanisms are common in the different kinds of cells, it seems that the cell nature, as well as the physiological/pathological state, could determine a different sensitivity to the NPs treatment.

Many authors investigated the NPs' fate when administered to cell cultures, taking into account the cell physiology in terms of cell growth and division. Different articles inquired about the NPs' inheritance after the mitosis process and reported that the NPs internalized by the cell are inherited by the daughter cells, during the cell division. Interestingly, this event could result in a NPs' dose dilution potentially affecting the therapeutic efficacy. A particular attention to this phenomenon is devoted in the field of cancer therapy where cancer cells rapidly proliferate. In particular, Summers et al. demonstrated that NPs are partitioned by the cell in an asymmetric way and this concept is stressed also by other authors, reporting that it could be a way of the cells for protecting themselves from the toxins (32). In addition, other than the different NPs partitioning between the daughter cells, other evidences suggested the importance of the cell cycle on the NPs toxicity behaviour, connecting the different state of the cell cycle with the NPs uptake rate. In 2016, Patel et al., demonstrated that ZnO NPs were uptaken at a higher extent by epidermal cells in G2/M phase compared to other phases. This ability of the cell cycle phase to affect the NPs uptake was also reported by other authors (33). However, a recent review published by Nature in 2019 refutes this idea, demonstrating that the cells' rate of uptake is independent by the cell cycle. In particular, Rees et al. reported that this difference is abolished when using a model that removes the variability related to the different cell area and time integration of dose (34).

The effects on the ZnO NPs uptake could be evaluated also taking into account the biological variability in terms of cell type. Several authors reported that different kinds of cells could uptake the NPs in different ways. Actually, prokaryotic cells harbour a rigid cell wall and are not able to perform the endocytosis, thus suggesting a less extent of NPs internalization by these kinds of cell. Indeed, this event is maybe related to an increased permeability or to damages of the cell membrane induced by the NPs treatment. In fact, about the ZnO NPs, Ivasks et al. reported two works in which their surface modifications promote the uptake in bacterial cells (35).

The difference in uptake is evidenced also comparing phagocytic versus non-phagocytic cells, or cells of different species, these harbouring different preferential uptake mechanisms (36). In addition, Kettle et al. suggested that cancer cells could be able to uptake the NPs in a more efficient way due to a major expression of receptors-mediate endocytosis on their surface and due their high metabolic activity. However, contradictory results are reported in the literature about this internalization mechanism. Furthermore, the same authors underly that the uptake differences could be the results of different physical cell characteristics, like the cell volume and size, the available surface area and the composition of the cell membrane (37).

Considering the ZnO NPs specifically, a comparative study assessing the relationship between the ZnO NPs uptake and the kind of cell line used is missing in the literature. Despite this gap of knowledge, the ZnO NPs cytotoxicity behaviour was related to a specific cell characteristic, namely the cell proliferative potential. Hanley et al., in 2008, actually demonstrated that the ZnO NPs treatment's susceptibility directly correlates with the cell proliferation. Indeed, they demonstrated that the high proliferative T cancer cells were dramatically more sensitive to the ZnO NPs treatment, than their healthy counterparts. In addition, comparing the sensitivity of the healthy T resting cells with the same cells activated trough the T cell receptor, they demonstrated an higher susceptibility to these last cells, maybe related to their increased proliferation induced by their activation (13). These findings were supported by the work of Taccola et al.. In fact, by testing the ZnO NPs cytotoxicity on healthy cells, before and after the cell differentiation, the authors confirmed the ZnO NPs' preferential killing of undifferentiated and rapidly dividing cells (38). These evidences suggest the importance of an in depth investigation of the ZnO NPs' effects on the stem cells, largely utilized for disease

therapy, due to their self-renewal and differentiation ability. Accordingly to that, a recent study reports a ZnO NPs role in stem cell differentiation, maybe ascribable to the release of the zinc ions, essential for the tissue regeneration (39).

Other important parameters to take into account concern the cell culture conditions, first of all, the cell density. Hong et al. demonstrated that different cell densities used in an experiment could influence the ZnO NPs cytotoxicity. The authors showed that confluent cells monolayers at high density are consistently more resistant to the cytotoxic effects of the ZnO NPs compared to the sparse monolayers, independently by the cell line used. Therefore, for this reason, they underlined the importance of the homologation of the test systems between the different laboratories. In addition, they investigated the effect of ZnO NPs tested at fixed concentrations using different redispersion volumes, concluding that the mass or number of NPs per unit culture area is a key parameter more significant than the NPs concentration for the measurement of the cytotoxic dosage (15).

This concept was analyzed also by Teeguarden et al. that showed how the different state of nanoparticles in solution could affect the extent of NPs that are able to reach the target cells. They assumed for *in vitro* systems that "the nominal median concentration of a test material is proportional to the cellular dose": this concept is valid for soluble chemicals but not for NPs. Actually, they proposed that particles are affected by the solution dynamics changing their nature and their transport. Therefore, they suggested to take into account the gravitational setting of every cell culture experiments, to assume the particle concentration in the target site (40).

Considering the experimental setting, the scientist must carefully choose and then validate the method used to measure the cytotoxicity. Indeed, the toxicity tests that measure the cell viability were developed for chemicals and could be not suitable for NPs or need to be adapted. Many studies in the literature report on the NPs interference with the cytotoxicity tests, mainly ascribable to the NPs interaction with both the reagent of the assay and the detection method. For instance, numerous viability assays (WST-1-MTT-XTT) rely on the cellular reduction of the tetrazolium salts to their formazan product, whose absorbance is correlated to the cell viability. This conversion is performed by cellular enzymes, but could also be elicited by the NPs themselves or by their bioproducts that act as reducing agents (41). In particular for ZnO NPs, the zinc ions released in the cell culture medium after the NPs dissolution could react with the test, giving rise to false positive results. In addition, due to their semiconductor properties, the ZnO NPs are able to absorb certain wavelengths, influencing the correct interpretation of the results based on the absorbance quantitation (42).

Therefore, to avoid erroneous interpretations, scientists must test the effect of the NPs in cell free conditions and correct the light absorption subtracting the NPs background (43).

Fig. 2 shows a scheme of the main cell related characteristics influencing the ZnO NPs cytotoxicity, like the effects of the cell culture conditions (cell density and gravitational setting).

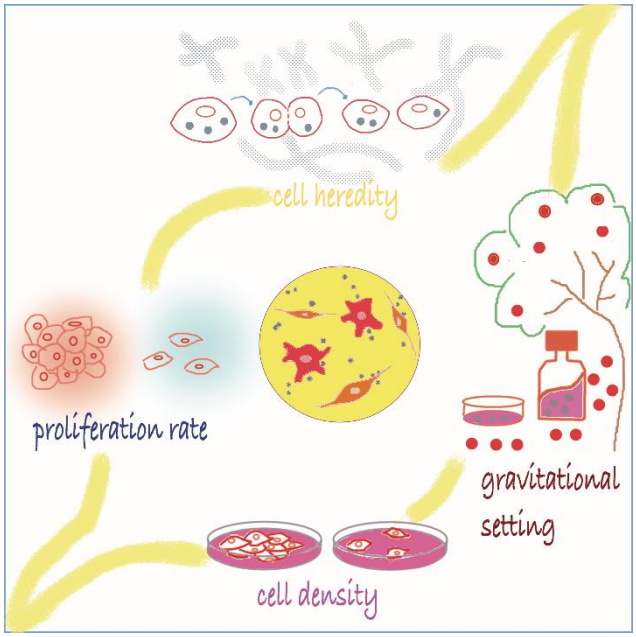

Figure 2 Scheme of the main cell related characteristics influencing the ZnO NPs cytotoxicity.

Table 2 Lessons and warnings obtained from the literature about the cell characteristics.

LESSONS FROM THE LITERATURE:
• **The kind of cells affect the NPs uptake related to the toxicity behaviour.** • **The cell cycle phase does not affect the NPs uptake.** • **Rapidly dividing cells are more sensitive to the ZnO NPs treatment, either healthy or cancer cells.** • **The ZnO NPs toxicity is influenced by the cell density, the NPs volume, and the gravitational setting.**
WARNINGS:
• **Carefully consider the kind of cell line used in the ZnO NPs cytotoxicity and uptake experiments.** • **Try to homologate as much as possible the culture conditions used in the different experiments to avoid results influenced by the experimental setting (i.e. cell confluence, cell density, gravitational setting,…).**

ZnO NPs interaction with biological fluids

Either working *in vitro* using cell cultures or performing *in vivo* tests, the scientists must have to consider the ZnO NPs interaction with the biological fluids. In *in vitro* settings, these fluids are the solutions used for the cell cultures handling and maintenance, namely the cell culture media supplemented with the factors needed for the growth sustainment, specific for the different cell lines used (serum, antibiotics, additional aminoacids, growth factors). In in vivo experiments, the fluids encountered by the NPs depend on the administration route, for instance, the intravenously injected NPs contact the blood first, while the orally administered NPs have to first face the saliva, followed by the gastrointestinal fluid. In addition, in both *in vitro* and *in vivo* settings, once the ZnO NPs are internalized by the cells, they encounter the intracellular fluids. They can vary depending on the internalization mechanisms, like the cytoplasm for the NPs internalized by passive diffusion and the endosomal and lysosomal fluids for the endocytosed NPs.

The importance of considering these parameters relies on the fact that the NPs interaction with the components of the biological fluids could dramatically change their physicochemical properties, which are in turn critical for the determination of the NP biological behaviour. Actually, the NPs could acquire in these fluids new characteristics different from the original ones, resulting in a new NP biological identity and different biological responses.

In order to predict, at least in part, this new NP behaviour, it is of high priority to understand what physically happens when NPs come in contact with the biological solutions.

The DLVO (Derjaguin, Landau, Vervey, and Overbeek) theory analyzed the forces that act on NPs in solution to predict the NPs stability in the colloidal system (44). This stability is regulated by the balance between intermolecular and surface forces. In particular, the attractive Van der Waals forces and the repulsive forces, due to the electrostatic double layer that forms around particles in solution, are considered. The formation and prevalence of one of these different forces determines the NPs behaviour in the solution. This behaviour not only depends on particle properties, but is also strongly influenced by the composition of the solution in which NPs are immersed.

The biological media used for the *in vitro* tests are usually formulated to resemble the blood composition. They are complex systems, made of aminoacids, ionic salts and especially proteins that potentially interact with the NPs surface. The behaviour of ZnO NPs in the most common cell culture medium was investigated in many articles that reported three main effects due to the interaction with the media components: the ZnO NPs aggregation, the ZnO NPs dissolution and the formation of a particular layer named “protein corona” around the ZnO NPs surface (Fig. 3). These three phenomena are often co-existent in the biological solutions, driving together to the NPs biological response. Here these effects are analyzed separately, for clarity purposes:

ZnO NPs aggregation

Many authors observe aggregation and even flocculation of the ZnO NPs when immersed in the cell culture media. In fact, in these complex media, the repulsive forces due to the electrical double layer surrounding the NPs are not sufficient for their colloidal stabilization. As reported by Moore et al, biological media are characterized by a high ionic strength due to their rich ions composition, that compresses the NPs electrostatic double layer, leading to aggregation due to Van der Walls forces.

The formation of aggregates of micrometrical size was reported for ZnO NPs immersed in cell culture media (45) but also in other biological fluids resembling those encountered by the NPs in the biological systems. For instance, the ZnO NPs behaviour was investigated in saliva (46), in the SBF, a simulated body fluid mimicking the inorganic composition of the human plasma (45), in the Gamble's solution, that simulates the interstitial fluid in the lungs (47) and also in the aqueous environment (48) for the evaluation of the ZnO NPs environmental toxicological impact.

The way by which these investigations were performed are different. The colloidal behaviour was investigated checking the formation of aggregates through the measurement of the NPs' hydrodynamic diameter in solution (by Dynamic Light Scattering, DLS, and Nanoparticle Tracking Analyses, NTA), screening the NPs morphology after their immersion in fluids (electron microscopy analysis) and assessing the NPs colloidal stability measuring the surface z-potential values in the related solutions. All these analyses allow the scientists to check the NPs aggregation extent in the different conditions.

But, why is the aggregation so important for the cytotoxicity studies?

As already mentioned, the aggregation changes the physicochemical properties of the NPs involved in their toxicity behaviour. First of all, the NPs size. Indeed, as discussed in the first paragraph, the NPs size strongly influences their cytotoxicity. Therefore, when investigating the effect of NPs in contact with biological fluids, a check of their original dimension after the synthesis process is not enough for the evaluation of their cytotoxic effect. It is in contrast mandatory the investigation of their real dimension after the NPs release (at a specific concentration) into these fluids, as the NPs will unlikely remain in their original form. As demonstration of that, Tripathy et al. investigated the cytotoxic effect of differently aggregated NPs in a murine macrophage cell line. They demonstrated that different concentrations of ZnO NPs induced the formation of aggregates with different sizes, by which the smallest ones were able to induce the most cytotoxic biological response (49).

In Fig3b Dumontel et al. compare the size of ZnO NPs in ethanol and water with those in biological fluids (a simulated body fluid and a common cell culture medium), demonstrating a strong increase in NPs size and thus aggregation in the biological fluids. In addition, the size of the ZnO aggregates was demonstrated to influence their cellular uptake, thus ultimately affecting their cytotoxic response. Condello et al. analyzed the ZnO NPs toxicity in human colon carcinoma cells, tracking the NPs uptake and intracellular path. They demonstrated that the extent of NPs agglomeration determined the route of NPs entry in the cell, with the smallest NPs entering by passive diffusion and the biggest ones being endocytosed. Moreover, they demonstrated that these different routes of entry affected the NPs fate inside the cells, producing different cytotoxic effects (50).

The extent of aggregation is also involved in the NPs biodistribution both in the *in vitro* and *in vivo* settings. The agglomeration and sedimentation of NPs could decrease the effective NPs available for the cells *in vitro*, thus modifying the real dose to which the cells are exposed. In fact, huge aggregates could be difficultly internalized by the cells and the sedimentation in the cell culture could modify the number of NPs/cell interactions.

In support of this idea, Wingett et al. demonstrated that the ZnO NPs cytotoxicity inversely correlates to different parameters, including the propensity for aggregation. They also suggested that the presence of intact nanoparticles, that do not sediment at the cell surface, could be a higher determinant for the cytotoxic effect than their uptake in the cellular model. (51). In contrast, Cho et al. reported that the sedimentation capacity could instead promote the cellular uptake, as demonstrated by these authors for gold nanoparticles (52). Therefore, independently from the different results obtained by the various laboratories, the aggregation and also the sedimentation must be taken in consideration in the cytotoxicity of *in vitro* experiments. Moreover, this consideration is fundamental for the prediction of the NPs biological fate in the *in vivo* setting. Indeed, as reported in the first paragraph, the size of the NPs is a critical determinant for their biodistribution, affecting the blood circulation time and clearance. For this reason, a precise control of NPs aggregation in the biological system is required, in order to potentially prevent undesired side effects. To this purpose, different mathematical models were developed by the scientists to predict the ZnO NPs behaviour in the *in vivo* settings due to their biological interactions. In particular, Tailor et al. proposed an interesting modelling approach, that analyzes the change of the ZnO NPs pysico-chemical properties during the different treatment stages. Combining multiple theories and models with experimental data, they were able to estimate the ZnO NPs behaviour in the different biological environments, taking in consideration the changes in nanoparticle size, z-potential and surface charge density, as crucial parameters in NPs interactions (53). Therefore, with their work, they demonstrated the ability of the ZnO NPs to modify their physico-chemical properties in response to the different biological contexts, underlying the importance to consider these changes in the nanomedical field.

ZnO NPs dissolution

As mentioned in the first paragraph, is essential taking into account the role of the ZnO NPs dissolution in the cytotoxicity studies, because the dissolution of NPs and release of zinc ions is reported as one of the main ZnO NPs cytotoxicity mechanisms. Indeed, even if zinc is an essential element for the human health, the level of free zinc ions in the cytoplasm of the cells are kept really low, in the order of picomolar

concentrations, and tightly regulated by many homeostatic mechanisms. Therefore, an unbalanced amount of zinc ions inside the cells, induce by the NPs dissolution, results in many cytotoxic effects.

The dissolution of ZnO NPs is demonstrated to be highly dependent from the pH, that greatly varies in the different cellular structures. In particular, a high intracellular ZnO NPs dissolution is observed when the ZnO NPs are located inside the lysosome, following the endocytosis process. In this cellular organelle, protonic pumps produce an acid pH of around 4, that induces a strong NPs dissolution and zinc ions release inside the cells, producing detrimental effects for the cell viability.

Moreover, even if not agreed by the all scientific community, many evidences in the literature demonstrated a determinant role in cytotoxicity of the ZnO NPs extracellular dissolution. Actually, many studies investigated the dissolution of ZnO NPs in different types of cell culture media used for *in vitro* toxicity tests. The results demonstrated the different extents of ZnO NPs dissolution depending on the cell culture composition, pH and temperature (54,55). In addition, an enhanced release of zinc ions was observed when buffers are added to the cell culture media. For instance, Eixenberger et al. demonstrated that the addition of HEPES to the RPMI cell culture medium produced an increase in the dissolution kinetics of the ZnO NPs, as illustrated by the ICP/MS measurements in Fig.1c. This increase in zinc ions significantly decreased the cell viability, (56).

Following the dissolution in the cell culture media, the zinc ions could enter the cells or interact with solution components, leading to the formation of insoluble metal hydroxides and salts. In particular, zinc ions interact with phosphate and carbonate ions present in relatively high concentrations in the common cell culture media, originating different types of insoluble phosphate species. As reported by Turney et al., other than reducing the availability of the potential cytotoxic zinc ions, the formation of these species adds a layer of complexity to the cytotoxicity studies. In fact, these kinds of newly formed NPs agglomerates, in the range of the 30-60 nm, could be able themselves to elicit a cytotoxic response (57).

The presence and level of the zinc ions in the extracellular solutions were detected by the authors through different techniques, i.e. using fluorescent zinc probes, or the ICP/MS, UV/vis and fluorescence spectroscopy analyses. Furthermore, many efforts were devoted to prove the zinc ions role in the ZnO NPs cytotoxicity behaviour. For instance, the effects on the cell viability due to the extracellular zinc ions were demonstrated comparing the ZnO NPs cytotoxicity with those of an equimolar amount of zinc, usually added in culture as inorganic salt, i.e. $ZnCl_2$ (58), or comparing the cytotoxicity of the ZnO NPs' suspensions with those of their supernatants (29,59). Some authors also used an indirect correlation, by testing the inhibition of the ZnO NPs toxicity after the addition in the cell cultures of zinc chelating molecules (60).

Due to the demonstrated relationship between the ZnO NPs dissolution and their cytotoxicity, other than a deep understanding of the ZnO NPs dissolution kinetics in the different biological fluids, a control of the *in vivo* NPs release of zinc ions is fundamental for determining the desired toxicity, avoiding undesirable effects. Indeed, as for the aggregation, pharmacokinetics study about ZnO NPs devoted many attentions to their dissolution, studying their biodistribution and elimination patterns in the *in vivo* setting. Actually, the ability of ZnO NPs to biodegrade in natural zinc ions was considered a promising feature for their use in biomedical application (61) and *in vivo* investigations will be needed to explore this intriguing characteristic. In addition, the rapid dissolution of the ZnO NPs in the acid environment was suggested by Sasidharan at al. as an exploiting feature for their preferential killing of the tumor tissues, characterized by an acidic microenvironment(62). Considering all of these facts, the ZnO NPs dissolution not only confers an intrinsic cytotoxicity to these NPs, but also interesting biodegradability features and an exploitable targeting selectivity.

Protein corona

The term protein corona indicates the layer made of different biological species that passivate the NPs surface when in contact with biological fluids.

The kind of species constituting the corona can be various, comprehending not only proteins but also lipids, sugars and other biological components. Despite that, the name "protein corona" is due to the fact that proteins are the most abundant species in these fluids, in particular the albumin, that is the most common protein found both in blood as well as in the bovine serum typically added to the cell culture media.

The formation of the corona depends on several factors both intrinsic to the NPs, like size and surface charge, or due to the exposure media composition. It is a dynamic structure that evolves during time and once encountering different bio-interfaces. The adsorption is governed by NP-protein and protein-protein interactions. The kind and amount of proteins in the corona do not compulsory reflect the composition and relative concentrations of the exposure media. Regarding the corona formation, it seems that immediately upon the exposure to the fluids, the most abundant proteins decorate the nanoparticles surface. These proteins are then gradually displaced and substituted by the higher affinity proteins, that irreversibly bind on NPs forming the "hard corona". Low affinity proteins adsorb then on these layers, forming a second layer called "soft corona". Fig.3d reports the corona composition of four various polymeric functionalized NPs, demonstrating differences in abundance and kind of proteins for each NPs.

As reported by Xiang Lu et al, an in-depth comprehension of the effects induced by the protein corona formation is a key point for the translation of nanomedicines. In fact, the protein corona is demonstrated able to affect the NPs behaviour, modifying the interaction with the biological systems and, for this reason, a critical parameter to explore in the NPs cytotoxicity assessment (63). As affirmed by Vo Van Giau et al, the adsorption of proteins on the NPs surface could modify their hydrodynamic size, shape, charge, and interfacial characteristics, conferring to NPs a new biological identity, thus critically affecting the NPs toxicity (64). For instance, the protein corona could change the way the NPs interact with the cells, masking some targeting ligands or

conferring NPs new targeting abilities, modifying in this way the NPs cellular uptake. In addition, the presence of this layer around the NPs surface could affect the NPs pharmacokinetics, resulting in different circulation time and biodistribution (63),(65). Moreover, it must be taken into account that the NPs-protein interactions change not only the surface's characteristics of the NPs, but also those of the adsorbed proteins. This effect could result in protein conformational changes, leading to protein fibrillation, loss of enzymatic activity, protein crowding and other detrimental effects (30).

Vo Van Giau et al. pointed out how the study of the protein corona is useful and can confer benefits for both *in vitro* approaches and therapeutic applications. For these reasons, in their study, they analyzed the formation of the protein corona around the surface of ZnO NPs following the incubation in a commonly used cell culture media. They did not only identify the different proteins belonging to the protein layer, but they also tried to give them a biological interpretation, predicting their properties in the *in vivo* and *in vitro* systems through the use of a Cytoscape plug in. Similarly to these authors, many other scientists investigated the effects of the protein corona on the ZnO NP cytotoxicity behaviour. Hsiao et al, underlined the importance of the choice between serum-free or serum-containing media in the *in vitro* tests with ZnO NPs, recommending the use of a serum-containing medium for the evaluation of NPs toxicity. Actually, the same authors analyzed the effects of the serum addition on different events connected to the ZnO NPs cytotoxicity, like the aggregation and sedimentation, the release of zinc ions and the rate of cell growth, determining in this way the effects on the protein corona formation on ZnO NPs cytotoxicity behaviour.

Independently from the specific obtained results, whose description is not the aim of this review, all these authors showed a remarkable critical and careful approach, providing good examples for the studies aimed to assess how ZnO NPs interactions with the biological fluids affect their cytotoxicity behaviour.

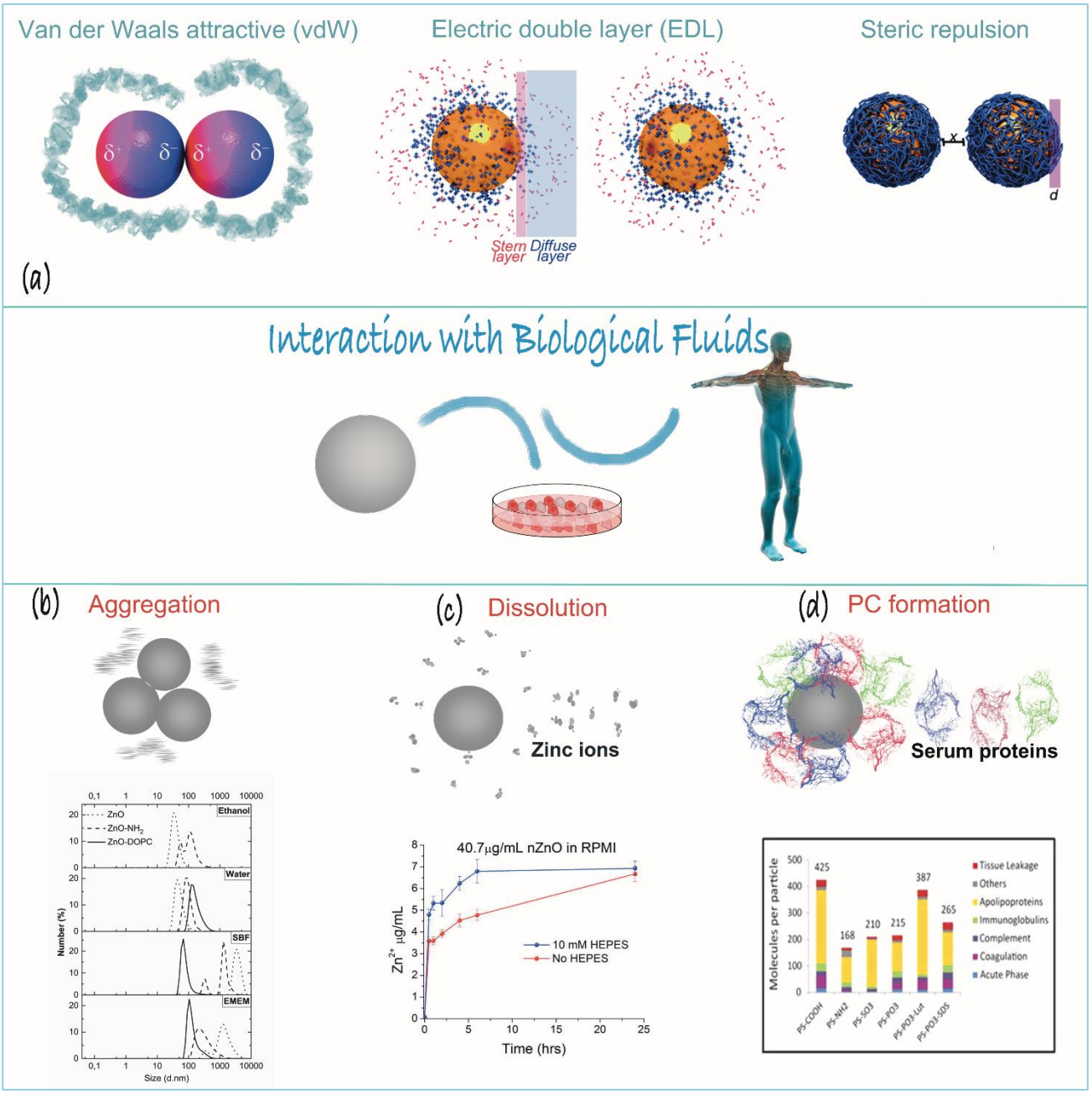

Figure 3 Scheme of the NPs interaction with biological fluids. **(a)** The various interactions of the colloidal NPs in solution. Adapted under a Creative Common Licence CC-BY 2.0. Copyright 2020. From (23); (**b**) Scheme of ZnO NPs aggregation and Dynamic Light Scattering measurements of different ZnO NPs formulations in ethanol, water, simulated biological fluid (SBF) and Minimal Essential Eagle's medium (EMEM). Adapted under a Creative Common Licence CC-BY 3.0. Copyright 2020. From (24); (**c**) Scheme of ZnO NPs dissolution and ICP of the amount of free zinc ions from nZnO present in RPMI-1640 media with and without 10mM HEPES over 24 h. Adapted with permission from (33). Copyright 2020. American Chemical Society; (**d**) Scheme of the formation of the protein corona and an example of LC-MS analysis of the corona proteins. Adapted with permission from Sandra Ritz et al., Biomacromolecules 2015,16,4,1311-1321. Copyright 2020 American Chemical Society.

Table 3 Lessons and warnings obtained about the interactions with biological fluids.

LESSONS FROM THE LITERATURE:
• **The NPs interaction with biological fluids induces the NPs aggregation, dissolution and formation of the protein corona, all phenomena affecting the NPs cytotoxicity behaviour.** • **The new sizes resulting from NPs aggregation affect the NPs interaction with cells, modifying the NPs cytotoxicity.** • **The ZnO NPs dissolution is one of the main determinant factor of the ZnO NPs cytotoxicity, taking place in both the intracellular and extracellular setting.** • **The formation of the protein corona around the NPs surface in biological fluids dramatically changes the characteristics of both NPs and adsorbed proteins, affecting their biological behaviour.**
WARNINGS:
• **For the evaluation of the NPs cytotoxicity, try to investigate the effects due to the real dimension of NPs following the aggregation in biological fluids and take into account the sedimentation phenomena (toxic effects related to the size, promoted or inhibited interactions, modification of the NPs dosage).** • **Analyze both the extracellular and intracellular zinc ions contribution to the toxic effects for a complete evaluation of ZnO NPs cytotoxicity.** • **Carefully consider the use of serum free or serum-containing cell culture media in the cytotoxicity tests.** • **Try to predict the effect to the NPs interaction with the biological fluids on the pharmacokinetics and biodistribution in the *in vivo* setting.**

ZnO NPs surface modifications

When starting the literature research about the ZnO NPs cytotoxicity, scientists have to face with a plethora of articles about ZnO NPs. These NPs, not only differ in their physico-chemical properties, resulting by the synthesis procedure, but also in surface modifications conferred by the authors to modulate the NPs behaviour. Considering the importance of the NPs surface chemistry on their cytotoxicity, it is fundamental to look at these surface modifications for a correct evaluation of the results obtained by the cytotoxicity tests. Indeed, specifically for ZnO NPs, the effects on the cytotoxicity behaviour produced by the surface modifications can be summarized in three main ones: (i) the increase/decrease of the NPs surface area able to interact with the cell membrane, namely the modification of the NPs surface reactivity and interaction with cells, (ii) the change of the NPs colloidal behaviour in terms of aggregation and interaction with the biological components, (iii) the modification of the dissolution kinetics and consequent release of zinc ions.

Here, these effects are schematized separately, but it is important taking into account that a single kind of surface modification is able to induce more than one effect, and that they often co-exist in the biological setting.

Many examples are reported in the literature, in which the authors faced with these biological effects, trying to elucidate the mechanisms by which the specific surface modifications act. In addition, as analyzed in the last part of this paragraph, the surface modifications are often introduced by the scientists for modulating the NPs cytotoxicity behaviour, according to their different purposes.

Starting from the analysis of these biological effects, Yin et al. investigated the effects of the surface chemistry on different aspects of the ZnO NPs toxicity behaviour, suggesting that it is possible to obtain a control over the ZnO NPs cytotoxicity just modifying their surface chemistry. Indeed, in their work, the properties of different NPs surface coatings constituted by oleic acid, poly(methacrylic acid) or components adsorbed from cell culture medium, were demonstrated the main determinants of these NPs toxicity, more than the ZnO chemistry itself. In addition, the same authors investigated different mechanisms connected to the ZnO NPs toxicity, finding a correlation between this toxicity and the ROS production at the NPs surface (66). About this mechanism, the literature well describes how the semiconductor properties of ZnO NPs allow them to directly generate ROS after UV light (67) or ultrasound activation (68). In fact, in the presence of radiation energy of more than 3.3 eV, the promotion of the excited electrons from the valence band to the conduction band leaves holes or unoccupied states able

to generate hydroxyl radicals, and free electrons in the conduction band able to react with oxygen to produce superoxide radicals (30) (7) (69) . However, it was reported that these events can take place even in absence of an external stimulus and the mechanism seems to be related to the structural changes that happen when ZnO assumes nanometric dimensions. Indeed, the shrinkage in particle size creates crystal defects that alter the electronic properties of the particles surface, resulting in an increase of reactive electron-hole pairs able to generate different types of ROS (7). Considering that, Yin et al suggested that the presence of the coatings on the NPs surface decreased the number of active electron donor-acceptor sites, masked by or complexed with the coating molecules, thus diminishing the ROS generation at the NPs surface and the consequent cytotoxicity (66).

The modification of the NPs surface area able to interact with the cell, not only affects the NPs surface reactivity, but also the NPs interaction with cells that drives their internalization mechanisms. In particular, the effect of the surface coating on NPs uptake and cytotoxicity were explored by Luo et al., that functionalized the ZnO NPs surface with the Polyethylene glycol (PEG), a biocompatible polymer highly used in biomedical applications. In fact, the literature reported that NPs surface functionalization with this polymer reduces their aggregation, enhances their hydrophilicity and masks them to the immune systems, producing the so called “stealth effect” (70). In their work, Luo et al. reported a reduced cytotoxicity conferred by PEG to the ZnO NPs and correlated this effect only to a reduced NPs uptake (Fig.4c). Indeed, these authors remembered that the preferential cellular uptake route for NPs is the endocytosis and that this process largely depends on the protein bound to the NPs surface forming the protein corona. Therefore, taking into account their experimental results, they postulated that the reduction of the NPs cytotoxicity was the result of a decreased uptake that possibly arose from a minimal protein corona. The presence of PEG, in fact, inhibited the protein adsorption from the serum, that was instead detected by the authors on the more cytotoxic 3-aminopropyltriethoxysilane (APTES)-functionalized and uncoated ZnO NPs (71). Analyzing the effects of the NPs surface modifications on their cellular internalization, these authors pointed out the already discussed dependence of the NPs uptake on the protein corona formation, underlining the importance of the surface modifications for the interaction with the biological components.

This aspect was explored by Ngaryun et al. that analyzed the modulation of the NPs interaction with the biological environment induced by the surface modifications. In particular, they studied how the modifications of the surface physico-chemical properties affect the NPs behaviour in the *in vivo* setting. They investigated the biological effects and pharmacokinetics of neutron activated ZnO NPs coated with an amorphous silica and injected in Wistar Han rat. Comparing these silica-coating NPs with their uncoated counterparts, they demonstrated that this coating modified the ZnO NPs pharmacokinetic behaviour in circulation, improving their clearance and uptake in the liver. In particular, they demonstrated that this effect was connected to a different protein corona formation around the two kinds of NPs surface (silica coated vs uncoated NPs), underlying the dramatic importance of the surface properties for the ZnO NPs colloidal stability and biological interactions (72).

The silica coating was employed also by Mohankandhasamy et al. to investigate the effect of this surface coating on the NPs toxicological response, especially on the NPs dissolution. Actually, the aim of these authors was to obtain a better control of the ZnO NPs toxicity behaviour, insulating their cytotoxic core with a shell of non-toxic silica, enhancing in this way the NPs biocompatibility and improving their solubility. To better assess the involvement of the coating on NPs toxicity, the effect of two kinds of silica coating, either a thick or a thin one, were explored by the authors. They demonstrated that even less toxic, the thin silica-coated NPs showed a cytotoxicity behaviour similar to the uncoated ones, whereas the thick silica-coated NPs displayed a remarkably less toxicity. The authors explained this less toxicity, demonstrating that the thick coating reduced the NPs dissolution and release of toxic ions, while, at the same time, prevented the direct interaction of the NPs core with the cell membrane, thus limiting the cellular damage. In addition, they investigated the internalization of these different kinds of NPs demonstrating that the NPs coated by the dense layer of silica were highly internalized by the cells and suggested a different intracellular fate of these NPs with respect to the thin silica-coated or the uncoated counterparts (73). Fig.4a shows the ZnO surface functionalization with a silica coating and the reduced cytotoxicity of these ZnO NPs compared to their uncoated counterparts.

From this work it emerges that other than a critical consideration of the cytotoxic effect due to the different surface composition, the intention of the authors is to tailor the ZnO NPs surface to obtain the desired cytotoxicity behaviour. Specifically, for the ZnO NPs, many studies were devoted to modify the ZnO NPs cytotoxicity, controlling other than the surface reactivity, the ZnO NPs dissolution. Indeed, a precise control over the ZnO NPs dissolution allow to selectively kill the cell targets, limiting the side effects related to possible uncontrolled release of zinc ions. This control was obtained by the authors not only making different surface coatings, but also doping the ZnO NPs surface with different atoms, such as iron. In their work, Burk et al. analyzed the toxicity of ZnO NPs doped with different levels of Fe in normal and cancer cell lines. These authors demonstrated that the Fe doping of the ZnO NPs resulted in a reduction of the NPs cytotoxicity and that this doping level inversely correlated with the cell death. In addition, they identified the best concentration and Fe doping level for obtaining a selective toxicity for cancer cell but sparing normal ones, demonstrating that the precise tuning of the NPs properties, other than the choice of the toxic NP concentrations, is fundamental for the design of an effective therapeutic strategy (74).

Another method employed by the authors to obtain the desired NPs toxic response is the modification of the ZnO NPs with biological molecules able to enhance their biocompatibility and confer them a selective targeting ability. The class of biological

molecules widely employed for these aims is the one of phospholipids. These molecules are present in all cells in a high amount, because they are the main constituents of the biological membranes, hence they harbor intrinsic biocompatibility and biodegradability features (75). Dumontel et al. demonstrated that the shielding of the ZnO NPs surface with a lipid coating improved the colloidal stability of these NPs in the biological environment, preventing unwanted NPs aggregation and limiting their dissolution (45). In addition, Ancona et al. demonstrated that the lipid coating improved the ZnO NPs uptake in HeLa cells after 24, 48 and 72 hours of incubation, as reported in Fig.4b (67). Actually, in the last years, the literature reported some works in which the authors tried to modify the surface of the NPs with lipids derived by the membrane of biological structures called extracellular vesicles (EVs). In their works, Susa et al. demonstrated that the coating of ZnO NPs with extracellular vesicles extracted by the cell culture supernatants, improved their colloidal stability, while preserved their cytotoxic potential for cancer cells. In addition, they suggested a possible surface modification of these EVs with targeting molecules, in order to confer to the NPs a precise targeting ability, hence a selective toxicity for the desired cells (76). These works confirmed the important role of the surface modification in the ZnO NPs cytotoxicity, pointing out the importance of considering the biocompatibility and toxicity features not only of the ZnO NPs themselves, but also of the surface modifications adopted.

Fig. 4 shows a scheme of the biological effects produced by three common ZnO NPs surface modifications.

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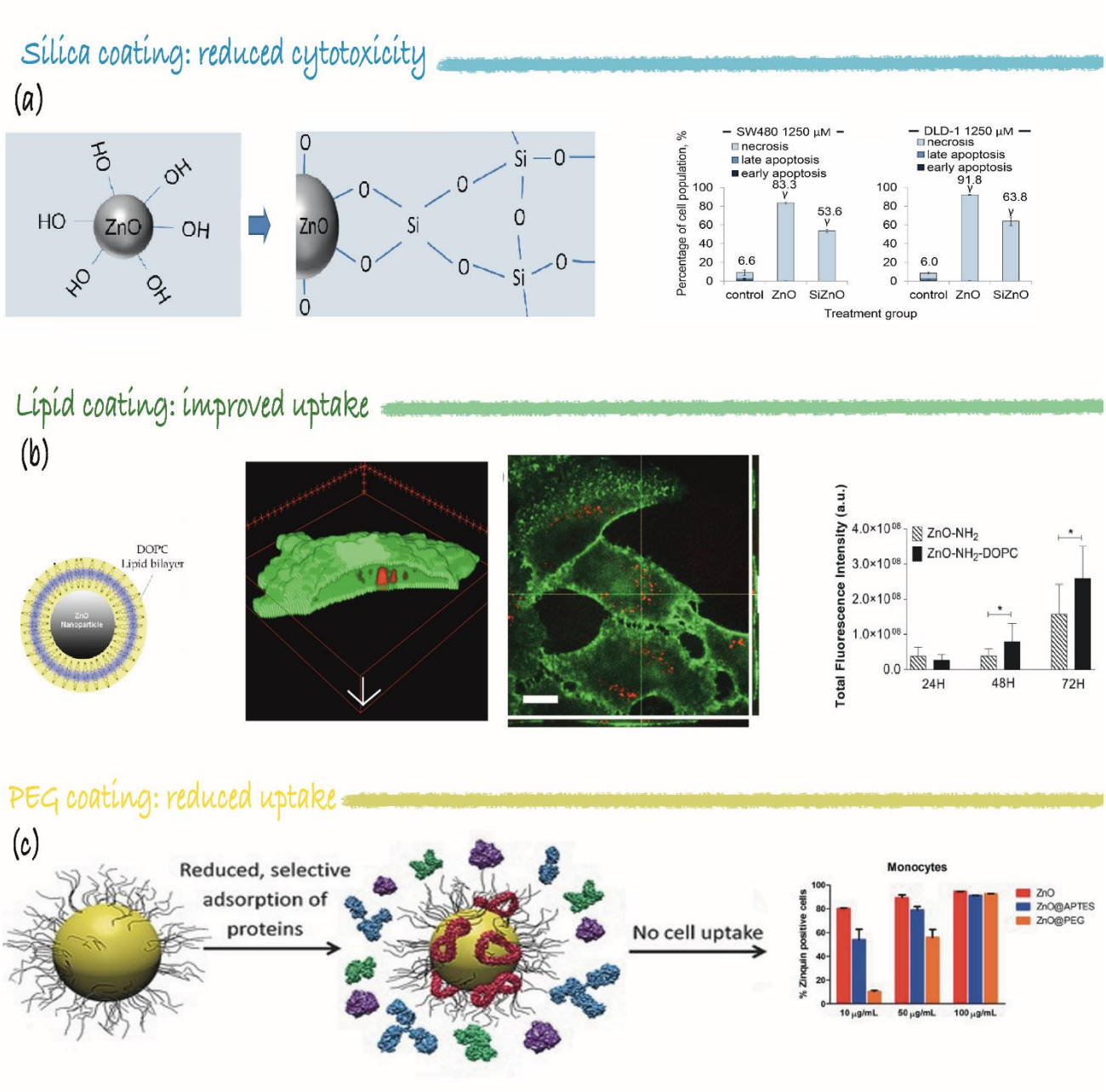

Figure 4 Scheme of the biological effects produced by three common ZnO NPs surface modifications: (**a**) the silica coating (SiZnO) reduced the cytotoxicity of ZnO NPs to colorectal cell lines. Reprinted from "Reducing ZnO nanoparticle toxicity trough silica coating", Vol 2, Sing Ling Chia, David Tai Leong, start page e00177, Copyright 2020, with permission from Elsevier; (**b**) the lipid coating (ZnO-DOPC) improved the ZnO NPs uptake in HeLa cells after 24, 48 and 72 h incubation. Adapted under a Creative Common Licence CC-BY 4.0. Copyright 2020. From Ancona et al. Nanomaterial (Basel) 2018;8:(3):143 doi: 10.3390/nano8030143 and from (24); (**c**) the PEG coating (ZnO@PEG) reduced the uptake of ZnO NPs, illustrated in the graph by the percentages of monocytes containing different levels of zinc ions after 24 h exposure to bare or surface modified ZnO NPs. Republished with permission of Luo et al. from (46); permission conveyed through Copyright Clearance Center, Inc.

Table 4 Lessons and warning obtained about the role played by ZnO NPs surface modifications.

LESSONS FROM THE LITERATURE:
• **The NPs surface modifications critically affect their toxic behaviour, changing the NPs surface reactivity and interactions with the biological systems.** • **The two main mechanisms of ZnO NPs toxicity, namely the ROS formation and the release of zinc ions, are strictly related to the NPs surface properties.** • **It is possible to obtain a more precise control over the ZnO NPs toxicity tailoring their surface properties.**
WARNINGS:
• **Carefully evaluate the role of the surface modifications in the NPs toxicity behaviour, including the toxicity of the modification itself.** • **Consider that the NPs surface modification probably affect more than one aspect of the NPs toxicity behaviour (ion release, ROS generation, cellular interaction and uptake).** • **When introducing a new surface modification, re-establish the cytotoxic effect of the NPs comparing them with the uncoated counterparts.**

Conclusions

ZnO NPs prompted in the last years as promising candidates for biomedical applications, especially for the development of therapeutic strategies in cancer therapy.

However, with the huge number of published studies, many discrepancies in results appeared between the laboratories, that induced the scientific community to deeply investigate the parameters affecting the NPs cytotoxic behaviour. As widely described in this review, the ZnO NPs toxicity is determined not only by the intrinsic NPs characteristics, but also by the external conditions, like the experimental setting. Considering the intrinsic characteristics, the effects of the NPs size and surface charge were deeply investigated by the scientists, that demonstrated their importance for the interaction with the cell and the associated cytotoxicity mechanisms, mainly related to the release of zinc ions and the ROS production. The biological effects were also analyzed both in the *in vitro* and *in vivo* settings, correlating the NPs characteristics with their pharmacokinetic behaviour and biodistribution. However, as stated earlier, from these studies it emerged that the ZnO NPs are not static and unalterable, but instead reactive particles that change when in contact with the environment, in particular the biological one. This discovery modified the ideal image of ZnO NPs, perceived now as dynamic entities that evolve during time in response to the environmental conditions. Actually, ZnO NPs are described by the scientists able to acquire a "biological identity", i.e. a new identity when in contact with the biological environment. Indeed, the interactions with the components of the biological fluids that the NPs encounter both in the *in vitro* and *in vivo* systems, produce a modification of the NPs physico-chemical properties, critical for the determination of the NPs toxicity. As deeply described above, the NPs interaction with the biological fluids, produces three main phenomena, namely the NPs aggregation, dissolution and the formation, at the NPs surface, of the protein corona. These effects, as well as the NPs characteristics, must be carefully taken in consideration by the scientists not only to predict the nanoparticles cytotoxicity behaviour but also to avoid potential undesired effects in both the *in vitro* or *in vivo* settings. However, as mentioned in the previous paragraphs, the NPs cytotoxicity behaviour strongly depends on the biological system, such as the kind of cell line used. For instance, cancerous cell lines were demonstrated more sensitive than their healthy counterparts and this different sensitivity was attributed to a different cell rate of proliferation. Other cell characteristics, like the cell specific internalization mechanism or the cell-related gravitational setting, strongly affect the NPs cytotoxicity, because they influence the way how the NPs interact with the cells and could modify the administered NPs dosage.

All these results, extensively covered in the review, state the importance for the scientists of a careful planning of the NPs cytotoxicity experiments, pointing out the attention on the NPs identity, on the choice of the suitable cell model and on the experimental conditions.

Moreover, the last part of the review describes how the scientists, taking into account all the parameters affecting the NPs behaviour, took advantage of the NPs variability, to produce ZnO NPs with the desired toxic effects. Indeed, this acquired knowledge provided them the ability to modify the NPs characteristics to tailor their cytotoxic response. They obtained different cytotoxic effects just modifying the NPs surface, thus demonstrating the high versatility of the ZnO NPs, useful for the different ZnO NPs applications.

Considering all, it is mandatory that scientists take the results of the cytotoxicity experiments "with a grain of salt", screening the ZnO NPs toxicity with more than one assay and evaluating the robustness of the results, checking the experimental reproducibility also versus time. Furthermore, scientists must take into account that the passage from the in vitro to the in vivo setting could completely change the NPs cytotoxicity behavior, particularly influenced by the biological identity that the NPs acquire in the different environments. Therefore, before the in vivo application, it is important to obtain the most stable and homogeneous ZnO NPs formulation and analyze their behavior in different conditions, in order to reduce the variability associated to this experimental setting.

In conclusion, this review summarizes the most recent scientific works on the ZnO NPs cytotoxicity, because fundamental for the comprehension of the NPs toxicity behaviour. However, even the general statements about the ZnO NPs toxicity produced by the literature are really useful for approaching the cytotoxic experiments, we think that it is fundamental for the ZnO NPs toxicity to be re-evaluated every time, for the specific NPs and experimental setting.

The aim of this statement is not to depreciate the value of the published literature, but instead to remind the importance to share the increasing knowledge about these NPs, thanks to the different scientists' contribution. Indeed, at this point of the research, an extension and a continuous updating are necessary for a complete understanding of the ZnO NPs cytotoxicity mechanisms, as well as of the parameters affecting the NPs cytotoxicity behaviour. Moreover, this review suggests a new and different approach for the study of NPs toxicity, aimed at minimizing the experimental variability and maximize the parameters analysis in order to increase the control on the NPs behaviour, producing reliable and concrete results.

The ZnO NPs are nanoparticles with a huge potential, harboring a completely new cytotoxic behaviour, exploitable for the development of new efficacious therapeutic strategies. An accurate and careful study of the NPs toxicity could make this potential real. Therefore, despite the scientific road is long and full of adversities, the use of this guide aims to shorten the distance and reduce the difficulties that the researches can meet, thus helping the nanoscientists in the choice of the right paths.

Conflicts of interest

"There are no conflicts to declare".

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