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How toxic are gold nanoparticles? the state-of-the-art

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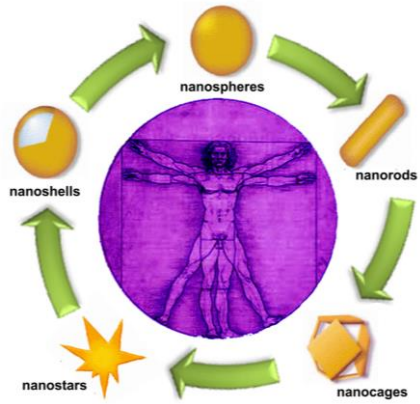

Toxic or beneficial effects of gold nanoparticles on human health depend on their shape, surface charge and functionalization and biological viability. Widely accepted laboratory research protocols are recommended to overcome the spread of controversial results.

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How Toxic are Gold Nanoparticles? The State-of-the-Art

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ABSTRACT

With the growing interest in biotechnological applications of gold nanoparticles and their effects exerted on the body, the possible toxicity is becoming an increasingly important issue. Numerous investigations carried out, in the last few years, under different experimental conditions, following different protocols, have produced in part conflicting results which have leaded to different views about the effective gold nanoparticle safety in human applications.

This work is intended to provide an overview on the most recent experimental results in order to summarize the current state-of-the-art. However, rather than to present a comprehensive review of the available literature in this field, that, among other things, is really huge, we have selected some representative examples of both *in vivo* and *in vitro* investigations, with the aim of offering a scenario from which clearly emerges the need of an urgent and impelling standardization of the experimental protocols. To date, despite the great potential, the safety of gold nanoparticles is highly controversial and important concerns have been raised with the need to be properly addressed. Factors such as shape, size, surface charge,
surface coating and surface functionalization are expected to influence interactions with biological systems at different extents, with different outcomes, as far as gold nanoparticle potentiality in biomedical applications is concerned.

Moreover, despite the continuous attempt to establish a correlation between structure and interactions with biological systems, we are still far from assessing the toxicological profile of gold nanoparticles in an unquestionable manner. This review is intended to provide a contribution in this direction, offering some suggestions in order to reach the systematization of data over the most relevant physico-chemical parameters, which govern and control toxicity, at different cellular and organismal levels.

KEYWORDS

Gold nanoparticles, nanospheres, nanorods, nanocages, nanostars, toxicity

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INTRODUCTION

Gold in its bulk form has long been considered an inert, nontoxic, bio-compatible, noble metal with some

therapeutic (and even medicinal) properties. However, when the size of the typical objects decreases into nanoscopic dimensions (nanometer dimensions), gold behaves very differently than in bulk and its safety, as far as a promising material for biomedical applications is concerned, is no more unquestionable and many important concerns in the risk assessment for humans have been raised. The *a priori* assumption that gold nanoparticles are intrinsically bio-compatible must be rejected.

On the other hand, the term itself of *toxicity* is rather vague. From a theoretical point of view, toxicology is related to the adverse effects that a generic substance exerts on living organisms. In this context, it must be recognized that, if exposure occurs in sufficient quantities, all materials are toxic (this *basic* principle of toxicology was expressed by Paracelsus more than five centuries ago, i.e., many drugs that are beneficial at low doses are toxic at high doses) [1]. Hence, a very important aspect is the identification of the most relevant dosimetry for particle toxicity [2,3].

The basic question to be addressed is: how toxic are gold nanoparticles at the potential concentrations at which they might be used for therapeutics ? At present, even if a considerable number of reports have appeared, the problem remains basically unsolved.

Gold nanoparticles [AuNPs] have been widely used in current medical and biological research, including targeted delivery of drugs [4], optical bio-imaging of cells and tissues [5], imaging and diagnosis of many diseases [6], intravenous contrast agent for imaging and noninvasive detection of lung cancer and many other topics [7] and many labs have tried to investigate, from different points of view, their safety.

Along this line, much experimental work has been done, which confirms the non-toxicity of gold NPs [8,9,10]. However, on the contrary, as much conflicting researches are also present, which revealed the toxicity of gold NPs [11,12,13].

An emblematic example of this confuse and intricate situation can be found in the work of Villiers et al. [14], who analyzed viability of dendritic cells generated from bone marrow, extracted from C57BL/6 mice, after their incubation in the presence of gold NPs (with a mean size of 10 nm and a zeta potential of -13.0 mV at pH 7.4). These results showed that these AuNPs are not cytotoxic, even at high concentrations. However, the analysis of the cells at the intracellular level revealed important amounts of AuNPs amassing in endocytic compartments, where the secretion of cytokines was significantly modified after such internalization, indicating a potential perturbation of the immune response. In other words, these gold nanoparticles, even if not toxic, are not completely bio-inert (and bio-compatible).

Beyond the wide variability of the experimental conditions and the substantial discrepancy of a considerable part of published results, the general opinion is that *naked* AuNPs (i.e., as synthesized) are significantly toxic both *in vitro* and *in vivo*, while appropriate coating may partially prevent their harmful effects [15].

This intriguing scenario is even more confusing if one considers that different factors might influence the potential toxicity of gold nanoparticles and that toxicity is directly related to the five following factors: *i)* surface chemistry, *ii)* coating materials, *iii)* size, *iv)* shape, and *v)* biological target tested [16].

For each of these physico-biochemical parameters, gold nanoparticles offer extraordinary wide possibilities, since, for example, size may vary from few nanometers to some hundreds of nanometers, gold nanoparticles have been found in different shapes (nanospheres, nanorods, nanocages, nanoshells, nanostars) and, finally, the gold surface can be functionalized in a wide variety of ways, due to a straightforward synthesis, where a variety of coating agents can be used, including small molecules such as citrate, surfactants such as cetyltrimethylammonium bromide [CTAB] or polymers and proteins. Recently, polyvinylpyrrolidone [PVP] has been added to this class of molecules, having opened the possibility of

producing safer nanomaterials [17].

Moreover, the kind of effects that nanoparticles may induce must be also considered. Even if we confine ourselves to the cellular toxicity, there are two specific forms of cell death which have received increased attention in relation to cell gold NP exposure [18]. The first of these processes is the controlled cell death (apoptosis). The latter one is a pathological process that occurs in response to externally induced toxicity (necrosis) which can differently influence cell death.

To date, due to the different experimental methods employed, the extraordinary variety of sizes and functionalities of gold nanoparticles, and, finally, the variability of cell lines, there is a lack of general consensus on nanoparticle toxicity. Moreover, a fundamental question exists, whether toxicity arises associated with the chemical functionalization of nanoparticles or simply is due to the decreasing of the particle size, that favors cell internalization. There is a lack of correlation between both fields and there is no clear understanding of intrinsic nanoparticle effects.

Consequently, standardization in experimental set up, such as choice of model (cell lines, animal species), exposure conditions (cell confluence, exposure duration, nanoparticle-concentration ranges and dosing increments) and physico-chemical characterization of AuNPs is necessary, in order to compare different investigations conducted by different researchers in a conclusive and comprehensive way.

At present, the major obstacle is the significant discrepancy in experimental conditions under which toxicity effects (and bio-distribution, too) have been evaluated by individual non-correlated studies, where only few specific parameters have been monitored, without a systematic control of the others, following a well-pondered protocol.

On the contrary, in order to achieve an effective comparison of the different experimental results, it is extremely desirable a standardization of the protocols used, as far as size, shape, purity, intracellular stability and NP surface charge and chemistry are concerned, beside the cell types, which can react quite differently for the same type of nanoparticles.

An interesting progress in this direction comes out from the work by Pompa et al. [19], who proposed a systematic and reproducible evaluation of nanoparticles toxicology in living systems, based on a physical assessment and quantification of the toxic effects of AuNPs by the experimental determination of the key parameters affecting the toxicity outcome. These authors were able to define different regions in the multi-parametric space of toxicity. This approach may pave the way to a systematic classification of nanomaterials, leading to important developments in risk assessment in a wide range of nanomedicine applications.

Numerous excellent reviews on gold nanoparticle toxicity have been published so far [15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30] and in particular an important review dealing with the nanotoxicity evaluation has recently appeared [31]. As pointed out by these authors [31], the assessment of AuNP safety results rather complicated due to a great variety in: *"i) types of AuNPs, ii) stabilizing coating agents, iii) physicochemical parameters of the NPs (diameter, surface charge, surface topography, surface area), iv) incubation conditions (time and concentration), v) type of cells used, vi) type of assay used or vii) possible interference of the NPs with the assay readout"*. We have reproduced here exactly the sentence reported by these authors [31] because it captures in a striking way the core of the problem, but, at the same time, makes it rather difficult to try and define the optimal method to study AuNP cytotoxicity.

In this review, we present a series of rather recent experimental results on the *in vivo* and *in vitro* cytotoxicity of gold nanoparticles of diameter from 1 nm (a cluster of few atoms) to 200 nm, including nanospheres, nanorods, nanoshell, nanocages and nanostars. Rather than offering an exhaustive coverage of the most recent works, our choice has been oriented to evidence how the experimental differences under

which toxicity effects have been evaluated prevent the possibility to reach a general conclusion regarding the effective safety of gold nanoparticles. On the basis of this scenario, there is the strong need of a common protocol and of a predictive paradigm to screen multiple overlapping factors. In light of the these considerations, we have summarized the main physico-chemical parameters associated with gold nanoparticles which influence, on the basis of the data published to date, their toxicity. We have furnished a series of suggestions with the aim of going towards a standardized protocol, yielding a systematic and reproducible assessment of gold nanoparticle toxicology with a precise control of the different parameters which govern this complex phenomenology.

Parameters affecting toxicology evaluation. General considerations.

Before entering the core of the problem, we will examine, under a critical point of view, some of the parameters whose ill definition contributes to conflicting results.

The toxicity of nanoparticles is commonly expressed as the particle concentration causing 50 % of growth inhibition in cell culture (IC_{50}). As far as this parameter is concerned, in order to compare the toxicity of various AuNPs, it should be determined as equimolar doses instead of numerical particle concentration, since nanoparticles tend to aggregate, rendering the particle number concentration practically meaningless. Close attention should moreover be given to the interaction of nanoparticles and biological fluids, which might favor particle aggregation [32,33].

Commonly used media include cell culture medium (with or without serum), phosphate buffered saline [PBS], 0.9% sodium chloride, plasma, and, sometimes, whole blood. Nanoparticles are typically exposed in media containing no serum, or a reduced amount of serum; however, this is sometimes not possible since the cells require certain serum levels to maintain normal viability. Presence of serum greatly favors particle aggregation, making numerical concentration even more meaningless.

Cytotoxicity *in vitro* is studied with various animal cell cultures, most commonly the fibroblast of the human skin (HeLa), human leukemia (K562), human hepatocarcinoma (HepG2), human breast carcinoma (SK-BR-3), and others. Immortalized cell lines like HeLa cells are commonly used to compare the cytotoxicity of nanoparticles varying in size and surface chemistry [34, 35]. Cytotoxicity *in vivo* has been recently reviewed by Johnston et al. [30], who present a detailed analysis of the particle characteristics and of the different mechanisms responsible for the observed toxicity.

Selection of the appropriate cytotoxicity assay is vital to the accurate assessment of nanoparticle toxicity. Various assays can be used to study the toxic effects of nanoparticles on cell cultures, including lactate dehydrogenase (LDH) leakage, 3-(4,5-dimethyl -thiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay and identification of cytokine/chemokine production.

For gold nanoparticle-treated cells, the dead cells were imaged with the commonly used fluorescent propidium iodide [PI]. Normally, the fluorescent PI molecules cannot penetrate the cell membrane. However, in most part of the experiments, the PI molecules entered the cell during the endocytosis of the nanoparticles and resulted in a false-positive toxicity result [36].

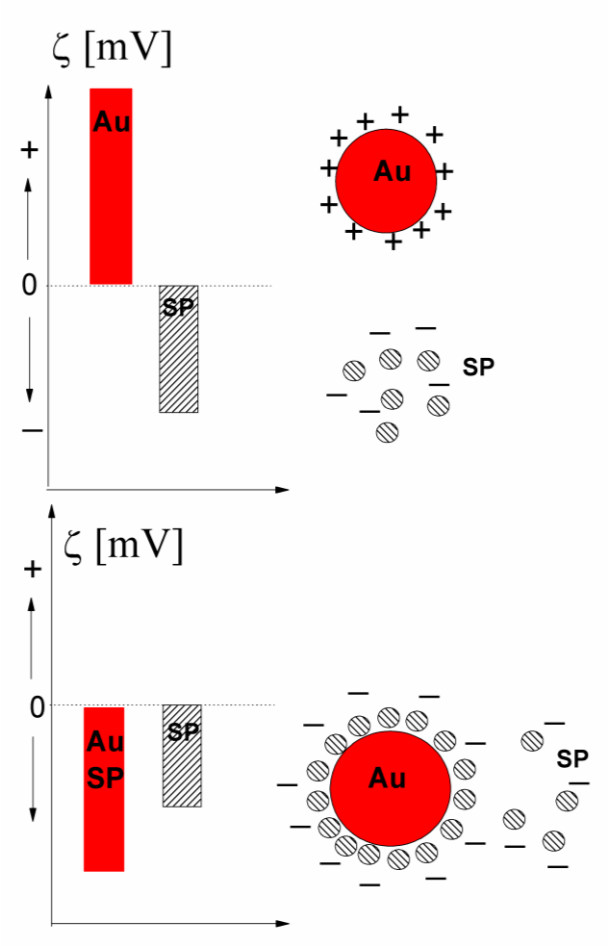

Figure 1: Cartoon demonstrating the formation of protein *corona* on a gold nanoparticle surface. Adsorption of serum proteins [SP] onto the surface of gold nanoparticles flips their effective surface charge from positive value (upper panel) to negative value (bottom panel). On the left, the change in the ζ-potential is shown.

Nanoparticle charge is a key parameter. Cationic nanoparticles are able of binding to negatively charged DNA. When DNA binds to highly positive nanoparticles, it wraps around the nanoparticle and bends. This bending generally causes damage to DNA. Usually, hydrophobic ligands bind to the minor grooves and charged ligands can bind to minor and major grooves where high electrostatic and van der Waals interactions are required [37]. Positively charged nanoparticles, with high enough surface charge densities, may attach to DNA irreversibly [38].

Anionic nanoparticles can be internalized within a cell through endocytotic pathways [39]. One important effect of the particle surface functionalization is the change in the particle charge, since electrostatic interactions influence cellular uptake much stronger than hydrophobic or wan der Waals interactions.

Variability in the data among different assays was found to be the result of interferences such as nanoparticle dye interactions and absorption by the nanoparticles. Different experimental results have indicated that toxicity is highly dependent on the physico-chemical properties of nanoparticles. Moreover, the occurrence of false-positive and false-negative results highlights the importance of cross-checking the data with alternative assays to ensure reliability of the results.

Specifically, particle size is an important parameter that affects the agglomeration, sedimentation and diffusion of nanoparticles and, in turn, the transport of nanoparticles into the cells during toxicity assay. On the other hand, researchers also reported that gold nanospheres were not as cytotoxic as gold nanorods. These findings emphasize the importance of correlating specific size and shape with toxic biological responses.

An aspect often undervalued concerns with the clearance of nanoparticles from the body after that their therapeutic effect is completed. Different studies suggested that nanoparticles might be retained for example in liver and spleen in mice for long period of time or also permanently. For example, Huang et al. [40] and Haimfeld et al. [41] observed the persistence of particles larger than 10 nm in liver and spleen in mice for up to six months with no observed consequences. It is to date unknown if gold nanoparticles completely clear from the body and what undesirable consequences their retention may provoke on long time limit. Some of the most recent aspects for human toxicology have been reviewed by Gerber et al. [42], who, once again, strengthen that the data are still largely limited to predict hazard potential of AuNPs for humans.

In the following, we will summarize the main

results concerning gold nanoparticle cytotoxicity outcoming from some selected rather recent works. We have ordered these effects taking into account the nanoparticle shape.

Gold nanospheres.

The synthesis, the characterization and the functionalization of differently shaped gold nanoparticles have been extensively reviewed by Dreaden et al. [43] and by us [44]. Some particular faces of these techniques have been presented and discussed, in the last few years, in a series of papers from our group [45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56].

An important effect, occurring when the effect of surface charge on toxicity and cellular uptake is considered, is that nanoparticles have a positive effective surface charge upon preparation but they are no longer cationic in the cellular media, since many different plasma proteins adsorb on nanoparticles surface spontaneously, so that the surface chemistry of the nanoparticles in growth media/plasma is not the same as the originally synthesized materials. Instead, the nanoparticles adopt the physico-chemical properties of the adsorbed protein shell: a protein corona [57, 58, 59]. A sketch of this relevant phenomenon is shown in Fig. 1. Although a complete understanding of nanoparticle-protein interaction is lacking [60], the adsorbed protein layer strongly influences cellular uptake and particle biodistribution, ultimately conditioning particle toxicity. The relevance of the protein corona in the biological impacts of nanoparticles *in vivo* and *in vitro* has been discussed by Monopoli et al. [61] in the case of hydrophobic particles (sulfonated polystyrene, $PSOSO_3$) and hydrophilic particles (silica, SiO_2). The general findings and the conclusions can be easily extended to gold nanoparticles.

The effect of spherical gold nanoparticle size on toxicity has been deeply investigated by various authors.

Water-soluble AuNPs stabilized by triphenyl-phosphine derivatives in the range from 0.8 to 15 nm were investigated by Pan et al. [34]. They found that, according to the IC_{50} values in MTT assays, cytotoxicity in different cell lines representing the principal barriers and lining cells of the body (connective tissue fibroblasts [L929], epithelial cells [HeLa], macrophages [J744A1], and melanoma cells [SK-Mel-28]), markedly depended on size. The particle size was varied from 0.8 nm (cluster with eight gold atom) to 1.8 nm (cluster with 150 gold atom). Cytotoxicity was investigated in both actively dividing cells (in the logarithmic growth phase) and quiescent cells (in the stationary phase). Fig. 2, as an example, shows cytotoxicity during the logarithmic growth phase of cell lines.

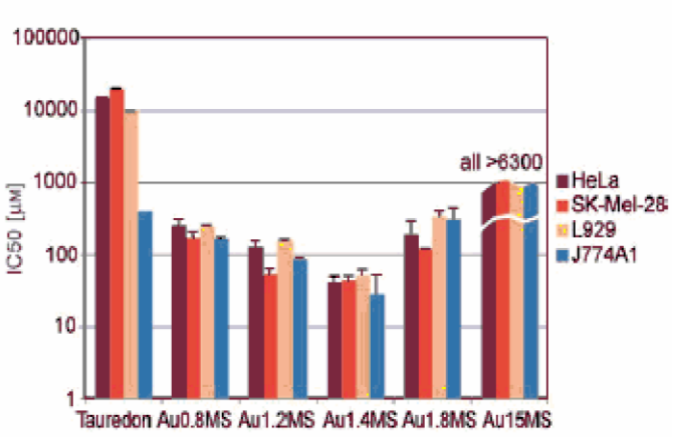

Figure 2: Cytotoxicity of AuNPs (of different sizes, 0.8, 1.2, 1.4, 1.8, 15 nm) during the logarithmic growth phase of four cell lines: Hela cervix carcinoma epithelial cells [HeLa], melanoma cells [SK-Mel 28], mouse fibroblasts [L929] and mouse monocytic/macrophage cells [J774 A1]. The IC_{50} values of AuNPs 1.4 nm in size were lowest across all cell lines and the Au compounds of smaller or larger size were progressively less cytotoxic. Data reproduced with permission from Ref. [34], copyright Informa UK, Ltd, 2012.

Particles 1.4 nm in size resulted the most toxic with IC_{50} values ranging from 30 to 56 μM, while particles of 0.8, 1.2 and 1.8 nm in size are less toxic at up to 60-100 fold higher concentrations. Moreover, 1.4 nm particles led to cell necrosis after 12 hours of incubation. These results suggest a stringent and undubitable size dependency of cytotoxicity, although it remains rather obscure why a particular size produces more toxicity than the others.

One of the stringent examples of the dependence of the toxicity on the gold nanoparticle concentration has been provided by Pernodet et al. [62] who investigated the interaction of fibroblast cells with citrate-coated nanoparticles 1 nm in size at different concentrations, from 0.2 to 0.8 mg/ml. The different accumulation in vacuoles resulted in a damage of the actin fibers, whose density (at the top of the cell) varied from 0.65 μm^{-1} in control to 0.1 μm^{-1} at a particle concentration of 0.8 mg/ml.

As gold is one of the most electronegative metal, it is easily attracted to DNA grooves which have a negative environment. Furthermore, AuNPs of about 1.4 nm diameter almost perfectly match with the size of the major DNA groove, leading to strong potential toxic effects of AuNPs, especially for those in the smaller size range [63].

Even if not directly connected to cytotoxicity, the intracellular uptake of spherical AuNPs depends critically on particle size.

While it is well-established that small size of AuNPs plays a major role in the mechanism of entry into cells, relatively little is known about their health effect in human. Chithrani et al. [64] incubated HeLa cells with citrate gold nanoparticles with various sizes (diameters of 14, 30, 50, 74, and 100 nm) for 6 hours in Dulbecco Minimum Essential Media [DMEM] plus 10% serum and the uptake effectiveness was determined by means of Inductively Coupled Plasma Atomic Emission Spectroscopy [ICP-AES]. The maximum uptake by a cell occurred at a nanoparticle size of 50 nm, with uptake ranging from 500 to 6000 particles per cell, depending on the degree of protein adsorption and the cell line used. In this case, the uptake is mediated by nonspecific adsorption of serum proteins onto the gold surface via the mechanism of receptor-mediated endocytosis.

Connor et al. [7] and later Murphy et al. [65] have examined the uptake and the potential toxicity of a series of gold nanoparticles in human leukemia cells. Gold nanospheres varied in both size (4, 12, and 18 nm diameter) and surface modifiers, including a range of anionic, neutral, and cationic groups: citrate, cysteine, glucose, biotin, and cetyltrimethyl-ammonium bromide [CTAB]. The K562 leukemia cell line was exposed to the nanoparticles for three days and the cell viability was determined using a colorimetric MTT assay. The results (see Fig. 3) suggested that none of the spherical gold nanoparticles were toxic to the human leukemia cells, without no detrimental effects in cell functionality, up to about 150 μM in gold atom concentration, even though they were being taken up into the cells (confirmed by transmission electron microscopy of cell slices)

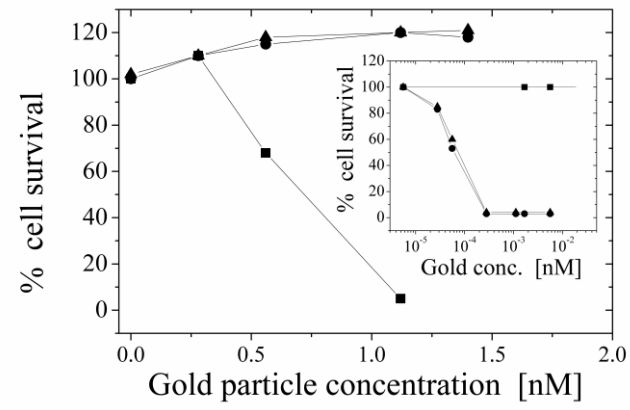

Figure 3: Percentage survival of human K562 cells exposed to differently functionalized, 18 nm in size, gold nanoparticles for three days. Cells exposed to gold nanoparticles containing citrate (▲); cells exposed to gold nanoparticles containing biotin (•); cells exposed to $AuCl_4$ precursor (▪). Inset: cell exposed to gold nanoparticles containing CTAB (▲); cells exposed to CTAB alone (•); cell exposed to gold nanoparticles containing CTAB washed three times prior to incubation (▪). Data replotted with permission from Ref. [7] Copyright

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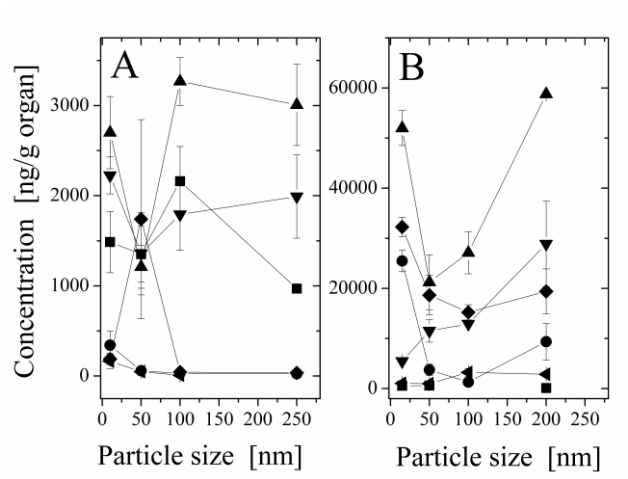

Figure 4: Concentration of gold nanoparticles measured in different rat organs (expressed as ng/g organ): (▪): Blood; (▲): Liver; (▼): Spleen; (♦): Lungs; (●): Kidney; (◄): Heart. Left panel A: Data redrawn from Ref. [66]. Right panel B: Data redrawn with permission from Ref. [67], copyright M.A.K. Abdelhalim and Elsevier 2013 .

The influence of size on the *in vivo* tissue distribution of spherical-shaped gold nanoparticles in rats has been recently investigated by De Jong et al. [66]. Rats were intravenously injected with gold nanoparticles with a diameter of 10, 50, 100 and 250 nm and after 24 h gold nanoparticle concentration was quantitatively measured with inductively coupled plasma mass spectrometry [ICP-MS] methods. Qualitatively similar results have been reported by Sonavane et al. [67] for the tissues and organs of albino mice after 24 h of dose administration. In Fig. 4, we show a cumulative synthesis of De Jong et al. [66] and Sonavane et al. [67] results which, taken together, confirm that accumulation of gold NP in various tissues was found to be depending on particle size. However, it is rather difficult to find a well-defined behavior and the only conclusion we can draw is that relatively small particles (10-15 nm) revealed higher amount in all the tissues including blood, liver, lung, spleen, kidney, heart. Relatively larger particles (200-250 nm) showed very minute presence in organs including blood, brain and spleen.

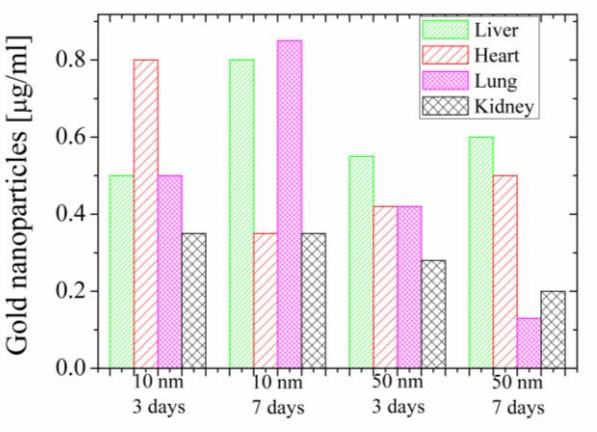

Figure 5: Concentration of gold nanoparticles (10 and 50 nm in size) in different organs of rats after intraperitoneally administration of 50 µl for 3 and 7 days. Data redrawn with permission from Ref. [68] copyright Abdel Halim MAK, 2012.

Even if rather indirectly linked to toxicity, it deserves to be mentioned here the work of Abdel Halim [68], who investigated the accumulation of spherical (and spheroidal) gold nanoparticles in several organs *in vivo* of rats. The level of particle accumulation is considered as an indication of nanoparticle toxicity. The main results are summarized in Fig. 5, where it is evident that more marked toxicity effects are induced by smaller AuNPs, somehow confirming the results of De Jong et al. [66] and Sonavane et al. [67].

The effect of 15 nm citrate capped gold nanoparticles on the model system *Drosophila melanogaster* has been investigated by Pompa et al. [69]. In this animal model, they observed, upon ingestion of 12 µg/g per day, a strong reduction of life span and fertility, the presence of DNA fragmentation as well as a significant over-expression of the stress proteins. This example highlights how nanoparticles introduced into a complex systems, such as a living system is, are able to modify its behavior.

PEGylated nanoparticles are commonly used to lower cytotoxicity. However, PEG can lead to a lower cellular internalization efficiency, then reducing the potential for using gold nanoparticles as therapeutics.

Simpson et al. [70] have suggested that glutathione

may be an attractive alternative to PEG in the design of gold nanoparticle therapeutics. Mice injected with glutathione-coated gold nanoparticles did not experience any clinical signs of illness nor did not cause morbidity (checked through histological analysis) at any concentration over the course of 6-weeks. Therefore, glutathione-coated gold nanoparticles presumably do not cause any toxic effects in passing through the kidneys, contrarily to what observed in a previous tiopronin monolayer protected cluster [TMPC] study at the same concentrations [71].

AuNP stabilization with chitosan has already been reported [72, 73]. Recently, Stefan et al. [74] present a study to evaluate the effects of gold nanoparticles (12 nm 22 nm in size) capped with chitosan on brain and liver tissue reactivity in male Wistar rats, exposed to lipopolysaccharide (LPS from Escherichia coli serotype) upon 8 daily sessions of intraperitoneal administration. Their results demonstrate that smaller size of chitosan-capped AuNPs shows protective effects against LPS-induced toxicity.

A detailed analysis of all these metabolites (Fig. 6) in the serum of animals treated with LPS, as compared to that of the control, showed that LPS induced toxicity, suggesting symptoms of kidney dysfunction, as evidenced by the significant decrease in the levels of urea nitrogen.

Conflicting results could arise from the variability of the used toxicity assays, cell lines, and nanoparticles chemical/physical properties. For example, cytotoxicity results can vary with the used cell line. Citrate-capped gold nanoparticles (13 nm in diameter) were found to be toxic to a human carcinoma lung cell line but not to human liver carcinoma cell line at same dosage [9].

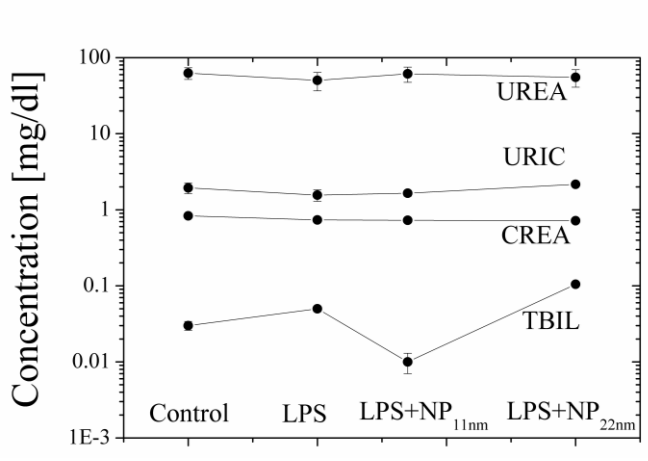

Figure 6: Biochemical parameters in the serum of rats treated with AuNPs (11 nm) and AuNPs (22 nm) after exposure to LPS. Data redrawn with permission from Ref. [74] copyright 2012 Elsevier B.V.

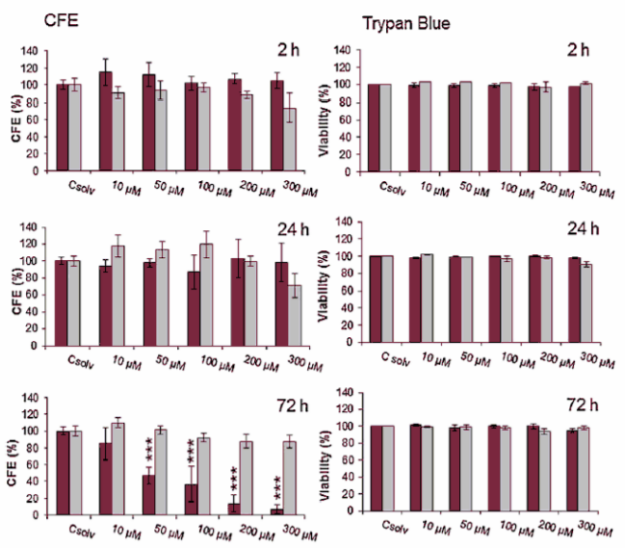

Figure 7: Toxicity of AuNPs 5 nm (red histogram) and 15 nm (gray histogram) in Balb/3T3 cells exposed for 2, 24 and 72 h to increasing concentration of AuNPs (10-300 µM) for two different assays, Colony Forming Efficiency [CFF] (on the left) and Tryptan Blue exclusion test (on the right). AuNPs 5 nm induced cytotoxicity in Balb/3T3 cells at 72 h of exposure at concentration higher than 50 µM. In the range of concentration and time-points tested no cytotoxicity was found in Balb/3T3 cells exposed to AuNPs 15 nm. Data reproduced with permission from Ref. [75] copyright Elsevier 2013.

Another point to be addressed is that *in vitro* and *in*

vivo investigations are the basis of a different methodology. *In vitro*, three-dimensional (3D) cell culture models have been used as a bridge between the *in vitro* two dimensional (2D) plated cell culture and the *in vivo* models [76]. In this context, Lee et al. [77] compared the toxicity of gold nanoparticles in both 2D and 3D cell culture constructs. They used hydrogel inverted colloidal crystals as a cell growth substrate and human hepato-carcinoma cells to construct the 3D cell culture environment. They found that toxicity of both citrate (anionic)- and CTAB (cationic)- capped gold nanoparticles was significantly reduced in the 3D environment compared with 2D one [77]. These results point out that *in vitro* studies alone are not adequate to assess the toxicity of nanoparticles.

The effect of nanoparticles 5 and 15 nm in size and at different concentrations (10-300 μM) on Balb/3T3 mouse fibroblast cells *in vitro* has been investigated by Coradeghini et al. [75]. Cell cytotoxicity was evaluated by Colony Forming Efficiency [CFF] assay [78] and by Tryptan Blue assay. The main results, summarized in Fig. 7, show that toxicity was observed only in the case of AuNPs 5 nm at concentrations higher than 50 μM, at 72 hours exposure time in the case of CFF assay, while no toxicity was observed, even at the highest concentrations (300 μM) and at longest exposure time (72 hours), when Tryptan Blue assay is employed.

The above stated example is emblematic into two different aspects. First, it highlights how particle size plays a relevant role. Although the difference in size of AuNPs employed is very small, the overall biological response is significantly different. Second, the different cytotoxicity results derived from the two different assays can be better understood when combined with ones by different techniques. In this particular case, TEM analysis showed that NPs remained confined in vesicles without entering the nucleus and inductively coupled plasma-mass analysis [ICO-MS] revealed that the total Au content in cells increased in a time-dependent manner. These additional findings justify, at least partially, the different cytotoxicity behaviors these authors [75] observed.

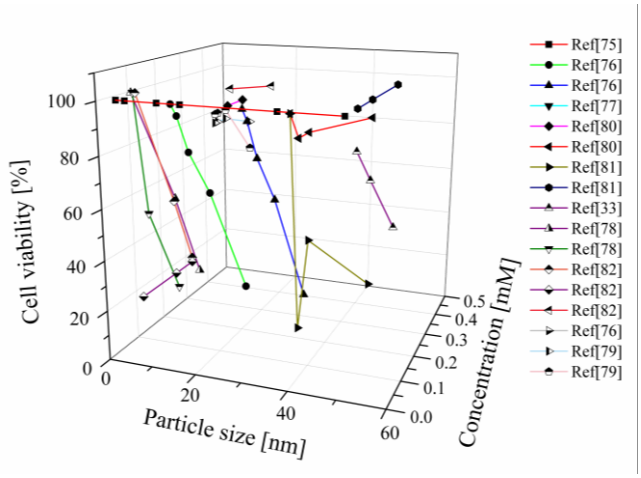

Figure 8: Viability (derived from MTT assay) collected from recent literature data of HeLa cells incubated with gold nanoparticle of different sizes and at different concentrations. Gold nanoparticles are differently functionalized. Data taken from Refs. [79, 80, 81, 34, 82, 83, 84, 85, 86]

No toxicity of either non-functionalized or polyacrylamide-coated gold nanoparticles 18 nm in size was found by Salmaso et al. [87] for human breast adenocarcinoma cells. Likewise, no toxicity was found by Qu et al. [88] for citrate-coated AuNPs, 10 and 50 nm in size, for embryonal fibroblasts up to a concentration relatively high of 300 μM.

From these examples, it comes out that, in order to enhance the understanding of AuNP induced cytotoxicity, there is an urgent need for standardization of the different protocols employed. A typical example that sustains this need is shown in Fig. 8, where different results concerning the viability of Hela cells from the MTT assay, scattered in the recent literature, are collected together. However, it is difficult to organize the available data in a fully intelligible way, because of the many parameters involved, which are different in the different studies. Here, the data are ordered by increasing the size (that is a very important parameter in both cellular internalization efficiency and cytotoxicity) and the concentration of the gold nanoparticles. This choice

implies that each gold nanoparticle formulation is characterized by a different surface functionalization, ranging from *naked* nanoparticles to PEG-coated nanoparticles. As can be seen, the large scattering of the data prevents the possibility to find any reasonable correlation among them, with the exception that larger particles are more toxic than smaller ones.

A further example is reported in Fig. 9, where Patra and Dasgupta [89] summarize the response of cancer cells with respect to the hydrodynamic diameter and zeta potential for a varying class of nanoparticles in terms of percentage cell survival, using the MTT assay. The left panel shows the dependence on AuNP size, and the right panel on the zeta potential. As can be seen, it is evident that the percentage cell survival with AuNPs smaller than and larger than 50 nm in size is comparatively similar, although the abundance of the nanoparticles is higher with small hydrodynamic diameter (Fig. 9, left panel, zones A and C). However, the zeta potential has slightly more linear influence on the cell survivability. The bottom left quadrant (left panel, zone B) has very few points with respect to the bottom right quadrant. This implies that even if the nanoparticles are larger than 50 nm, the nanoparticles can interact with the cells if the zeta potential is in the permissible range.

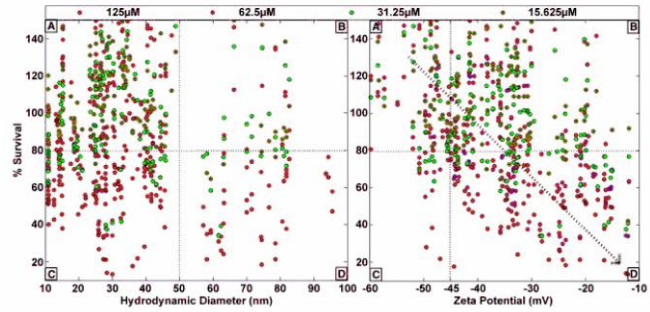

Figure 9: Cell survivability reported by Patra ans Dasgupta [89] summarizing the different influence of size and ζ-potential. Each point represents the mean percentage cell survival of a triplicate experimental set for a given nanoparticle size (left panel) or for a given zeta potential (right panel). The colors representing the corresponding concentration of nanoparticles are indicated at the top of the figure. Reproduced with permission from Ref. [89] copyright elsevier 2012.

This kind of analysis of the different cytotoxic results highlights the importance of the data comparison and is devoted to the possible characterization (from a phenomenological point of view) of the influence that different parameters exert, when nanoparticles interact with cells.

Gold nanorods.

Gold nanoparticles having a rod-like morphology (gold nanorods, AuNRs) are of particular interest because of their anisotropic shape. Due to their non-spherical geometry, these particles have both a transverse and longitudinal plasmon [90]. The absorption profile includes two absorption bands: one due to light absorbed along the short axis (transverse) and the other due to absorption along the long axis (longitudinal). As the rod length increases, so does the longitudinal band red shift together with an increase in the extinction coefficient.

Among the different shapes, rod nanoparticles have been reported to demonstrate more toxicity than their spherical counterparts [21]. However, the mechanism of more toxicity of nanorods compared with spherical nanoparticles is yet to be understood.

An interesting study was conducted by Takahashi et al. [85] who investigated the cytotoxicity of gold nanorods [AuNRs] to HeLa cells after 24 h of incubation. Hexadecyltrimethylammonium bromide [CTAB], a cationic micellar surfactant which is necessary for the preparation of gold nanorods, was substituted by phosphatidylcholine [PC] and PC-NRs showed low cytotoxicity in comparison with CTAB-NRs. The cell viabilities are shown in Fig. 10. In the case of PC-NR solutions, little cytotoxicity was observed up to a concentration of 1.45 mM, where viability was more than 80 %. This situation must be compared with the viabilities observed in the case of CTAB-NR particles, where a drastic decrease is observed with increasing particle concentration. These

authors [85] concluded that, because the PC is not inherently toxic to living cells, the PC-NRs show reduced cytotoxicity.

The same basic phenomenology occurs when CTAB is substituted by poly(acrylic acid) [PAA] or poly(allyamine hydrochloride) [PAH] polymers (molecular weight 15 kD) [91]. In this case, cell viability of a human colon cancer cell line [HT-29], measured by the MTT assay, after four days exposure to a particle concentration of 0.4 nM, displayed a significant reduction of cytotoxicity, the viability being increased from 30% in the case of CTAB-NRs to more than 90 % in the case of PAA-NRs and more than 80 % in the case of PAH-NRs.

It is worth nothing that, in the conditions of exposure to growth media with serum proteins (containing 10% bovine serum albumin), the three types of gold nanoparticles (nanorods coated with CTAB, PAA, and PAH, respectively) present approximately the same value of the ζ-potential (~ -20 mV), i.e., the same effective surface charge, and have an effective size in the range 30-40 nm in diameter. This means that, at least in this case, the surface functionalization, rather than size and surface charge, influences cytotoxicity.

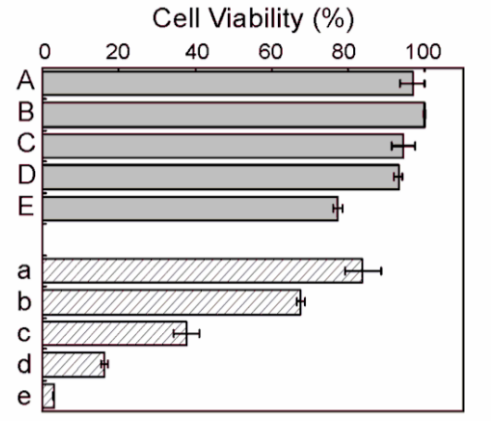

Figure 10: Viabilities of HeLa cells after contacting with the PC-NR solutions (A-E) and twice-centrifuged CTAB-NR solutions (a-e). NR concentrations: 0.09 mM (A, a), 0.18 mM (B, b), 0.36 MM (C, c), 0.72 mM (D, d), and 1.45 mM (E, e)). Reproduced with permission from Ref. [85], copyright American Chemical Society, 2006 .

Huff et al. [92] exposed KB cells to CTAB-coated gold nanorods to examine their internalization, monitored by two-photon luminescence (TPL) microscopy. The CTAB-coated nanorods were found localized near the perinuclear region within the KB cells and, after five days, the cells appeared unaffected by the internalized nanorods, as they grew to confluence over that period. This study, among others, suggests that CTAB promotes nanorod uptake by cells, which could explain, on the other side, the cytotoxicity observed by Niidome et al. [84] with CTAB stabilized nanorods.

These latter authors [92], in order to reduce the strong cytotoxicity observed in gold nanorods stabilized with CTAB, developed PEG-modified gold nanoparticles that showed a nearly neutral surface, with little cytotoxicity *in vitro*. However, as pointed out by Khlebtsov and Dykman [24], the difficulty in assessing the toxicity of CTAB-coated gold nanoparticles is that these particles tend to aggregate inducing a release of CTAB in the surrounding medium, which, by itself, is toxic.

The influence of the surface modification of gold nanorods [AuNRs] administered via direct injection into the circulation on potential adverse effects on blood vessels has been investigated by Alkilany et al. [93]. Surfactant-capped AuNRs were synthesized and either coated with a polyelectrolyte [PE] to prepare PE-AuNRs, or modified with thiolated polyethylene glycol [PEG] to prepare PEG-AuNRs. These authors demonstrated that therapeutic concentrations of PE-AuNRs, but not PEG-AuNRs, are toxic to the vascular endothelium, suggesting that the difference in toxicity (and cellular uptake too) of PE-AuNRs versus PEG-AuNRs could be linked to free surfactant molecules and protein adsorption. Finally, the authors pointed out that toxicity (and cellular uptake) in the vascular endothelium in blood vessels produces potential adverse effects of systemically administered AuNR solutions, which can be prevented by an appropriate surface functionalization.

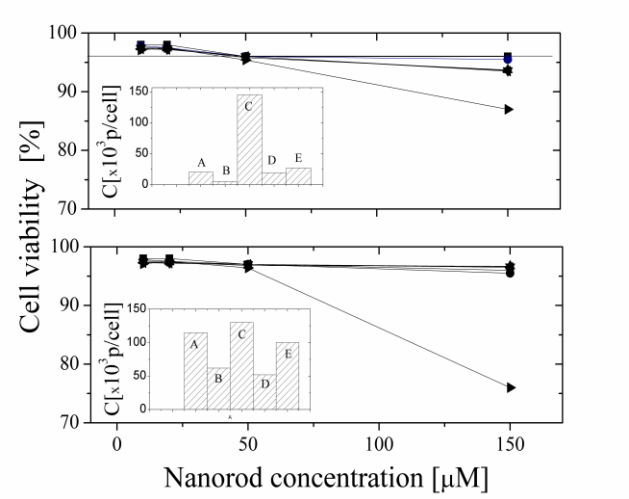

Figure 11: Toxicity of gold nanorods in a medium containing fetal bovine serum (upper panel) and in serum-free medium (bottom panel), at different gold atom concentrations and different surface coatings: (•):PSS1; (•):PDADMAC; (▲):PHA; (▼): PSS2; (▶):CTAB. The two insets show the cellular uptake of nanorods in media containing serum (upper panel) and without serum (bottom panel). (A): CTAB; (B): PSS1; (C): PDADMAC; (D): PHA; (E): PSS2. Data redrawn with permission from Hauck et al. [86] copyright Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2008.

In another study, Hauck et al. [86] produced nanorods (18x40 nm in size) exhibiting different surface charges through layer-by-layer coating with different polyelectrolytes, i.e., hexadecyltrimethyl ammonium bromide [CTAB], poly(4-styrene sulfonic acid) [PSS1], poly(diallydimethyl ammonium chloride) [PDADMAC], poly(allylamine hydrochloride) [PAH] and poly(4-styrene sulfonic acid) [PSS2]. Their ζ-potential varied from very negative to very positive values (from -69.5 to 52.2 mV). The uptake of these nanorods by HeLa cervical cancer cells, together with their toxicity using a dye-exclusion cell viability assay, is shown in Fig. 11. As can be seen, for all the nanorods investigated, with the exclusion of CTAB-coated nanorods at the concentration of 150 μM in serum-free medium, the cell viability was about 95%, not significantly different from control cells.

These findings, together, confirm that a high concentration of CTAB, such as the one employed in nanorod synthesis, provokes some concerns regarding their toxicity [94, 95]. The cytotoxicity of CTAB-coated AuNRs in serum-free media has been also confirmed by Hauck et al. [86], who at the same time found that in serum-containing media, the viability of the cell was greatly increased, becoming similar to that of control. This effect has been attributed to the protein adsorption to the surface of the nanorods, thus reducing the CTAB cationic surface. In the case of nanospheres, however, recent studies have shown that CTAB-coated gold nanoparticles by themselves have minimal in vitro cytotoxicity, if the surfactant concentration is reduced [7].

Gold nanoshells.

Gold nanoshells are a class of nanoparticles composed of a silica dielectric core coated with an ultrathin metallic gold layer, displaying tunable optical resonances. This core/shell structure, with diameters ranging in size from 10 to 200 nm, allows for the gold nanoshells to be made, by either preferentially absorbing or scattering in the visible and near infrared (NIR) regions of the spectrum, by varying the relative core and shell thicknesses.

As novel nanostructures, they possess a remarkable set of optical, chemical and physical properties, which make them ideal candidates for enhancing cancer detection, cancer treatment, cellular imaging and medical biosensing.

As far as the toxicity of these nanoparticles is concerned, it deserves to be mentioned here the investigation carried out by Hirsch et al. [96] who incubated human breast epithelial carcinoma SK-BR-3 cells (ATCC) with gold-silica nanoshells (core 55 nm, shell 10 nm) suspended in serum-free medium (4.4 x 10^9 particles per ml) at the temperature of 37 ˚C. Cells maintained their viability, suggesting that not even therapy by itself is cytotoxic.

Nanoshells with dimensions of about 130 nm, providing peak optical scattering and absorption

efficiencies in the NIR (~800 nm), were fabricated by Loo et al. [97]. SKBr3 breast adenocarcinoma cells were added to the nanoshells at a volumetric ratio of 1:9, for 1 h incubation. Comparison of cells incubated with nanoshells and control cells not exposed to nanoshells, yielded that no difference in viability was observed. The nanoshells used in this work are silica-gold core-shell nanoparticles, which are nominally 110 nm in core size with 10 nm thick shell [98]. In order to provide the steric repulsion to reduce their aggregation in blood, as well as a deterrence to protein absorption, the nanoshells were stabilized by coating with polyethylene glycol [PEG].

Healthy female albino mice of more than 6 weeks age and about 15 g body mass were anesthetized via isoflurane, then injected with 100 µl of PEGylated nanoshells suspended in 0.9% NaCl via tail vein.

Although nanoshells were quickly scavenged from the blood after 28 days, elevated levels of gold were still present within the liver and spleen with still three orders of magnitude higher than pretreatment levels with no physiological complications due to the presence of these elevated levels.

In a recent study, Khlebtsonet al. [24, 99] investigated toxicity effects induced by PEG-coated silica/gold nanoshells administered intravenously to rats at 75, 150, 225, and 300 mg kg^{-1}. Fifteen days after injection, some macroscopic changes in the liver and spleen, as well as multiple macrofocal effusion of blood, were observed for the highest concentrations. (225 and 300 mg kg^{-1}). However, these results are not definitive since such morphological changes were detected only in some of the treated groups. For the majority of the rats, necrosis of hepatocyte cells with pyknosis of the nucleus, and other histological modifications, in comparison with normal samples, were observed [99].

Gold nanoshell particles with an average diameter of about 30 nm consisting of a thin gold wall with a hollow interior have been synthesized by Melancon et al. [100] and employed in photothermal ablation therapy for destruction of epidermal growth factor receptor. These particles, which display a strong resonance absorption peak tunable in the NIR region, did not show observable effects on cell viability, probably due to the absence of the silica core.

Au_3Cu_1 (gold and copper) nanoshells showed a promising magnetic resonance [MR] contrast effect for *in vitro* MR images, [101]. For *in vivo* MR imaging, these agents enhanced the contrast of blood vessels and suggested their potential use in MR angiography as blood-pool agents. Au_3Cu_1 nanoshells with an average diameter of about 50 nm and a shell thickness of about 6 nm have been prepared by Su et al. [101]. The zeta-potential measurements indicated that these Au_3Cu_1 hollow nanoparticles had a negative surface charge of -18 mV, which could be further engineered to assemble with multilayer polyelectrolytes on their surfaces as nanocapsules.

These authors used a WST-1 assay on a Vero cell line (monkey kidney cell line) to measure mitochondrial dehydrogenase activity known to be associated with cell viability. Nanoshells were bio-compatible at all dosages between 0.1 to 10 µg/ml, while cell survival decreased as dosage increased. At 200 µg/ml, viability dropped out to about 15% after 24 hours of treatment. Authors hypothesized that viability reduction could be associated, at least in part, with the nanoshell occupancy of the space for the cell growth in the culture wells.

Gold nanocages.

Gold nanocages [AuNCs], which represent a new class of nanoscale agents for applications in bio-imaging, photothermal therapy and drug controlled release, are hollow porous gold NPs, with size basically in the range between 10 and 150 nm [102]. These particles, consisting of hollow interiors and porous walls, are characterized by extraordinarily large cross-sections

for both absorption and scattering.

Due to their tunable SPR peaks, the rather simple synthetic methods and their hollow-porous structure, gold nanocages represent a new class of nanoscale agents which provide a marked synergic effect in the cancer treatment. Nanocages in the range 40-50 nm are optimal for the uptake into cells because their plasmon resonance is in the biological tissue optical window (~ 800 nm) [103].

Nanocages have been employed as photothermal agents for the selective destruction of cancerous or diseased tissue and served as drug delivery vehicles for controlled and localized release in response to external stimuli [102]. Upon NIR irradiation [104], the photothermal of gold nanocages leads to a rapid rise in the local temperature. This effect favors the uncapping of the thermal sensitive gatekeeper, allowing the release of the interior content.

However, serious issues, like toxicity and in some cases stability, need to be addressed.

Wang et al. [105] explored the *in vivo* biodistribution of the ^{198}Au doped AuNCs in mice bearing EMT-6 tumors. The gold nanogages, 33 nm in edge length and covered by poly(ethylene glycol) [PEG] chains of 5000 kD in molecular weight, were intravenously injected into mice and bioluminescence images were sequentially captured using the IVIS living imaging system. This breast cancer model is known to allow for accumulation of nanoparticles in the tumor through the enhanced permeability and retention [EPR] effect. These PEGyated nanogages rapidly accumulated in the tumor post injection with a significant accumulations in the spleen and liver. It is worth mentioning that no adverse reaction was observed at the administered doses (1.7×10^{12} particle/mice) during all experiments.

Gold nanocages of different sizes and different surface functionalities have been employed by various researchers in different biomedical applications. For example, Kim et al. [106] used gold nanocages with an outer edge length of 46 nm and a wall thickness of 7 nm with surface functionalized by poly(ethylene glycol) in B16 melanomas *in vivo* as contrast agents for photoacoustic tomography. Roughly the same PEGyated gold nanocages (outer edge length of 48 nm and wall thickness of 3.5 nm) were employed by Chen et al. [107] as photothermal therapy against human glioblastoma cell line in mice.

As a further example, the antibody-conjugated Au nanocages were attached to the surface of the cells through antibody-antigen binding [108] and then internalized into the cells via receptor-mediated endocytosis. No morphological change or plasma membrane damage was observed. These works highlight the novelty of the structure of these gold nanoparticles which forecast exciting perspectives in cancer therapy.

Gold nanostars.

Gold nanostars (AuNSs), that are the prototype of anisotropic particles, with tunable morphology (number and length of the branches controlled during the synthesis procedure) are characterized by unusual optical properties that render them particularly suitable in various biomedical uses. Their peculiar shape provides a large surface area on which a higher concentration of drug molecules can be loaded, so that less gold particles would be required, with a reduced toxicity.

Only few studies have concerned AuNSs, mainly because the use of nanostars is greatly limited by the toxicity of CTAB and other surfactants employed in their formulation and, moreover, by the formation of aggregates that this particular morphology favors [109]. A novel method to produce surfactant-free monodisperse gold nanostars has been recently proposed by Yuan et al. [110] with an easy surface functionalization, offering particles with a great potential in diagnostic applications, with a reduced toxicity.

A randomly branched gold nanostructures with a core of 26 to 220 nm and a branch length of 8 to 114

nm have been recently produced by Trigari et al. [111] with extinction properties that can be tuned from visible up to 1500 nm.

Star shaped gold nanoparticles of 180 nm in average width with 70% yield have been recently applied by Salinas et al. [112] to mouse hippocampal slices during the recording of the action potential activity of neurons in the CA3 area. While the firing rate is markedly increased, no adverse functional effects on neurons have been observed, opening the possibility of using star nanoparticles for neurobiology applications.

The biocompatibility of PEGylated gold nanostars (and bipyramides, too) incubated with melanoma B16-F10 cells has been investigated by Navarro et al. [113] and the uptake was analyzed by dark-field microscopy. These particles revealed to be rather monodisperse with a tip-to-tip distance of 130 nm with a spherical core of about 60 nm and with a number of branches of about 10 per gold core. These authors [113] were able to remove CTAB and myristyl bromide replacing the double layer surrounding the particles by PEG, a biocompatible polymer. Taking advantage of their peculiar shape, resulting in specific photo-physical properties, it is possible to easily locate these particles within the cell, making them suitable agents for bioimaging.

The increase of loading density on AuNSs, favored by their asymmetric shape, provide a simple means to improve uptake in cancer cells. Dam et al. [114] demonstrated that a dense packing of DNA aptamer drug AS1411 [Apt] on AuNSs 37 nm in size favors the internalization of these nanoconstructs in a wide range of cancer cells. These particles were taken up by pancreatic cancer cells and fibrosarcoma cells at a faster rate, producing an increase in cancer cell death. Interestingly, treatments of these nanoconstructs on normal cell lines had no adverse effects [115].

The cytotoxicity of irregularly shaped urchin gold nanoparticles (with an average volume equal to that of a sphere 77 nm in diameter) was investigated by Hutter et al. [116] in a microglial (N9) cell line and the results compared with those obtained with nanospheres and nanorods covered with the same surface coating (either CTAB and PEG). In contrast to spherical, rod and urchin AuNPs were relatively innocuous.

Finally, the biodistribution of gold nanostars (56 nm in size) in mouse liver, spleen and blood vessels has been investigated by Li et al. [17] by means of a quantitative photoacustic microscopy technique. Results showed that AuNSs accumulated preferentially in liver from blood circulation with a moderate toxicity.

Towards a standardized protocol. Opportunities and recommendations.

The goal in toxicity assessment is to relate the physico-chemical properties of gold nanoparticles to their toxicity so to predict potential risks in biomedical applications in humans or, at least, to give a recipe in their design with minimal toxicity. In this connection, a recent review is appeared [118] dealing with this important topic, where an attempt to find a correlation of parameters including size, shape, charge, stability, material concentration and ability to adsorb biological compounds is reported. Unfortunately, at present, as the same authors claim [118], this correlation with the whole toxicity is not straightforward. In view of the few examples we have listed in the previous sections, it is quite obvious that cytotoxicity of gold nanoparticles remains an open question and that there is an urgent need for standardization of the protocols employed. Some views on the need of a standardization of the experimental protocol including the species or the cell type used and the method of particle administration have reviewed by Johnston et al. [30]. More recently, a rather complete analysis of the potential adverse implications of nanomaterials,

based on the findings from the research project (named PARTICLE_RISK) funded by the European Commission, has been reported by Johnston et al. [119]. These authors analyze the physicochemical characteristics of nanomaterials from a general point of view and identify the possible nanomaterial attributes responsible for any observed toxicity. These aspects include nanomaterial selection, dispersion of nanomaterials, their relevant doses and concentrations, identification of the relevant cellular model, the target sites and, finally, nanomaterial distribution following exposure. These authors [119] have provided potential solutions to overcome uncertainties that nanomaterial risk pose. We have taken advantage of these indications that now we have directed towards gold nanoparticles.

Below we shall consider separately the different parameters that experimentally have been proved to influence the toxicity of AuNPs and for each of them we will briefly discuss some aspects that it is necessary to take into account to tend towards a standardized protocol.

Nanoparticle characterization.

Shape and size of nanoparticles are generally determined by means of two common techniques, i.e., dynamic light scattering [DLS] and electron microscopies, such as scanning electron microscopy [SEM] and transmission electron microscopy [TEM]. However, different artifacts may arise since TEM and SEM operate under vacuum conditions and for highly polydisperse systems, DLS analysis of the autocorrelation function is certainly not an easy task, since DLS overestimates mean particle size due to the high scattering intensity of larger objects, which makes the data difficult to interpret. In order to overcome this limitations, to image samples in aqueous environment, the cryogenic temperature electron microscopy (cryo-EM) should be used.

The surface charge of nanoparticles can be obtained from the measurement of the ζ-potential using the technique of laser Doppler electrophoresis. Its value is generally affected by different factors such as the pH of the solution, the electrical conductivity, that defines the thickness of the double layer, and the concentration of the particles present in the solution and finally the aggregation effects that occur in solution. Since the ζ-potential can be strongly influenced by the surrounding medium, an accurate characterization of the sample under investigation is highly recommended. In this respect, it deserves to be mentioned the attempt made by the International Alliance for NanoEHS Harmonization [IANH] in order to carry out an inter-laboratory comparison of size and surface charge measurements on nanoparticles prior to biological impact assessment [120].

A further significant factor to be considered is that, to a large extent, the chemical (and biological) activity of nanoparticles is exerted by the atoms at the particle surface. Since the ratio of the surface to total atoms increases exponentially with decreasing particle size, a direct correlation between particle size and toxicity is misleading and more caution in the interpretation of the data is worthwhile [121].

Trace of impurities within the nanomaterial formulation may lead to additional toxic effects. For example, as we have reported above, free cetyltrimethylammonium bromide [CTAB] in solution, derived from nanorods formulation, might cause toxic effects in human colon carcinoma cells [91].

Often, modifications of AuNP surfaces by means of coating with polyelectrolytes or proteins cause undesirable ionic interactions with biological systems [122], which may play a significant role in their toxicity. As pointed out by Kong et al. [123], these surface-bound molecules can greatly influence cell-nanoparticle interactions giving rise to unintended toxic effects which are basically unrelated to the nanoparticles.

Finally, when aggregated, AuNPs show modified surface charges which influence cellular environment, altering the cellular behavior and cellular toxicity

[124].

The nanoparticle concentration and the effective dose.

Nanoparticles have a tendency to aggregate in different dispersion media and, especially, in biological media, which display a relatively high ionic conductivity, favoring the screening of the nanoparticle surface charge. Nanoparticles may aggregate in cell culture media due to ions and proteins. The formers produce a decrease of the screening length of charged chemical groups at the particle surface and the latters cause a thermodynamically favored replacement of surface-associated molecules with themselves [125]. The formation of particle clusters prior interaction with cells or during the adsorption on the cell membrane can justify, at least partially, the different results obtained on the size-dependent behavior of particle uptake and demonstrate the need for real-time single-particle techniques, in order to better understand the effects of particle clustering.

The extent of this effect depends on the nanoparticle size, shape, charge and on the viscosity and density of solutions. This means that the effective number of nanoparticles which interact with cells remains, in most cases, largely undefined. As pointed out by Elsaesser et al. [126], the most appropriate metric is object of considerable discussion [127]. A fully quantitative interpretation of data requires three primary physical metrics, i.e., metric of mass, surface area and particle number [128]. An almost general consensus has been reached on a dose metric related to the number of particle per each cell or each sub-cellular compartment, even if this number can be difficult to estimate [129]. However, a different opinion has been expressed by Wittmaack [130] who considers particle number per unit volume to be the best dose metric.

In some circumstances, the total surface of nanoparticles may be a preferable metric, in particular when the chemical reactions occurring at the particle surface assume a dominant role. In this case, particle size and shape must be known, since mass alone cannot predict the total surface area. Along with this opinion, surface area per unit volume is preferred by Joris et al. [131] since both particle size and particle number are contained in this metric and, furthermore, toxicological response depends on the surface properties of nanoparticles and the surface area exponentially increases with the decrease of the particle size.

In this context, it deserves to be mentioned here that a significant correlation was found between the in vitro oxidative response and the in vivo inflammatory response for different NPs with different physicochemical properties when NP concentration was expressed in terms of surface area per unit volume [127, 132].

Consequently, it is desirable the measurement of the number of particles per cell that can be obtained by analytical tools, such as liquid chromatography mass spectrometry [LC-MS] and radioactive isotopes [133].

The content of gold in tissues can be also determined by means of Inductively Coupled Plasma-Mass Spectrometry [ICP-MS] which ensures a sensitivity of about 1 ng/Kg of animal [134]. Finally, for the localization of AuNPs in tissues, histology and SEM and TEM techniques can be applied [135].

As pointed out by Rivera-Gil et al. [118] in the case of gold nanoparticles, the entity, whose toxicity must be ascertained, is a hybrid object consisting of an inorganic core (gold in this case) and an organic coating. Since increased surface area is generally accompanied by increased chemical reactivity, greater attention might be paid to the particle surface rather to its core. However, it does not make sense to say that toxicity must be attributed to the surface coating or conversely to the metallic core. In order to prove this position, it deserves to be mentioned here the two examples reported by Rivera-Gil et al. [118]. The first deals with AuNPs capped with CTAB developed by

Qiu et al. [136], where the toxicity was due to the shell of CTAB and not to the gold core. The second example deals with nanoparticles with different cores (Au, FePt), but with the same surface coating (a carbon-terminated polymer). In this case, the cytotoxicity effects are attributed to the inorganic core rather than to the surface coating [137]

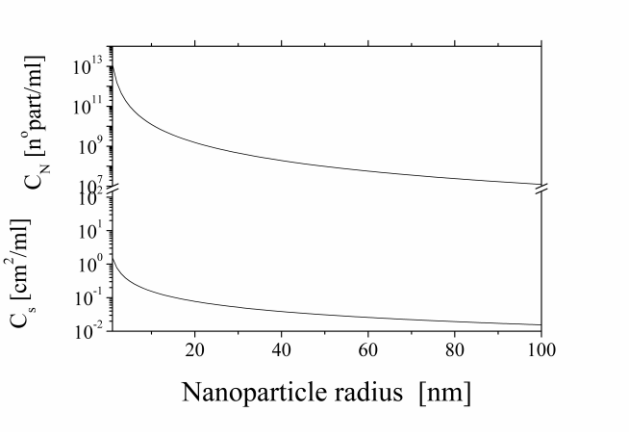

Figure 12: The numerical particle concentration C_N (upper curve) and the total area concentration C_S (bottom curve) as a function of the nanoparticle radius R for a given value of the mass concentration C=1 μg/mL.

The comparison of different results is also made difficult by the use of wide-ranging nanoparticle concentrations. As suggested by Johnston et al. [30], it may be useful to introduce the use of threshold doses, such as the *no observed adverse effect level* (NOAEL) concentration that may be relevant to separate the behavior at low concentrations, where AuNPs may mediate protective responses from the one at higher concentrations where toxic responses become evident.

Frequently, in order to indicate the amount of nanomaterial which interacts with cells, the particle concentration is expressed as mass per unit volume (μg/mL). However, in doing so, the particle number concentration and the total surface area can differ by several orders of magnitude [138]. As a matter of fact, the smaller the diameter of the spherical particle the more the surface-to-volume ratio increases. In Fig. 12, the behavior of the particle number concentration C_N and the total surface concentration C_S is shown as a function of the nanoparticle size for a fixed value of the nanoparticle concentration expressed as mass per unit volume. As can be seen, depending on the particle radius, these quantities vary rather strongly. As toxic effects have been expected to be associated with the available surface area [139], this can lead to a undefined effective concentration, rendering the particle number practically meaningless.

To overcome this difficulty, in cytotoxicity assay, the concentration of particles introduced into the cell cultures should be expressed in terms of mass per unit surface area of the culture dish (μg/cm^2) or in terms of mass per cell numbers (μg/10^6 cells), instead of mass per unit volume (e.g., μg/ml), or in number of nanoparticle per unit volume, as usually done.

Gold nanoparticle stability.

Nanoparticle stability is assured by the organic coating which imparts electrostatic or steric repulsion. As we have above stated, the protein corona plays an important role. However, its presence contributes to make things complicated. As found by Monopoli et al. [61] in the case of SiO_2 nanoparticles, the protein corona evolves passing from protein concentration appropriate to *in vitro* cell studies to that present for the *in vivo* studies. This implies a further intricacy in the interpretation and in the comparison of different experimental results.

Cellular uptake.

In *in vivo* experiments, the size, and shape too, of the nanoparticles should be chosen with care since the cell uptake, and its kinetics and internalization, are strongly size-dependent. Moreover, beside the specific cell-nanoparticle interaction, it must be considered

that large particles, which will be detected by the immunologic system, will be rapidly removed and delivered to the liver and the spleen and, on the contrary, very small nanoparticles will be easily excreted through the kidney, by renal filtration. As suggested by Rivere-Gil et al. [118], the optimal particle size is in the range from 10 to 100 nm.

We have already mentioned the uptake by HeLa cells of spherical and rod-shaped AuNPs of different sizes investigated by Chithrani et al. [64]. This work suggested that uptake was mediated by the adsorption of serum proteins onto the particle surface favoring particle entry via clathrin-mediated endocytosis. This picture is largely supported by the fact that at low temperature (4˚C, where ATP generation is reduced) this active uptake mechanism is greatly reduced. These important results and the hypothesis that the saturation of NP uptake may be a result of the extent of protein binding were subsequently confirmed by Chithrani and Chan [140], who investigated the uptake of transferrin coated nanoparticles by epithelial, fibroblast and neuronal cell lines. These studies confirmed the involvement of endocytosis and highlight the importance of protein adsorption in NP uptake. However, beside uptake induced by active mechanisms, such as endocytosis, particle entry may be also induced by simple diffusion (passive mechanisms).

Electrical surface charge and particle hydrophobicity are two key parameters that determine cellular uptake of functionalized gold nanoparticles. Whereas interactions between cationic NP and negatively charged groups at the cell membrane due to the presence of sialic acid are easily understood on the basis of electrostatic attraction, the influence of hydrophobicity in this process is less clear.

The importance of the chemical structure of NP surface functionality has been highlighted by Green et al. [141] who employed nanoparticles coated with cell penetrating peptides, such as the ones containing the amino acid sequence Arg-Gly-Asp. These structures were found to have favorable characteristics including near-neutral ζ-potential and a relevant stability in serum. A more complex structure was used by Kang et al. [18], who demonstrated that, after endosomal escape, penetration to the nucleous occurs from AuNPs coated with poly-ethylene glycol [PEG] and conjugated with Arg-Gly-Asp acid peptides.

A general scheme illustrating uptakes and translocation routes of nanoparticles (including but not only gold nanoparticles) is shown in Fig. 13

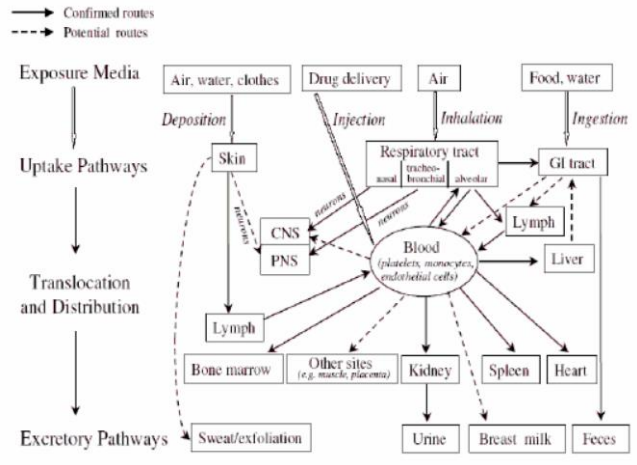

Figure 13: A general scheme of biokinetics of nanoarticles, including uptake and translocation routes already ascertained (full harrow) and others still hypothetical (dotted harrow). Reproduced with permission from Ref. [121], copyright Environmental Health Perspectives 2005.

However, further *in vivo* studies are necessary to elucidate pathways and entry routes of AuNPs in complex subcellular organisms [126].

Cytotoxicity assay.

Various assays have been used to study the toxic effects of nanoparticles on cell cultures, depending on the cellular parameter of interest. Viability assay looks for the overall dose-dependent toxicity of nanoparticles on cultured cells after their exposure [142]. These methods include lactate dehydrogenase leakage [LDH] (which measures the release of lactate dehydrogenase into the culture medium, as an indicator of the cell membrane disruption) or trypan

blue or propidium iodine assay for cell membrane permeability, 4-[3-(4-iodophenyl) -2-(4-nitrophenyl) -2H-5-tetrazolio]-1,3- benzene disulfonate [WST-1] or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay (the *gold standard* for *in vitro* toxicology studies) that measures the enzymatic activity of cellular mitochondria, calcein AM for intracellular esterase activity and, finally, fluorescent Annexin V or caspase substrates for apoptosis indicators.

However, cell viability is a quite general term and each of these methods, which determine one or more cellular parameters, cannot be compared directly with the other as they basically measure different parameters [143]. As a general advise, to avoid misinterpretations of the results, cytotoxicity should be verified with at least two independent assays.

A further problem concerns with the occurrence of false-positive and false-negative results and cross-checking the data with alternative independent assays to ensure reliability of the results is certainly desirable and would help to avoid errors. For example, NPs with optical properties can alter the results from assays based on spectrophotometric measurements or NPs with high absorbance capacity and catalytic activity may interact with enzymes or substrates [144, 145]. A further example deals with the case of gold nanoparticle-treated cells, where the dead cells are imaged with the commonly used fluorescent propidium iodide [PI]. Normally, the fluorescent PI molecules cannot penetrate the cell membrane. However, in some experiments, the PI entered the cell during the endocytosis of the nanospheres and resulted in a false-positive toxicity result [36].

Immortalized cell lines, like HeLa cells, are commonly used to compare the cytotoxicity of nanoparticles varying in size and surface chemistry (see, for example, Fig. 8). However, one must take into account that in immortal cell lines both their genome and proliferation pattern deviate from the ones of normal healthy cells. Cells in the logarithmic growth phase are more sensitive than those in the stationary phase [34].

As a final comment, it must be noted that there is often a lack in the correlation between *in vivo* and *in vitro* results, indicating that the design of better assays with physiologically relevant end-points must be strongly encouraged.

The "toxicity factor".

In the paper by Pompa et al. [19], a systematic evaluation of AuNP toxicity is introduced by means of a multiparametric space of nanotoxicology, where different variables are related to the chemical-physical characteristics of the NPs (namely, composition, shape, surface chemistry, and surface charge). By this approach, these authors were able to elucidate whether size-dependent toxic effects observed *in vivo* are due to the NPs dimension, to their exposed surface area, or if toxicity is mainly determined by the total number of up-taken NPs.

After defining a dose-response curve for AuNPs ingestion in the model system *Drosophila melanogaster*, Pompa et al. [19] characterized such specific type of NPs by a "toxicity factor", based on some specific parameters derived from the mathematical fitting of the observed toxicity in the model organism defining a universal toxicity scale, ranging from highly toxic to bio-compatible nanomaterials (Fig. 14).

A more general three dimensional phase diagram of toxicity based on three independent variables such size, ζ-potential and dispersibility (hydrophobicity) is show in Fig. 15. In this case, the data were obtained from qualitative biocompatibility trends revealed after *in vivo* screening of about 130 nanoparticles, which now include, beside gold particles, fullerenes, metal oxides, polymers, liposomes, dendrimers and quantum dots [146]. This diagram evidences some general trends, common to all nanoparticles investigated. For example, cationic particles are more likely to be toxic than the larger relatively hydrophobic, which can be

easily removed by the reticuloendothelial system. Particles that have mid range sizes and relatively neutral surface charges promote enhanced permeation and retention effects.

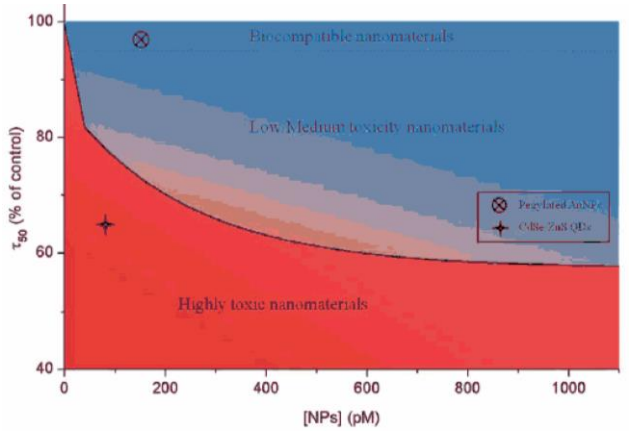

Figure 14: Schematic picture representing the different toxicity regions of the different nanomaterials (highly toxic, low/medium toxic, biocompatible). The black line represents the reference toxicity curve (bi-exponential fit) of the 15 nm citrate-capped AuNPs. The toxicity levels of pegylated AuNPs (150 pM) and carboxyl terminated QDs (85 pM) are also shown. Reproduced with permission from ref. [19] from Royal Society of Chemistry, 2011.

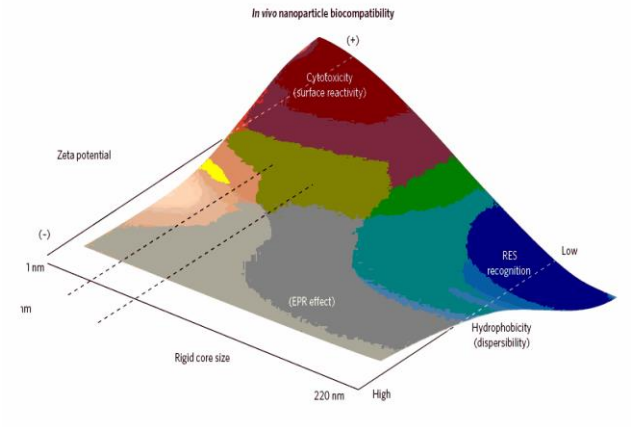

Figure 15: Biocompatibility three dimensional phase diagram after *in vivo* screening of different nanoparticles employed in therapeutic. The independent particle variables are size, zeta potential and dispersibility (particularly the effect of hydrophobicity). Biocompatibility is reflected in the colour spectrum, with red representing likely toxicity, blue likely safety and blue–green–yellow intermediate levels of safety. Figure taken with permission from Ref. [146] copyright Nature Publ. Group 2009.

Gold nanoparticle-membrane interactions.

A final comment is in order. Interactions of nanoparticles with the cell membrane, in the proximity of the membrane surface, influence the mechanisms by which nanoparticles attach to the membrane itself, this being the initial process that leads to cytotoxicity. Since both biological membranes and nanoparticles bear a surface charge, electric double layer and van der Waals interactions are of preeminent importance. The importance of electrostatic interactions between charged gold nanoparticles and phospholipid monolayers assumed as model membranes has been recently stressed by Torrano et al. [147], who investigated differently charged nanoparticles (either negatively charged particles coated with citrate anions or positively charged particles functionalized with cationic poly(allylamine hydrochloride)). However, hydration and undulation forces originated from membrane dynamic fluctuations must be considered as well.

Because of the complexity of the phenomenology involved in the case of biological membranes, it may be suitable to employ model membranes, where the known composition allows, at least in principle, a systematic investigation of the key parameters that control the attachment of nanoparticles to the membranes. Relatively few studies have been carried out until now and, among these, it deserves to be mentioned the analysis of this problem presented by Nel et al. [146] and by Negoda et al. [148]. More recently, a critical insight into these mechanisms has been provided by Chen and Bothun [149].

Effects of defects and impurities

It must be noted that the inevitable presence of defects and impurities, favoured by the reduction in particle size with the huge increase of the surface-to-volume ratio, was found to alter nanoparticle toxicity. Podila and Brown [150] have recently discussed this

important and frequently neglected aspect.

The presence of surface defects, ranging from topological defects, presence of vacancies and dislocations, grain boundaries and surface states may alter, to a different extent, interactions with cells and subcellular structures, producing cytotoxicological effects.

While these effects have been studies in a variety of nanostrucured and differently engineered materials, less attention has been directed to gold nanoparticles. For example, the electronic properties of insulator and semiconductors monooxides (SiO_2 or CuO, respectively) have proven to play an important role in cytotoxicity, as recently evidenced by Xu et al. [151]. By introducing intentionally structural defects in multiwalled carbon nanotubes, Fenoglio et al [152] have demonstrated that these structures may induce acute effects in lung toxicity.

The influence of structural defects on AuNP toxicity has not yet been throughly explored. A possible interrelation among different causes of cytotoxic effects, including defects, is shown in Fig. 16.

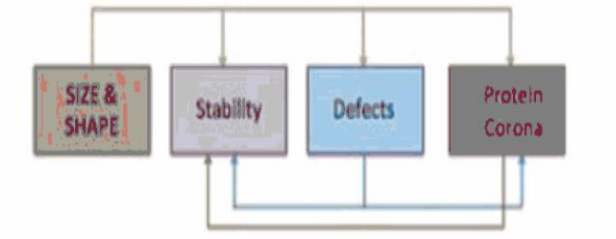

Figure 16: The scheme shows how size, shape and defects are strongly interrelated in determining protein corona eliciting different cellular or subcellular effects resulting in a cytotoxic response. Reproduced with permission from Ref. [150], copyright Wiley Periodicals, Inc 2012.

Conclusions and Outlook.

The statement that gold nanoparticles have been thought as basically non toxic, since bulk gold has been deemed as such, is controversial. To date, on the basis of the fast growing utilization of AuNPs in diverse biomedical applications, recent studies have raised some concerns regarding the toxicity of gold in nano-sized range. The potential toxic impact of AuNPs is certainly multi-sided and is hard to predict [42]. Additionally, the lack of a common toxicity database limits comparison between research results.

Despite the promising future of gold nanoparticles in different biomedical fields, there are many fundamental issues that need to be addressed. Some of them concern with the therapeutic doses and, more importantly, it is mandatory to define the therapeutic window within which nanoparticles can be employed in the absence of side-effects. Even more, nanoparticles must be rigorously purified, this control being extended to each reagent employed in the nano-gold formulation. A relevant question deals with the long-term fate of gold nanoparticles in the organism and, finally, the long-term effects upon nanoparticle exposure, which need to be thoroughly investigated in the widest possible scenario before gold nanoparticles can be used in humans without health risks. In this respect, it is also important to mention here that there is a difference between toxicity (cytotoxicity) and cellular damage and that this difference must be further clarified. Nanoparticles that have little or no cytotoxicity (as ascertained by standard assays) may cause cellular damage. As an example, Perdonet et al. [62] found that 13 nm in size citrate-capped gold nanoparticles induced the formation of actin filaments, with a consequent decrease of cell proliferation, adhesion and motility, while they were non toxic to skin cells.

A further point that deserves attention is if *in vitro* investigations are representative enough of *in vivo* observations. The need for more reliable in vitro models with a high predective power has been recently discussed by Joris et al. [131]. As stressed out by Oberdorster et al. [121], the lack of definitive toxicology data, at present, does not allow for an adequate risk assessment and the precautionary

principle should not be used in view of the potential hazard of AuNPs when administrated intentionally for improving human health.

On the basis of this scenario, the urgency to investigate the toxicological impact and the development of early indicators to detect possible adverse health effects is well documented. At present, the conflicting data in the literature regarding gold nanoparticle bioactivity on the basis of differences in the laboratory research protocols makes it difficult to evaluate and generalize important aspects and does not allow to reach a definitive conclusion and a shared opinion about gold nanoparticle cytotoxicity.

In this review, we have selected from the wide current recent literature some relevant works dealing with both *in vivo* and *in vitro* cytotoxicity effects of gold nanoparticles, with the aim of highlighting the urgent need to have a common platform to investigate the effective extent of toxicity in different experimental frameworks.

Coordination between different research groups in order to establish the proper correlation between the different parameters describing the physical chemical properties of AuNPs and the effects in biological structure of different complexity is greatly desirable.

This coordination can be attained only if standards are introduced in all the different steps encountered in the toxicity analysis, beginning from the characterization of the nanoparticles (size, shape, charge surface functionalization), the experimental protocol (animal model, organs, tissues and cells) up to the choice of the method for the toxicity assessment.

As much desirable it should be to develop a predictive paradigm that, starting from the physico-chemical properties of gold nanoparticles and on the results of *in vitro* experiments, may lead to prediction about their possible outcome effects *in vivo*.

This approach has been attempted in the UC Centre of Environmental Implications of Nanotechnology [UC CEIN] and the UCLA Centre for Nanobiology and Predictive Toxicology [CNPT] [153, 154] and recently reviewed by Sun et al. [155].

In the light of the experimental work done so far, in order to improve a more rigorous evaluation of gold nanoparticle bio-compatibility versus cytotoxicity and to obtain reliable and realistic data, it is critical to employ a standardized protocol. In the last part of this work, we review the most significant factors that must be taken into consideration and summarize the more critical issues that should be addressed when designing the experimental protocol to assess toxicity of gold nanoparticles.

A final comment is in order. Of considerably interest, even if not directly connected to the problem of toxicity, are studies on the properties of oligonucleotide-modified AuNP conjugates that can play a relevant role as intracellular gene regulation agents, opening new possibilities in the development of therapeutic and gene delivery systems. For example, Kim et al. [156] found that AuNPs functionalized with covalently attached oligonucleotides activate immune-related genes and pathways in human peripheral blood mononuclear cells. Moreover, transcription factor based gene regulation is a promising approach for many biological applications. Also in this case, gold nanoparticles, when functionalized by functional peptides, can perform some of the functions of natural transcriptional factors [157], for example to program stem cells to create specific tissues or, even more interesting, to revert cells back to earlier developmental state. Finally, cancer is regulated by a number of signaling pathways and the determination of protein expression provides a way to study the mechanism of tumor progression [158]. Using AuNPs functionalized with monolayer of hairpin DNA with a specific sequence for the key protein mRNA, Xue et al. [159] detected the expression of STAT5B, AKT and mTOR gene in living cancer cells. These examples demonstrate how interactions of multifunctional gold nanoparticles with mammalian cells is one of the hottest areas in current biomedical research.

Acknowledgements

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