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The puzzle of toxicity of gold nanoparticles. The case-study of HeLa cells

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Because of the growing interest of gold nanoparticles in biomedical and biotechnological applications, their toxicity is becoming an increasingly important issue and, in the last few years, there has been continuously expanding research activity in this field. However, due to the intrinsic complexity of the problem, together with the lack in the standardization of the experimental procedures, there is to date a large scattering of the results that still prevents reaching a general consensus of the possible toxic effects of gold nanoparticles in biological systems of increasing complexity (cell membrane, cells, tissues, organs and human body). The strong need to systematize the data suggests employing an appropriate metric, as far as the particle concentration is concerned, that could help in comparing and organizing the available data in a more intelligible scenario. In this note, some recent literature data on the viability of HeLa cells exposed to differently functionalized gold nanoparticles have been analyzed on the basis of a metric based on the numerical particle concentration (number of particles per unit volume of cell culture) that, to a certain extent, takes into account both the size and the shape of nanoparticles. This analysis offers a much more intelligible behavior than the one based on metrics that considers mass concentration (molar concentration) or particle size. At least, in the particular case of HeLa cells, the analysis of the data shows that differently functionalized gold nanoparticles behave similarly and that the different surface coating of the different nanoparticles considered defines the range of particle concentration where toxic effects begin. This kind of analysis could furnish some albeit preliminary suggestions towards an appropriate method to study gold nanoparticle toxicity.

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1. Introduction

In the last few years, an increasing number of scientific reports have appeared with the goal of understanding the interactions between different types of gold nanoparticles and biological systems of increasing degrees of complexity (cells, tissues, organs and whole body) and some excellent reviews have provided a comprehensive analysis of the most recent results.[1–9]

Unfortunately, the results are highly conflicting, in some cases inconsistent, and obtained without a thoughtful protocol, consequently no sure conclusions have emerged so far. This distressing situation is mainly due to two concomitant reasons. The former is inherent to the problem itself, since this rather complex phenomenology is governed by numerous parameters which span from the physical and chemical properties of the particle, cell types, dosing parameters, up to the biochemical assays employed. The latter, in contrast, is due to the overall methodology employed, which requires a standardization in the design and, moreover, in the execution of the experiment that is to date lacking.[10,11]

Nanoparticles [NPs], and in particular gold nanoparticles [AuNPs] due to their unique physicochemical properties, such as enhanced surface area, tunable size and modifiable surface chemistry,[12,13] possess tremendous promise to advance in biomedical applications, at a different level of complexity. However, these same characteristics have made NPs a potential health hazard, thus giving rise to the field of nanotoxicology [NT], which has become a prominent player in toxicological advancement and research over the past decade.

The analysis of the toxicity of AuNPs is difficult and requires a pedantic analysis of the data, conditioned by the apparent controversial results. As a matter of fact, it was shown that toxicity depends on multiple factors which include the inherent physico-chemical properties and the environmental conditions as well. The toxicity of these materials may vary according to numerous parameters[14] like nature, size, roughness, form, aggregation state, coating, *etc.*, making it difficult to provide general and shared results. For example, gold nanoparticles have been found to be *nontoxic* by Connor

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et al.[15] in human leukemia cell lines, or by Shukla *et al.*[16] in immune system cell lines. In contrast to these results, Goodman *et al.*[17] found that gold nanoparticles are *toxic* at certain doses in Cos-1 cells, red blood cells, and bacterial cultures (*Escherichia coli*).

We will illustrate, taking as a case-study a HeLa cell line, how most parts of the toxicological results appearing in the recent literature are largely conflicting, thus preventing the possibility of assessing in a clear and authoritative way the effective toxicity of gold nanoparticles. This confusing position is mainly due to the fact that different results are generated, in part by different experimental procedures, but in large part also by inappropriate analysis of the data.

In this note, we will show that, if a more appropriate analysis of the results is carried out, most parts of data originating from different experiments can be integrated together in a more rational and plausible way, offering an overall more intelligible scenario. A remarkable progress along this direction can be made if gold nanoparticle concentrations are expressed through the numerical concentration, instead of the mass concentration, as usually occurs. In this case, a plausible correlation between the size and numerical concentration, at least in the case of HeLa cell viability, can be found.

This approach should be extended to other cell lines to further enforce this attempt in the light of a unified and standardized behavior, with the aim of a stronger understanding of how to interpret and use epidemiology data in the weight-of-evidence analysis and risk assessments.

Obviously, caution is mandatory since cytotoxicity and cellular uptake, which is ultimately the final effect causing cytotoxicity, are in fact the two linked factors which are jointly controlled by the surface charges of the AuNPs[18,19] and the incorporated particles are surely more harmful than those remaining in the surrounding medium.[20]

2. The HeLa cells: a case-study

HeLa cells (human epithelial carcinoma cell line) have widely been used to investigate the toxicity of different compounds and to explore the rather complex processes involved in interactions with nanoparticles.

Toxicology is intimately connected to the issue of dosimetry and the need for adopting a universal standard, as far as nanoparticle concentration is concerned, has become a forefront topic.

Arriving at a consensus on this issue for the establishment of AuNP exposure limits has been so far critical but any attempt of clarification obliges to move towards this direction.

Dose for nanoparticles *in vitro* can be defined at various levels of specificity.[29]

Particle concentrations can be expressed through the numerical concentration (independently of the particle volume) or through the mass concentration (independently of the number of particles). This choice greatly influences the results and, at least partially, the interpretation of the results, too.

The number N_{NP} of gold nanoparticles in the sample volume V_{sam} [in mL] (numerical concentration) is given by

$$\frac{N_{NP}}{V_{sam}} = \frac{6MM_w}{\pi\rho r^3 10^3}$$

where M is the gold concentration expressed in molarity [mol l^{-1}], M_w the molar mass [g mol^{-1}] and r is the nanoparticle radius expressed in cm and ρ is the density [g cm^{-3}].

The mass M_{NP} of NPs in the sample volume V_{sam} (mass concentration) is given by

$$\frac{M_{NP}}{V_{sam}} = \frac{MM_w}{10^3}$$

In Fig. 1, we show the dependence of N_{NP} and M_{NP} on the gold atom molarity M, for different values of the radius r of the particles.

Both the above concentrations depend on the molarity M. The gold concentration (gold atom molarity M) can be relatively easily measured by atomic adsorption spectroscopy [AAS], inductively coupled plasma atomic emission spectroscopy [ICP-AES], inductively coupled plasma mass spectroscopy [ICP-MS] or, finally, in the case of trace amounts of gold, by neutron activation analysis [NAA]. The numerical concentration can be easily derived from the molar concentration provided that the size and the shape of the particle are known.

However, it must be noted that cells respond to particles that come in contact with them and are subsequently internalized, rather than to particles that remain suspended in the environmental medium over the course of the experiment.[29–31] In any case, a proportional regime can be reasonably conjectured.

To illustrate in real terms the above mentioned situation, we have collected, from the recent literature, a series of data

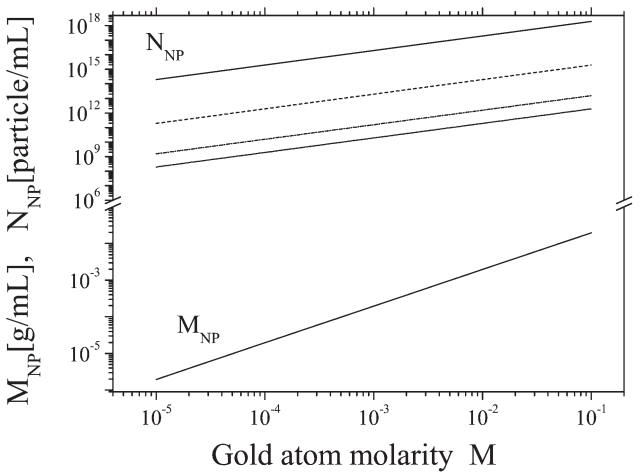

Fig. 1 The mass M_{NP} and the number N_{NP} of gold nanoparticles per unit volume as a function of the gold atom molarity M, for different values of the particle radius r. (full line): 1 nm; (dash line): 10 nm; (dash-dot line): 50 nm; (dotted line): 100 nm.

concerning the viability of HeLa cells incubated with gold nanoparticles under different experimental conditions.

Shapes, size and surface functionalization of gold nanoparticles were widely varied and, moreover, doses, physicochemical properties of the external environment and exposure duration changed from one experiment to another. However, in order to reduce the intrinsic scattering of the data, without influencing the effective particle–cell interaction, we have considered the results obtained only by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay.

In Fig. 2, we have shown some of the results taken from the recent literature on the viability of HeLa cells incubated with differently characterized (and functionalized) gold nanoparticles. The cell viability has been reported as a function of the particle concentration (expressed as molar concentration) for particles of different sizes (upper panel) and as a function of size for particles at different concentrations (bottom panel). In both cases, the exposure times, as well the particle coating and functionalization, vary from one experiment to another.

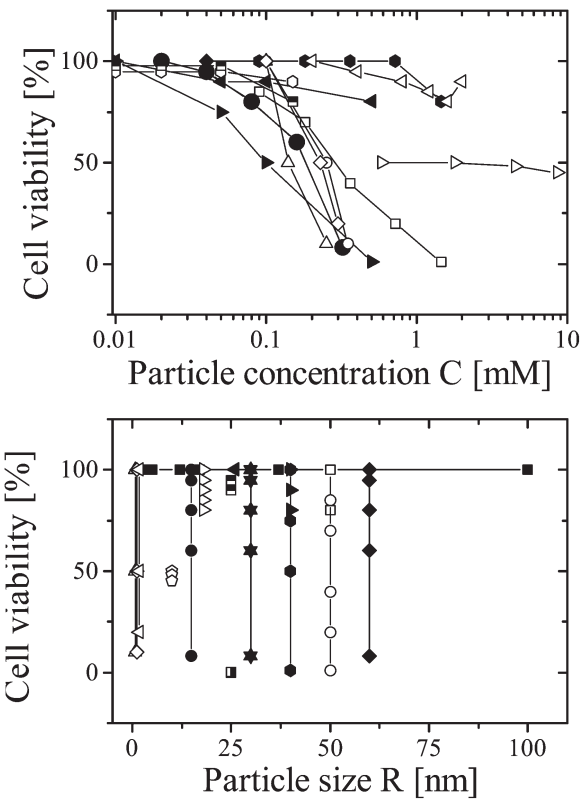

Fig. 2 Upper panel: viability of HeLa cells incubated with gold nanoparticles of different sizes as a function of concentration (expressed as molarity mM). Bottom panel: viability of HeLa cells incubated with gold nanoparticles at different concentrations as a function of size (expressed as [nm]). The particles are differently functionalized. Data taken from references: (black square): ref. 21; (bullet): ref. 22; (black triangle): ref. 22; (black triangle down): ref. 22; (black lozenge): ref. 23; (black triangle left): ref. 24; (black triangle right): ref. 24; (box dot): ref. 25; (square): ref. 25; (big circle): ref. 26; (big triangle up): ref. 26; (big triangle down): ref. 26; (triangle left): ref. 27; (triangle right): ref. 18; (lozenge): ref. 28.

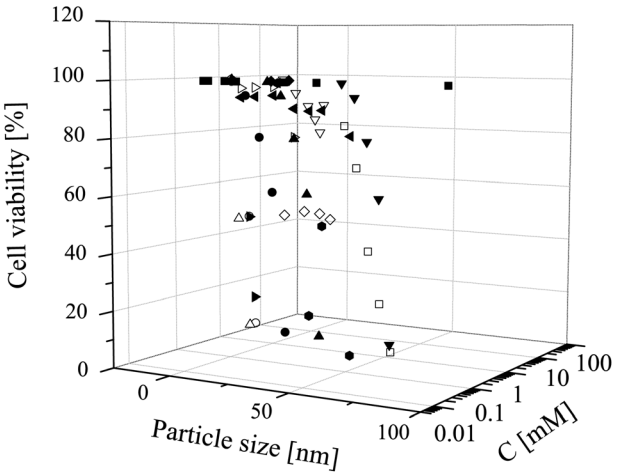

Fig. 3 Viability of HeLa cells incubated with gold nanoparticles of different sizes and at different concentrations (expressed as molarity M). The particles are differently functionalized. Data taken from references: (black square): ref. 21; (bullet): ref. 22; (black triangle): ref. 22; (black triangle down): ref. 22; (black lozenge): ref. 23; (black triangle left): ref. 24; (black triangle right): ref. 24; (box dot): ref. 25; (square): ref. 25; (big circle): ref. 26; (big triangle up): ref. 26; (big triangle down): ref. 26; (triangle left): ref. 27; (triangle right): ref. 18; (lozenge): ref. 28.

The same data are presented in a 3D plot in Fig. 3, where two independent variables have been chosen, *i.e.*, the particle concentration and the particle size. We remark once again that, for each experiment, there are specific parameters not taken into consideration, such as exposure time, that differ in the different experimental situations.

On the basis of these rough data, it is extremely difficult to identify any plausible behavior and one should honestly conclude that a common opinion concerning the toxicity of gold nanoparticle towards HeLa cells, on the basis of these behaviours, has not yet been reached.

However, if the same data are plotted taking into account, as far as the particle concentration is concerned, the numerical particle concentration N_{NP} rather than the molar concentration M_{NP}, this general scenario is greatly simplified. As an example, in Fig. 4, the overall behavior is much more recognizable. An inspection of Fig. 4 suggests that at least three different regions of the numerical concentration can be easily evidenced. The general trend is characterized by the fact that the cell viability, starting from the initial values of 100%, progressively decreases towards lower values with the increase in the number of nanoparticles. This decrease however occurs in different concentration regions, depending on the surface characterization of the nanoparticles (besides, to a lower extent, the exposure time and the nanoparticle shape and size). In the low concentration range, there are nanoparticles coated and stabilized by cationic surfactants (hexadecyl trimethylammoniumbromide [CTAB],[24,25] phoshatidylcholine [PC],[25] quaternary amines such as poly(diallydimethyl ammonium chloride)[18] or by peptides (for example CALNN and its derivatives[22]). In the high concentration range, we find nanoparticles stabilized by triphenylphosphine[26] and in the

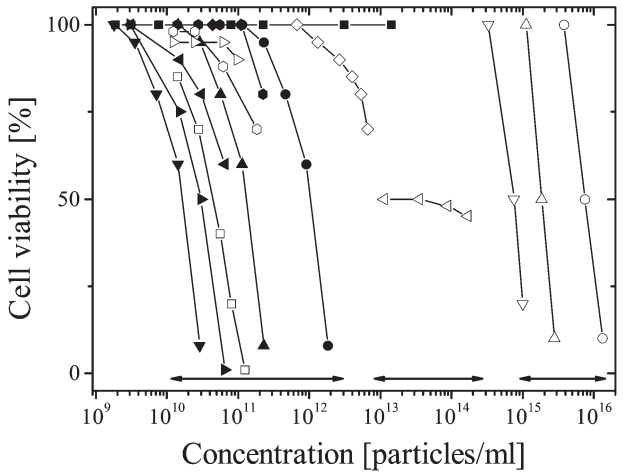

Fig. 4 Viability of HeLa cells incubated with gold nanoparticles of different size and at different concentrations (expressed as nanoparticles per ml). The particles are differently functionalized. Data taken from references: (black square): ref. 21; (bullet): ref. 22; (black triangle): ref. 22; (black triangle down): ref. 22; (black lozenge): ref. 23; (black triangle left): ref. 24; (black triangle right): ref. 24; (box dot): ref. 25; (square): ref. 25; (big circle): ref. 26; (big triangle up): ref. 26; (big triangle down): ref. 26; (triangle left): ref. 27; (triangle right): ref. 18; (lozenge): ref. 28. Three different behaviors, marked by the arrows, can be easily identified, corresponding to different surface nanoparticle functionalization.

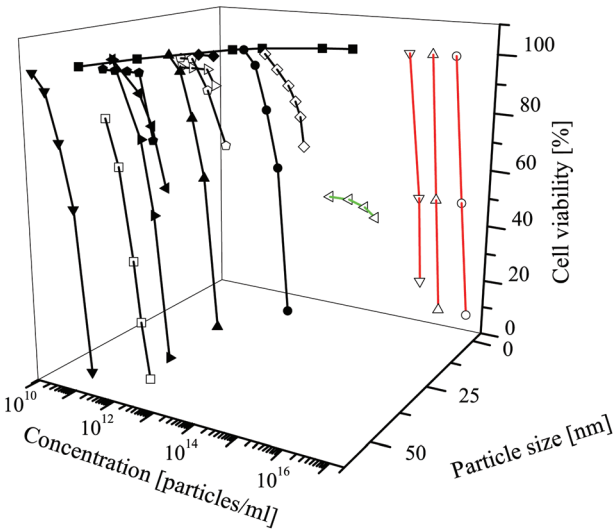

Fig. 5 Viability of HeLa cells incubated with gold nanoparticles of different sizes and at different concentrations (expressed as nanoparticles per ml). The particles are differently functionalized. Data taken from references: (black square): ref. 21; (bullet): ref. 22; (black triangle): ref. 22; (black triangle down): ref. 22; (black lozenge): ref. 23; (black triangle left): ref. 24; (black triangle right): ref. 24; (box dot): ref. 25; (square): ref. 25; (big circle): ref. 26; (big triangle up): ref. 26; (big triangle down): ref. 26; (triangle left): ref. 27; (triangle right): ref. 18; (lozenge): ref. 28.

range in between gold nanowires with surface coated by a monolayer of thiols with carboxyl end groups fall.[27]

The influence of surface coating has been recently well evidenced by Chen *et al.*,[32] who investigated the viability of HeLa cells after incubation with CTAB-coated gold nanorods modified with two successive layers of positively and negatively charged polyelectrolytes. The results clearly show that, contrarily to CTAB-coated nanorods, even at low dosage, the cell viability in the presence of polyelectrolyte-coated nanorods was greatly preserved (about 100%), even at the maximum dosage (200 μM). In this case, the modification of polyelectrolyte multilayers onto the surface of CTAB-coated nanorods makes a dramatic decrease in cytotoxicity.

The same phenomenon occurs in the case of folic-acid protected gold nanoparticles[33] after incubation of HeLa cells for 24 hours, where cellular viability is not significantly influenced. HeLa cells are known to express high levels of folic acid receptors and surface-confined folic acid at the particle surface imparts a full bio-compatibility. The two above stated examples, among many others, evidence that a complete biocompatibility can be assured only in the presence of an appropriate coating.

In Fig. 5, the cell viability is shown in a 3D plot, in analogy with the plot shown in Fig. 3, as a function of the numerical concentration and the size of the nanoparticles.

Data plotted in Fig. 4 and 5 show that the particle size dependence is much less important than the number of the particles per unit volume present in the sample.

It is worth noting that, in some cases, the data do not allow passing from the mass concentration to the numerical concentration or, in the presence of particle aggregation, for example induced by serum, the effective number of particles considered as isolated entities cannot be easily known, being largely overestimated. However, even if with large uncertainty, a more defined trend when the data are plotted as a function of the numerical nanoparticle concentration is observed.

This example visualizes the importance to quantify the administered dose of AuNPs by means of an appropriate metric, the numerical concentration, even if, in the case of heavily coated particles, due to their hybrid properties, the determination of this parameter is not a trivial task.

3. Conclusion

Investigations gathered so far on gold nanoparticle toxicity are currently limited and insufficient to definitely resolve the long-dated controversy (whether toxic or no-toxic).

This initial emphasis is appropriate because few conclusions regarding the comparative toxicity of nanoparticles can be drawn without a clear understanding of the physical and physicochemical characteristics of these materials.

However, the choice of an appropriate metric to characterize the parameters that influence cytotoxicity is fundamental.

We have shown here an example of a predictive toxicological approach evidencing how, in the case of HeLa cells, the number of nanoparticles per unit volume is the relevant parameter over other compositional ones, which allows a better characterization of the toxicological effects of differently functionalized gold nanoparticles.

The possibility of extending and generalizing this metric could have limitations in *in vivo* systems, where interactions involve multiple cell types, tissues and organs and further biological parameters could play a relevant role.

A final comment is in order. In addition to the lethal dosage of nanoparticles (LD_{50}), the effective therapeutic dosage should also be considered (ED_{50}, *i.e.*, the dose required to produce a therapeutic response in 50% of the population). This parameter allows one to use more realistic dosages to assess the toxicity of nanoparticles and would be highly beneficial in reducing the current scattering of the experimental results.[34]

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