Reactivity of Inorganic Nanoparticles in Biological Environments: Insights into nanotoxicity mechanisms

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Abstract.

A deeper understanding of the inorganic nanoparticles behavior in biological media is needed not only to fully control and develop the potential of these materials but also to increase the knowledge of the physical chemistry of inorganic materials when its morphology approaches that of molecular entities. Although this knowledge and control is not yet entirely acquired, industry and society are already using nanomaterials in greater quantities and in consumer products. As normally happens when something new arrives to society, the interest in the broader implications of this emerging technology has grown together with unfounded “nanoeuphoria” and “nanoscares”. In this context, only understanding the mechanisms of the nano-bio interaction will be possible to safely develop nanotechnology. In this review, we discuss on how nanoparticles behave once are naturally or intendedly produced and are exposed to humans and the environment. The response of nanoparticles inside organisms or released to the environment is complex and diverse, and depends on a variety of parameters involved. Mainly, they may i) be aggregated into microscopic particles or embedded in exposed materials; ii) the surfaces of the nanoparticles, which determine their bioactivity, experience constant modifications; and iii) nanoparticles may corrode and dissolve or they can suffer morphological modifications.

Keywords: Inorganic Nanoparticles, Agglomeration, Dissolution, Corrosion, Protein Adsorption, Nanosafety.

Introduction.

Nanomaterials have received enormous attention since, due to the control acquired over their properties, they can be applied in many fields, as biomedicine for diagnosis and therapy, catalysis, energy production and storage, and in environmental remediation, among others. Nevertheless, their unique bio-interacting behaviour may be associated with potential toxicity. Despite differences between macromolecules of the cellular machinery and NPs, such as the quantum monodispersity, the low density and the low electron density of the former respect to the latter, both have in common a reduced Brownian motion, multiple reactive sites, a three-dimensional structure and a high surface energy (which favour aggregation, a common issue in NPs and proteins). All these similarities enable the unique behaviour that NPs can display.

It is known that not only bacteria, viruses and parasites can be the cause of various pathologies, but also inorganic foreign bodies can lead to health-related problems such as silicosis, asbestosis and inflammatory reactions due to debris from worn out prostheses or related to diesel exhaust particles (DEPs) [1]. Similar problems account when considering other applications as in environmental remediation: the use of NPs could help to reduce the cost of remediation but the benefit obtained has to be balanced with the potential risks of introducing large amounts of NPs in the environment [2]. Thus, a present hot question is still whether the unknown risks of engineered NPs outweigh their benefits to society. To solve it, many efforts are devoted by laboratories and companies worldwide, and also by national and supranational organizations. So far, a huge amount of data on the toxicity caused by NPs has been generated and an exhaustive collection of them is not feasible in few pages. However, the question is not yet solved. In this work, we aim to review the main physicochemical features and processes that must be taken into account in order to assess NP induced effects in human health and the environment, along with some examples of their reactivity in those environments.

These studies do not start from scratch. NPs are not a completely new class of materials. In fact, different man-made nanomaterials and of natural origin have coexisted with humans since ancient times and have been described over the last century with the advancement of characterization techniques. Their health-related effects have been also

studied, and this knowledge is very valuable and must be taken into account when assessing the safety and risk of engineered NPs.

1. Nanoparticles Before Nanotechnology.

1.1. Nanoparticles of natural origin.

Knowledge on the NPs reactivity in different biological media, i.e., their evolution in those environments and their biological impact, is key to design NPs with improved safety profiles. NPs are not a completely new class of materials. There are plenty of NPs from natural origin and, in principle, they do not have a great effect on health, as we have evolved in their presence. Soil as natural matrix, natural waters, dissolved substances and biological activity conform a "natural laboratory" of synthesis of organic and inorganic nanostructures. Moreover, it is already well known that some incidental NPs are central to many natural processes such as marine aerosols, terrestrial dust storms, volcanoes and forest fires [3, 4]. One example is the mentioned aerosols, small particles in the submicrometric size regime down to few nanometers, suspended in the atmosphere which, despite their reduced size, affect the energy balance of the earth through the absorption and scattering of the sun radiation [5].

These natural NPs have a variety of morphologies and compositions due to different mechanism of nucleation, growth and aggregation. For instance, recent studies show how several types of NPs, similar to those synthesized in the laboratory, can be naturally formed in soils containing humic substances [6]. As known, humic substances (humic acids, fluvic acids, humin) are the major organic constituent of soils, surface and groundwaters, coal, dystrophic lakes and ocean water. These substances increase the water solubility of metal impurities [7]. In addition, they contain carboxyl and phenolate groups that play a critical role in the synthetic production methods of different types of nanostructures [8-10]. This is the case of naturally produced silver NPs (AgNPs). In the presence of humic acids, silver ions from dissolved silver metal impurities are reduced to AgNPs and the humic acids cover the NP surface preventing their aggregation [6]. TEM characterization showed that these AgNPs are nearly spherical in shape and polydisperse in size. Also, naturally occurring gold NPs (AuNPs) < 200 nm and Au nanoplates (6 nm thick) have been characterized recently [11]. These nanostructures are formed as result of weathering of gold deposits and are identical to some synthesized in the laboratory (see e.g. reference [12] and figure 1).

1.2. Biogenic Nanoparticles.

Nanomaterials can also be found perfectly integrated into biological structures. For example, biogenic NPs occur naturally in many organisms ranging from bacteria to protozoa and to animals [13-17]. These inorganic NPs usually have well-defined size, composition and morphology and play a critical role in the adaptation or metabolism of both prokaryota and eukaryote [18]. Different roles include: i) Chemolithotrophy, for energy production, where inorganic substrates are metabolized to obtain energy via aerobic or anaerobic respiration; ii) Participation in complex adaptative functions, wherein the NPs are integrated as multifunctional components (e.g. magnetosomes [19]); iii) Detoxification, when NP formation is used as a mechanism to regulate the intracellular concentrations of harmful metals ions.

One classical example of a biogenic NP is, as mentioned, magnetosomes. Magnetotactic bacteria found in the muddy bottoms of ponds and lakes use the Earth's magnetic field to distinguish up from down, allowing them to seek out optimal oxygen conditions for growth and survival. These magnetic sensors are called magnetosomes, each comprising microscopic crystal of iron oxide (magnetite) or iron sulfide (greigite) enclosed in a specialized pocket in the cell membrane. Magnetosomes are arranged in linear chains acting as compass needles that enable the cells to follow geomagnetic field lines. Magnetosome crystals are typically 35-120 nm, which makes them single-domain [20]. Regarding detoxification, a biological model of coated nanomaterial found in humans is ferritin, an iron storage protein of approximately 12 nanometers (nm) in diameter that contains 5 to 7 nm hydrous ferric oxide NP inside a protective protein shell [14, 21], lowering the amount of harmful free iron ions in blood.

Also through biological processes, it has been recently shown how AuNPs can be formed. It is known the existence of a natural cycle for gold, where microorganisms play an essential role. Studies in auriferous soils have shown that the resident microbiota can dissolve up to 80% (wt.) of the gold contained in the soil [22]. Bacteria associated with Au grains (e.g. metallophilic bacteria) can further reduce $AuCl_4^-$ and accumulate metallic Au in the form of NPs. This formation of natural NPs, that is known to be done by bacteria but no by eukaryote, occurs via an atom by atom aggregation process on inorganic or organic template. Other example is the formation of

iron oxyhydroxide nanocrystals (3nm diameter) by *Gallionella spp.* [23] through the oxidation of dissolved ferrous ions) in an acidic-near neutral solution [9]. A useful reference that recompiles the different types of NPs from natural and biological origin is the work of Banfield and Zhang [23].

1.3. NPs from anthropogenic sources.

Similarly, man made inorganic NPs have been in contact with humans since ancient times [24]. One of the classical examples are in creams for cosmetics [25]. It has been proved that lead-based chemistry, initiated in Egypt more than 4000 years ago, and made by the combination of naturally available minerals with oils, various creams, or water, results in the synthesis of lead sulphide (PbS) NPs with diameter of about 5 nm. The appearance of these crystals is quite similar to PbS Quantum Dots (QDs) synthesized by modern material science techniques [25]. Another case is the observation of nanometric TiO_2NPs in the alveoli of the Oetzi man, a 5.400 years old mummy [26]. It is believed that the TiO_2 was used then as white pigment in tattoos.

However, over the last century, human exposure to airborne nanosized particles has increased dramatically due to anthropogenic sources such as internal combustion engines, power plants, and other sources of thermodegradation of organic matter. It has been also observed that some nanomaterials, as fullerenes, are produced naturally as unintentional byproducts of combustion processes, as burning paraffin or diesel produces carbon nanotubes (CNTs) and micrometric carbon fibers of aspect ratios comparable to those of lung-retained asbestos [27]. In a recent example, both diamond NPs and fullerene molecules have been discovered in the centre of the flame of a candle, along with graphitic and amorphous carbon (thus containing a candle flame all four known forms of carbon). Professor Zhou's work [28] revealed that around 1.5 million diamond NPs are created every second in a candle flame as it burns. These are burned away in the process, and they are finally converted into CO_2.

Interestingly, this unstability and short lived nature is common in the most of the naturally generated NPs. This is because when reducing the grain size, the surface energy increases and thus, resulting particles search ways to minimize this energy, either by aggregation, becoming part of a larger entity, or dissolving into more stable

atomic species. The processes leading to decrease surface energy (mainly agglomeration and dissolution) are key to understand the reactivity (and associated potential toxicity) of NPs, as will be discussed throughout this work. These processes are associated with different health effects. For instance, when NPs aggregate and they are no "nano" any longer, this could lead to a similar scenario to that posed by incidental inorganic microparticles, extensively investigated during the last century. Particulate inorganic matter, as burning oil residues as the above mentioned or asbestos, has been found in diseased tissues and it is know that cause various pathologies, such as silicosis, asbestosis or inflammatory reactions [29, 30]. Also interestingly, after oral administration of TiO_2NPs, detectable amounts were found in the blood, glands and some organs with the highest concentrations being in the lymph nodes, liver, spleen and lungs [1]. In such case, it is believed that were the ionic species which were detected in those far places.

2. Engineered Inorganic Nanoparticles.

There are lots of proposed definitions of the term nanomaterial, principally driven by the need of providing a framework to the regulations laying down provisions on substances. The International Organization for Standardization has proposed the ISO TS 27687 Definition, which defines a nano-object as a material with one, two or three external dimensions in the nanoscale, where, in turn, nanoscale is defined as the size range from 1 nm to 100 nm. This idea is common in most of the definitions, which emphasizes the size aspect or even only consider it. However, this still lacks of consensus. For instance, the European Commission (EC) made in 2010 a public consultation for a definition of the term "nanomaterial" that the EC would intend to use as a reference term for communication and/or legislation on nanomaterial [31]. The reply of the European Comsumers Organization to this EC public consultation on nanomaterials proposed that the Commission recommendation would not only be restricted to the size range of 1-100 nm but also take into account the functional properties of nanomaterials [32].

If NPs are of great scientific interest it is because they are effectively a bridge between bulk and atomic or molecular species. This means that, at the nanoscale, material properties change abruptly respect to those displayed by either a material of the same composition but larger ("bulk"), the single atom isolated (ion), or the single atom forming part of a molecule. And, while a bulk material should have constant physical properties regardless of its size, this is often not the case at the nano-scale. Materials properties change as the percentage of atoms at their surface becomes significant. Size-dependent properties observed for nanomaterials include, among others, quantum confinement in semiconductor NPs, surface plasmon resonance in metal NPs and superparamagnetism in magnetic materials. Thus, as discussed in Casals et al [1], a NP could be defined as a small particle which present novel properties, which are size dependent, that therefore differ from the bulk material. Consequently, there is no a strict dividing line between NPs and non-NPs.

Beyond size discussion, another important feature that distinguish engineered NPs from other particulate matter is their monodispersity. In this sense, it is useful to consider NPs as molecular entities, despite their structural dispersion when compared to truly

molecular materials. Thus, what qualifies NPs to be considered molecules is their reduced size and size and shape depending properties (as in the case of biological macromolecules and their Structure-Activity Relationship) as their monodispersity. Without entering into a discussion about the physical limit of monodispersity i.e. which standard deviation threshold respect the mean size determines that a collection of NPs should be considered monodisperse, from a practical point of view, two NPs can be considered monodisperse if they respond undiscernibly to a determined test using the current characterization techniques (such as Electron Microscopies, XRD diffraction, UV-VIS spectroscopy, Dynamic Light Scattering, BET surface analysis, etc). It is worth noting that it has been accepted in the field that a standard deviation of the size distribution equal or below a 10% can be considered as monodisperse, while values as low as to the 4% have also been obtained [33, 34].

Last but not least, to provide an accurate description of the concept of nanomaterial, it is important to note again that many of them are truly nanometric once produced, but they tend to aggregate rapidly into micro or macrometric particles, thus loosing their nanoscale properties. Therefore, if engineered NPs need to be kept separate from each other, it is required surface engineering in order to provide them with repulsion forces to prevent aggregation. This can be done either by electrostatic repulsion, e.g. allowing the formation of a double electrical layer of inorganic ions around the surface of the NPs, or by steric means, e.g. through the conjugation of organic or biological molecules. This has been several times undeservedly neglected in the discussion about the concept of nanomaterials toxicity and is a key point when studying their reacivity in biological environments: in the majority of applications, an engineered inorganic NP must be understood as a dense collection of atoms that display different properties repect other formats of the same material (inorganic core) together with the sabilization layer (inorganic or organic shell) that prevents its agglomeration or aggregation into something bigger.

3. The Nanoparticle Evolution in Biological Media.

The health-related effects of naturally generated minute inorganic matter have been studied for long during last century and a significant body of research on the effects of particles from natural origin or as a result of human activities has been acquired. For instance, in the case of airborne dust particles, it is known that iron or other metal rich dust can generate reactive oxygen species (ROS) on the lung surface tearing the lung tissue [35]. Importantly, these harmful effects must be especially addressed in subjects with asthma and emphysema. Also, ash from volcanoes or forest fires also entail different effects on health such as respiratory problems (nose and throat irritation, bronchitic symptoms), and eye and skin irritation. Apart from these well known cases, few other health effects caused by those natural particles have been described. However, studies with engineered nanomaterials are still at early stages and along with contradictory data. For example, reports on strong biopersistence and accumulation of NPs for months to years and on their rapid expulsion, including renal clearance of too large NPs, coexist in the literature [36]. Furthermore, while reactions of cells and organs to NPs have been observed, the long term consequences of such effects are still not known. Also, reports about NP crossing tight-junctions (as the blood brain barrier) and mucosal barriers are also still unclear and under controversy. A clear example of that is the case of iron oxide NPs. The toxicity of iron oxide NPs has been studied for long [37] but still the reports of potential medical benefits appear constantly, together with the ones about its toxicity. Thus, while some find a new promise for nerve cell regeneration [38], other detect the toxic effect of this material to neuronal cells [39]. Importantly, what is already known are the consequences of the frustrated phagocytosis that occur when micron-sized particles enter to the body that are too large to be phagocytosed and trigger chronic inflammation. Some of these particles are related to nanotechnology since have one or two dimensions in the nanoscale. For instance, in Poland et al. [40] it is reported that CNTs larger than 20 microns induced chronic inflammation in mice. The immune cells responsible for the defense mechanisms (fixed macrophages in the lung walls in this case) are unable of phagocytate the strange body, what results in inflammation as the body is not degraded. This mechanism is properly described and understood. It is the case of asbestosis, silicosis and a number of granulomatosis as mentioned previously. Finally, in the same work it is reported that

CNTs smaller than 10 microns did not caused any appreciable effect in that particular experimental conditions.

During the first decade of this century, the efforts to design and produce monodisperse collections of inorganic NPs and carbon nanostructures has rapidly grown and an increased number of different types of NPs of increasing complexity and more artificial with time are being produced [41, 42]. The main differences between natural, unintentional NPs and anthropogenic, intentional NPs are: i) the polydisperse and chemically complex nature of the former in contrast to the monodisperse and precise chemically engineered characteristics of the latter; ii) the particle morphology, often a branched structure from combustion particles is in contrast with spherical forms of engineered NPs, and, of course, other intended geometrical figures that are appearing in the scientific literature, including tubes, wires, rings, disks, boxes, double boxes, hollow NPs, etc [41, 42], and iii) NPs of natural origin and man-made NPs agglomerate rapidly or tend to dissolve or to minimize their increased surface energy, but engineered NPs are synthesized to be stable over a much longer time period than NPs from natural origin. Despite these differences, the same physicochemical principles are likely to be applied to both types of NPs in order to understand the behavior of this class of materials. This is because, as mentioned previously, their reactivity is determined by their high surface energy and their final fate is the agglomeration or disintegration towards more stable phases. For instance, in geochemistry, a NP is described as the short-lived intermediate between the nuclei -which are forming and dissolving as a function of ionic concentration- and larger (micro)particle/composites. NPs -and their composite materials- can be understood as evolving objects strongly subjected to the physical-chemical and biological environment in which they occur. NPs tendency to aggregate follow Derjaguin and Landau, Verwey and Overbeek (DLVO) rules, or they dissolve as their surface energy increases as described in the Gibbs-Thompson effect and the Noyes-Whitney equation. Also, for the same reasons, the surface of NPs readily interacts with other particles, biological molecules (e.g. proteins) and/or synthetic materials. Taking into account these considerations, to examine the NPs stability and evolution in biological media is key to study the following aspects (figure 2):

i) Aggregation of the NPs: their colloidal stability determines their proper interaction with biological entities, e.g., the higher citotoxicity of unstable colloidal NPs that form

large agglomerates is not due to the material but rather its final micro or macrometric size (section 4). Also, NPs are prone to attach to organic matter. This is important since while much of the NPs function is due to their core composition, the surface coating defines much of their bioactivity. For instance, inside the body, NPs never travel alone, but they are constantly surrounded by a coating of different molecules, mainly proteins. This spontaneous coating evolves as time progresses enabling different biological responses at differents times of exposure [43] (section 5).

ii) Dissolution of the NPs: Metalic cations released when NPs dissolve can modify the morphology of these materials and can also be a source of detrimental health effects. Morphological modifications are clearly manifested in the Ostwald Rippening phenomenon [44]. This process first described by Wilhelm Ostwald in 1896, describes a thermodynamically-driven spontaneous process based on the atomic exchange between atoms in solution and atoms in a particle, in such a way that the redeposition of the "dissolved" species on the surfaces of larger particles is favoured. In the case of NPs, as concentration of ions increases moderately after the purification and, unless free atoms are removed from the equilibrium (due to complexation with molecules or precipitation), the following consequences should be devised: i) Ion concentration remains constant while the size distribution of the NPs broadens; ii) NP shape is modified most probably via selective etching; iii) The number of NPs decreases as the smaller ones are completely dissolved [45] (section 6).

Finally, The potential impact of these processes to biological systems is also discussed in the following sections.

4. Aggregation.

It is known how the aggregability of NPs suspensions depend on parameters such as the surface charge or coating and on the medium in which they are dispersed, as cell culture medium, physiological buffers, serum, plasma, etc. (with their different ionic strengths and compositions). For example, Xia et al. and Casals et al. [43, 46], reported how NPs showed a dramatic change in their state of aggregation, dispersibility, and charge upon transfer from a buffered aqueous solution to commonly used cell culture medium. In these cases, NPs are destabilized, agglomerate, and those agglomerates precipitate by weight (following Stokes law). Note that this is different from saturation (high concentration of NPs) where some NPs are expulsed from the solution by entropy, recalling the case of soluble salts where the excess sediments once an upper threshold is overcome (saturation limit). In such case, once the concentration of particles in solution decreases, the sediment spontaneously redissolves (what is also observed with NPs [47]). However, on the other side, in many occasions NPs may end up in irreversible aggregates that cannot be redispersed.

These phenomena determine some upper concentration limit for NPs. Considering that colloidal synthesis represents a major technique to get individual size and shape controlled NPs [33, 48], the standard concentrations to still obtain monodisperse collections of stable and morphological controlled NPs is up to 10^{16} NPs/mL (this is not a theoretical limit, but practical), depending on the material. This corresponds to an upper NP concentration of 10 μM and an average interparticle distance of about 100 nanometers for the case of 10 nm NPs. In the case of a dense material as gold (19,3 g/cm^3), this is translated to a mass concentration of up to few mg/mL. Even if concentrations may seem small, it is worth noting that they are biologicaly relevant. Several stable NPs at those concentrations (0.1-10 mg/mL) display toxicity either to viruses, bacteria or mammalian cells [49-51]. As an example, in these references, the toxic effect of AgNPs (15 nm) started between 5 and 10 mg/mL (working with 10^5 cells/2 mL well) with EC50 at 8.75 mg/mL regarding proliferation assay and EC50 at 2.5 mg/mL regarding membrane leakage. In those experiments, higher AgNPs concentrations could not be tested because of particle clumping and precipitation above 10 mg/mL. Aluminium NPs (30 nm) affect the plasma membrane of C18–4 cell line (mammalian germline stem cells) with a EC50 of 4.7 mg/mL, while Molybdenum oxide

NPs (30 nm) showed a very similar EC50 of 5 mg/mL. Regarding apoptosis tests, an increased number of apoptotic cells are observed at similar concentrations (1-5 mg/mL for cadmium oxide and 10-50 mg/mL for silver, aluminium oxide and molybdenum oxide NPs). Of course, different NPs have been analyzed using several biological assays and this data can be found in different articles and reviews [52, 53].

Either because NPs are destabilized in biological media or because they have exceeded their saturation limit, special physic-chemical properties that arise at the nanoscale (quantum confinement, superparamagnetism, extreme catalytic activity, etc.) are progressive/partially lost when NPs aggregate. Nor the properties, nor the dynamics are longer the same. Agglomeration leads to specific surfaces, concentrations, mobilites, etc, very different from the parent NPs dispersions. Therefore, intended applications in which NPs have been designed cannot be carried out. For instance, for the use of NPs in drug delivery in *in vivo* conditions, highly agglomerated NPs will be less mobile than the well stabilized ones in the transportation media (as blood). Consequences of this are diverse: concentration of NPs in different parts of the body is affected, they can be accumulated or trapped in special organs and they could not reach the target, as larger sizes are more immunogenic, among others. Of course, NP aggregation and precipitation are time dependent processes, which may take from fractions of a second to weeks, and this has to be considered when performing biological tests. For example, studies of ROS production are tipically done at 1 hour, the active response of the immune system is detected at short times (~30 minutes) and the cell viability assays are usually performed at 24 and 48 hours.

Following this reasoning, when evaluating NP toxicity, both NPs aggregation or sedimentation could be indeed a source of confusion: the larger size of the agglomerates yield to a different range of effective sizes and concentrations of particles, and consequently no comparable doses in terms of number of NPs and surface area, the dosing determining parameters [54]. Needless to say that the toxicity of a substance is related to its dose (Paracelsus's *dosis sola facit venenum*), in such a way that an accurate determination of the dose is critical to properly assess the potential toxicity of a material. For instance, in some reports the onset of toxicity in the viability experiments might be related to the onset of agglomeration, probably because unstable NPs and NP agglomerates "rain" on top of cells, thus, not only changing the proben species, but also

increasing the dose on the cell [49]. These large particles could be a (too large) stone in the cell machinery [55], or more difficult to be processed by the cells, as in the previously mentioned case of frustrated phagocytosis [40]. Thus even if NPs are not very toxic by themselves they may be risky because they are source of toxic aggregates.

In this context, an example that deserve special physicochemical characterization are commercial nanopowders. These type of materials are produced massively industrially and represent a broad range of the nanometric materials to be used in low tech applications, from textile to cosmetics. However, also this type of materials could enter in contact with biological entites, e.g. through the skin, by inhalation or accidental ingestion [54, 56]. To assess the biological impact of these commercial nanomaterials, the dry material has to be dispersed in water (or biological media) prior of their exposure to cell. However, if the resuspension protocol is not appropriate, the nanoparticles rapidly aggregate (see e.g. figure 3), losing their properties associated to the nanoscale. Thus, due to their dry origin, the control of the size distribution, aggregation degree and colloidal stability in water and biological media is even more necessary.

For instance, we have observed that resuspension of commercial Au, Co, Fe_2O_3,TiO_2 and CeO_2 nanopowders in an aqueous suspension of the inorganic salt TMAOH yield better stabilization than simply water or PBS. Resuspension of Au, Co, γ–Fe_2O_3, CeO_2 and TiO_2 commercial nanopowders of nominal sizes, according the supplier, of 40–100 nm, was carried out at different concentrations ranging from 10^8 to 10^{15} NP/mL. These concentrations were calculated according to the sizes given by manufacturer. Size distributions and colloidal stability were studied on the crude suspension in milliQ water and in the presence of additives/stabilizers such as Sodium Citrate, TMAOH, Phosphate Buffer Saline (PBS), Collagen and Cell Culture Media (CCM) consisiting on a mixture of DMEM salt and Foetal Bovien Serum at 10:1 (v/v). To compare different types and sizes of NPs, the parameter NPs/mL were chosen since number of NPs at a given size includes also the mass. Working volumes were 50 mL. Size distribution of the resulting suspended powders were characterized by TEM and their stability by UV-VIS measurements. UV–VIS measurements of loss of absorbance serve as indicator of concentration following Lambert-Beer law, $A = \varepsilon \cdot C \cdot L$, where A is absorbance, ε the dielectric constant of the substance, L is the optical path, and C the concentration.

Results of this characteriztion showed that, after resuspension in the different media tested (Sodium Citrate, TMAOH, PBS, Collagen and cCCM), the obtained colloids are of poor stability, size distributions are large, and shapes are randomly amorphous. Concretely, metal NPs (Au, Co) and metal oxide NPs (γ–Fe_2O_3, CeO_2 and TiO_2) display different behaviour: the former are not stable at all, and once resuspended irreversibly aggregate in seconds, while the later showed a slower sedimentation (few days until total sedimentation) (figure 4).

Interestingly, all commercial nanopowders at the tested concentrations (10^8 to 10^{15} NP/mL) were highly unestable in organic media as cCCM or collagen containing water, leading to the formation of a sediment immediatly after resuspension. Thus, as this stabilization is not enough, a process to fragment the orginal powder size distributions towards smaller sizes (getting rid of large aggregates) to focus on the most stable and nanometric particles was performed (supporting information) in order to obtain reproducible, controlled and monitorized samples of dry origin.

All these considerations are made assuming samples purity and the absence of poisonous ingredients. Different experiments showed that the solvents (NP-free aqueous solution in which NPs are dispersed) used to synthesize and stabilize the particles in solution sometimes had a toxic effect on different human cell lines, even when used at a final concentration of 9.1% v/v [52]. Examples of that include the NP contamination with the ubiquitous lipopolysaccharide (LPS), which will induce strong biological responses in many type of cells, or the use of Phenylphosphine, highly toxic surfactant used to control the synthesis of <3 nm AuNPs synthesis. These aspects have been neglected until recently.

5. Adsorption of (Macro)Molecules.

The NP interaction with its environment is through its surface, which experiments constant modifications. Besides, a living cell communicates to the exterior (to the rest of living entities and environment), predominantly through proteins. The two main characteristics that allow this interfacial role of proteins could be summarized as follows: i) they are macromolecules that have an amphipathic character, i.e. they possess polar and nonpolar residues enabling them to have a three dimensional structure to be in contact with different environments, and ii) the large number of hydrogen bonds and hydrophobic interactions that one single protein can perform allows them to interact to almost all the matter they get in contact.

Hydrophobicity and surface charge have been historically the factors taking into account to explain protein adsorption to functionalized inorganic surfaces. This is illustrated by the work of Prime and Whitesides [57] using self-assambled monolayers (SAMs) supported onto gold films. Those SAMs consisted of alkyl chains with different terminal groups, where the more hydrophobic the surface the greater the degree of serum protein adsorption. Similarly, in a later study [58], the binding of bovine serum albumin (BSA) to gold surfaces modified by alkyl thiol SAMs was found to decrease in the following order of terminal groups: C_6H_5OH > CH_3 > COO^- > NH_3^+ > OH^- > oligoethylene oxide. Moreover, it is obvious that the global charge of the protein can also drive its adsorption through electrostatic attraction. It was also observed by Norde and Lyklema [59] that there is a higher adsorption of positively charged proteins onto negatively charged polystyrene surfaces and vice versa, and other examples in this sense can be found in the literature [43, 60-62].

The interfacial chemistry between blood serum proteins and inorganic surfaces is a dynamic process governed by the Vroman effect [63]. Already in 1962, Leo Vroman reported how the exposure of hydrophobic inorganic powders to blood plasma resulted in the removal of coagulation factors, and the inorganic surface became more hydrophilic. This result manifested a competitive adsorption hierarchy: the highest mobility proteins arrived first and were later replaced by less motile proteins that had a higher affinity for the surface, mainly factor V and fibrinogen, in a process that takes up to few hours. This process is recognized as the general phenomenon governing the

competitive adsorption of a complex mixture of proteins (as serum) to surfaces, as pointed out by Slack and Horbett [64].

Furthermore, since the 1950's, studies of other interface phenomena involving proteins have identified the adsorption of proteins to inorganic surfaces as a process that evolves to an irreversible state (explaining the failure in fitting protein adsorption data to the Langmuir equation). Initially, the strongest argument for this irreversibility was that proteins are provided with multiple, although weak, anchor points. However, detailed studies suggest other mechanisms. The work of Alaeaddine and Nygren [65] pointed out that, contrary to what might be suspected, proteins do not distribute on surfaces randomly. Instead, once the first proteins are attached, an initial cluster of proteins forms around these, thereby stabilizing them, and this mechanism is repeated until the entire surface is filled. Thus, not only affinity protein-surface but also mechanisms such as molecular relaxation time or spreading, depending on the time that proteins remain on the surface, have been identified as determining factor in making the adsorption as definitive [60].

Accordingly, all these interfacial processes take place when NPs are dispersed in biological media, such as when injected into veins as drug delivery vehicles or incubated with cells in *in vitro* studies. However, some differences respect the behaviour previously described must be considered: NPs are not a fixed substrate but they move in solution, they have similar dimensions to proteins, and they possess a high curvature radii, thus changing accessibility to their inorganic surfaces. All this effects modify the kinetics of encounter between the NP surface and proteins, the mechanisms of attachment, and the biological outcome [43].

Recently grouped under the name of the Protein Corona (PC) formation, these processes become in the focus of the nanobiotechnology and nanotoxicology research fields. The increasing number of recent publications that cover different aspects of the NP-PC [66-72] reflects that interest. Largely, it is because the proteins forming the "corona" remain associated with the particles under normal conditions of *in vivo* and *in vitro* exposure, thereby conferring their biological identity to the NP-PC complex and determining the interactions between NPs and the host. In other words, this corona of proteins “expressed” at the surface of the particle is what is “read” by cells [73]. In such a way,

effects of NPs in contact with biological systems have to be analyzed together with the data about their PC formation process. To date, PC formation studies in the case of inorganic NPs smaller than few tens of nm (of interest since a new generation of diagnosis and therapeutic devices, based on such small engineered metal and oxide NPs are proposed for *in vivo* applications) have expanded to (Au [43, 66, 73, 74], Ag [66] and FePt [75]) metal oxide NPs (SiO_2 [76], Fe_3O_4, CoO and CeO_2 [66], TiO_2NPs [77] and TiO_2, SiO_2 and ZnO NPs [78]), and Quantum Dots (CdSe [72, 79] and CdSe/ZnS [75]) (see e.g. figure 5).

This association with proteins may indeed biocompatibilize foreign matter as NPs, which could result in detoxification of problematic particles, as in the case of albuminization of potentially toxic drugs [80, 81]. But, also it could entail detrimental effects, e.g. through the depletion of specific proteins or enabling a more intense or more specific interaction with living cells. The depletion of proteins is not expected, and really difficult, since NPs inside blood or organs will hardly reach high enough concentrations for protein depletion. But the triggering of a more intense or specific response could be due to avidity effects arising from the close spatial repetition of the same protein if accumulated on particle surface, or the possible alteration of protein conformation and exposure of novel epitopes. Also, a major hindrance in the use of liposomes or other nanoparticulated carriers for drug delivery results from the attachement of specific components of the immune system, which are proteins or protein derivatives, e.g., proteins of the complement system, antibodies or immunoglobulins, a type of glycoproteins that tag the vehicles for phagocytosis.

Finally, regarding the biological implications of the NP-PC, a crucial point to take into consideration is that the interaction between NPs and cells is mediated by their respective surfaces, and NPs surface nature in biological media is that conferred by their PC. Surface charge has been recognized as a key parameter that strongly influences the approaching of NPs to negatively charged biological membranes, and therefore determine internalization, immune response and toxicity. For instance, in pharmacology and toxicology it is known that positively charged macromolecules show an incremented immune and inflammatory response and eventually more toxicity than their neutral and negative counterparts [82, 83]. Also, together with NP size, surface charge not only yields to a concret interaction NP-cell membrane but also affect their

biodistribution and permanence in the body. Balogh et al [84], encapsulating AuNPs of differents sizes into dendrimers that provides them with negative and positive charges, found that the particles selectively accumulate in different organs depending on their size and/or charge alone. Also, Tan et al [85], in a study with dendrimers and liposomes found that the anionic PAMAM dendrimers did not induce cytokine secretion from human leukocytes, but cationic liposomes induced the secretion of pro-inflamatory cytokines as TNF, IL-12 and IFN-γ. Similarly, another cationic nanoliposome alone or in combination with bacterial DNA increased the expression of the dendritic cells surface markers CD80/CD86, which are important in the inflammatory response [86]. Finally, one should also take into account that a stable PC may help the NP to act as a "Trojan Horse" allowing a deeper penetration of the NPs into the biological systems, or even their noxious accumulation in cells or tissues. And, in addition, packing of proteins on the NP surface may increase their robustness against their natural metabolic degradation [87, 88]. However, after extended periods of time, the metabolic degradation of the corona, or a leaching of ions from the particles, could induce toxicity. Currently, some of these hypotheses are also an active field of research.

6. Dissolution and Corrosion.

Dissolution and corrosion processes have been widely studied for macro-sized materials of interest in the metallurgic industry, such as the well-known case of iron which corrosion (rust) is associated with degradation of iron-based tools and civil structures. Even more, these processes are of evident biological impact and the harms derived from the presence of metal ions in a variety of biological environments and ecosystems have been studied for long. Therefore, the ability of metallic particles to release metal ions and their induced toxicity has been often correlated. As an example, in any metallic implant, wear-corrosion greatly contributes to the release of ions which are responsible of health related problems [89]. Moreover, fairly stable oxides may continue oxidizing when exposed to biological environments, as in the case of some iron oxides [90]. In such biological environments, ions released may end up as different chemical species that produce different biological impacts [91-93]. The ions released from bulk metallic compounds , their speciation and their toxicity are also studied [94]. These processes also occur in inorganic NPs, and there is an increase of reports establishing that cations released from NPs are responsible of detrimental effects [91, 95, 96]. Moreover, the degradation of inorganic “non-degradable” matter is supposed to be magnified at the nanoscale. Accordingly, higher biological implications in the short-term are expected in the case of NPs.

In the case of NPs, due to their reduced size, high surface-to-mass ratio, high radii of curvature, and the corresponding low coordinated atoms at the surface, dissolution is enhanced. However, there are other many factors to take into account such as the metal solubility within a given environment, NP stability and aggregation states, functionalization of NPs with protective shells or coatings, as SAMs, or solvent properties such as pH, ionic strength and/or presence of adsorbing species. A NP may be subjected to a process of disintegration into its constituent atoms due to chemical reactions with its surrounding (merely from exposure to oxygen atmosphere [97] or to certain substances as chlorine or even enzymes [97-99], or simply because the process itself is thermodinamically/kinetically favorable. However, despite the importance it have, it still lacks a detailed study of the dissolution and corrosion processes for the different inorganic NPs [100].

First important consequence of this degradation, given the importance of size in the nanometric regime, is that dissolved particles are not in the same size range anymore when compared to the original ones. As concentration of ions increases after the synthesis, due to NP dissolution and unless free atoms are removed from the equilibrium (complexation with proteins, dilution), NP number concentration remains constant while the size distribution of the NPs broadens (due to Ostwald Rippening) and also their morphology changes (figure 6 and figure 7). Second, NP dissolution is of special relevance in the case of NPs dispersed in biological systems and the environment, where NPs are likely to be highly diluted. Under these conditions, a released ion loss its chances to return to a NP. And this would drive NPs towards higher or even complete dissolution in such environments over the due time. This could also be the case of breathed NPs (or the known case of fibers) which, in the alveoli, will not necessarily attain an equilibrium state in terms of corrosion since the ion-complexation/sequestration properties of these compartments enhance dissolution even further [44].

Thus, as mentioned previously, NPs may possess associated toxicity and environmental risk because they may dissolve becoming a source/reservoir of toxic cations. In this direction, different investigations on nanotoxicology showed the correlation between NP toxicity and the ions released from them [79, 95, 101, 102]. Maybe, the most paradigmatic example is the case of nanosilver where the bactericidal effect of AgNPs have found to be correlated to the amount of Ag^{+} released ions [103-107]. Another example is the case of Quantum Dots. Derfus et al [79] showed that the intracellular oxidation and toxicity of CdSe QDs was due to the release of Cd ions. Cadmium binds to sulfhydryl groups of critical mitochondrial proteins leading to cell death. Physiollogical levels of metallothionein, a protein found in the cytoplasm of hepatocytes which detoxifies cadmium by sequestering it into an inert complex, were not sufficient to cells exposed to the high levels of Cd^{2+} ions released from the QDs.

Similarly, to name a few other examples, toxicity studies performed on freshwater alga *Pseudokirneriella subcapitata* revealed comparable toxicity of ZnO (30 nm) and dissolved $ZnCl_2$ salts [95], thus presuming that effects must be attributed to Zn^{2+} ions. Also, it was found that the inhibition of wastewater nitrogen and pohosphorus removal induced by higher concentrations of ZnO NPs was due to the release of zinc ions from

ZnO NPs. It seemed that these Zn^{2+} ions caused an increase of the ROS production and an inhibitory effect of the responsible microorganisms of that removal [96]. Metabolization of magnetite/maghemite iron oxide NPs has also been described in the rat liver [108]. Also, it has been reported that CNTs can be biodegraded through enzymatic catalysis [109]. Finally, even "the most noble of the noble metals", AuNPs. Gold, is recognized as inert and not biodegradable and that is why it is used in medicine (stents) or dental restoration. However, also gold dissolves in biological environments. Larsen et al, [102, 110] showed that the extracellular liberation of gold ions from the surface of metallic gold implants reduced microgliosis and neuronal apoptosis and increased neural stem cell response after a focal brain injury. Interestingly, this last case shows that in the same way as the NP corrosion phenomenon could result in biological or environmental damage, this process could be harnessed for different applications, as the delivery of specifically desired compounds to specific targets.

Finally, in other cases, as in corrosion, were electrochemical processes are involved in the NP dissolution, this effect could be toxic by itself due to the release of electrons. Such redox activity could also be related to the general observation that NPs cause oxidative stress both *in vitro* and *in vivo* [54]. To name a few, ROS production, a non specific cell defence mechanism has been found induced by NPs as diverse as QDs, TiO_2, ZnO, C_{60}, SWCNTs and DEPs, especially under concomitant exposure to light, UV, or transition metals [79, 111-115]. The generation of ROS, as a result of NP reactivity, and the speciation of the released ions, play important roles when studying the potential toxicity and ecotoxicity of NPs to the extent that ROS induction is being un-officially established as a nanotoxicity paradigm [116]. Morevoer, the generation of ROS by NPs could be considered as a basal link between physics (electronic structure), chemistry (redox potential) and biology (bio-oxidation), and therefore taken as a model for bio-nano interaction.

Conclusion and Future Perspectives

To face the challenges presented by NP reactivity in biological environments, in the fields of nanotoxicology and nanomedecine, as in many others, there is a need for multidisciplinary integration. Biological applications of nanotechnological designed objects still suffer from gaps between the different disciplines. On one side, chemists, physicists, and engineers create new advanced materials of sophisticated functionality on a daily basis, but their understanding of biology is usually limited. This leads to biological studies using precisely engineered NPs while ignoring key biological facts such as the NP solvent (the medium in which NPs are dispersed) must be also biocompatible to truly determine the "NP" effects. On the other side, in biological contexts, effects of nanoparticles on cells are typically investigated with relatively undefined nanoparticles with large polydispersity, limited colloidal stability, unknown surface chemistry, etc. Thus, either NPs may be truly toxic or their smaller sizes have provided them with unique physico–chemical properties, these potential hazards and/or effects must be analyzed independently. For that purpose, appropriate NPs characterization is needed to understand the processess, otherwise conclusions may be weak.

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Figures.

Figure 1.

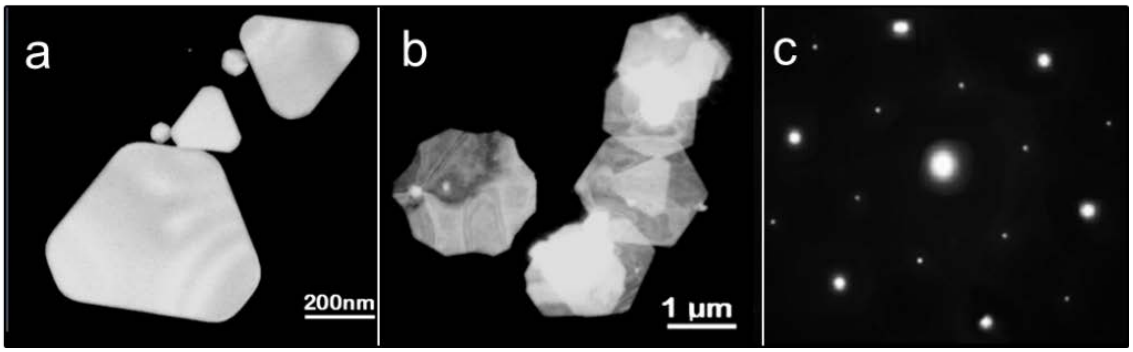

Figure 1. 1a and 1b) Scanning electron microscopy image of truncated triangular and hexagonal gold nanoplates synthesized by polyol method. **1c)** Corresponding electron diffraction pattern of b). The pattern show six symmetrical {220} spots that corresponds to face-centered cubic single crystals and the incident electron beam perpendicular to {111} facet of the observed plate.

Figure 2.

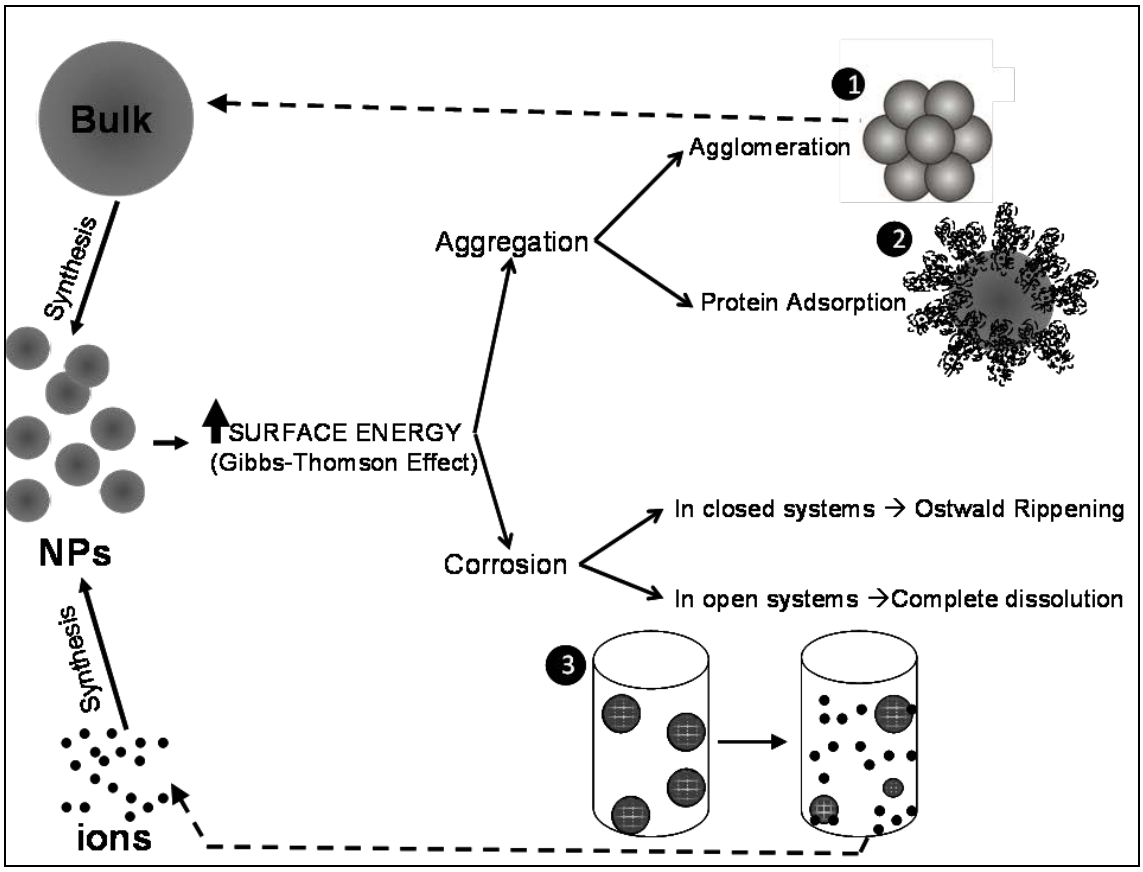

Figure 2. Physicochemical modifications of inorganic NPs in biological environments. Once NPs are produced they tend to minimize they high surface energy. The Gibs-Thomson effect refers to the observation that small crystals of a liquid melt at a lower temperature than the bulk. This is explained since as size decrease, surface tension increase. That is why NPs are systems in a metastable phase and their final fate is their aggregation or dissolution towards more stable phases. NPs can aggregate with other NPs (agglomeration, 1), NPs can adsorb organic or biological molecules on their surfaces (e.g. protein corona formation, 2), or NPs can be dissolved into its constituents atoms (corrosion, 3). This last process is translated in closed systems into Ostwald Ripenning, where the equilibrium between atoms in solution and atoms in the NP results in a broadening of the size distribution with time. In open systems, the dilution of ions in solution could drive the NPs to full dissolution.

Figure 3.

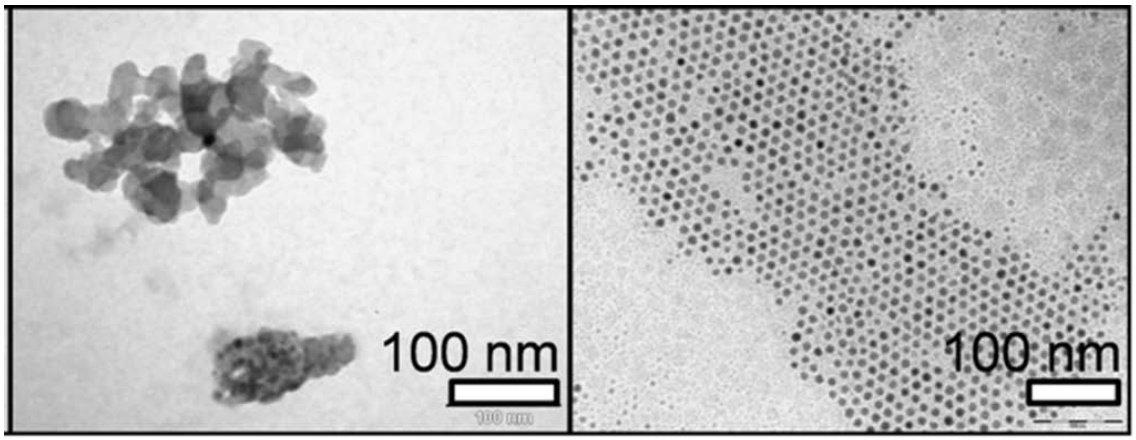

Figure 3. TEM images of commercial Co nanopowders resuspended in water(right) and CoNPs synthesized in the laboratory following reference [33] for comparison (left). Size, shape, surface state, additives and colloidal stability is critical and thus well defined in the syntesized ones while, in contrast, grains of nanopowders are polydisperse in size and shape, they could be oxidized and there is an intrinsic aggregation of the individual grains to a certain level.

Figure 4.

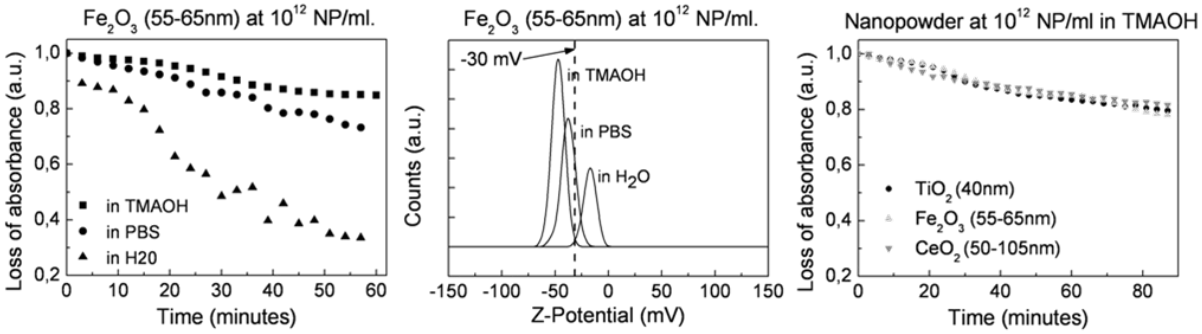

Figure 4. Stability of resuspended commercial nanopowders in different aqueous media: Left: Loss of absorbance for γ–Fe_2O_3 nanopowders resuspended in different media. TMAOH 10mM (squares), PBS (spheres) and H_2O (triangles). Resuspension in organic content biological media as collagen and cCCM result in immediate precipitation (data not shown). **Middle:** Z-Potential graphs of γ–Fe_2O_3 10^{12} NP/mL in TMAOH (-47 mV), PBS (-37,7 mV) and H_2O (-17,6 mV). Black line at -30 mV shows the theoretical limit of stability. Saline media (PBS and TMAOH) better stabilize NPs while H_2O resuspended nanopowders are below that limit. **Right:**Loss of absorbance of TiO_2, γ–Fe_2O_3 and CeO_2 nanopowders resuspended in TMAOH 10mM. It can be observed that all materials followed similar patterns of destabilization.

Figure 5.

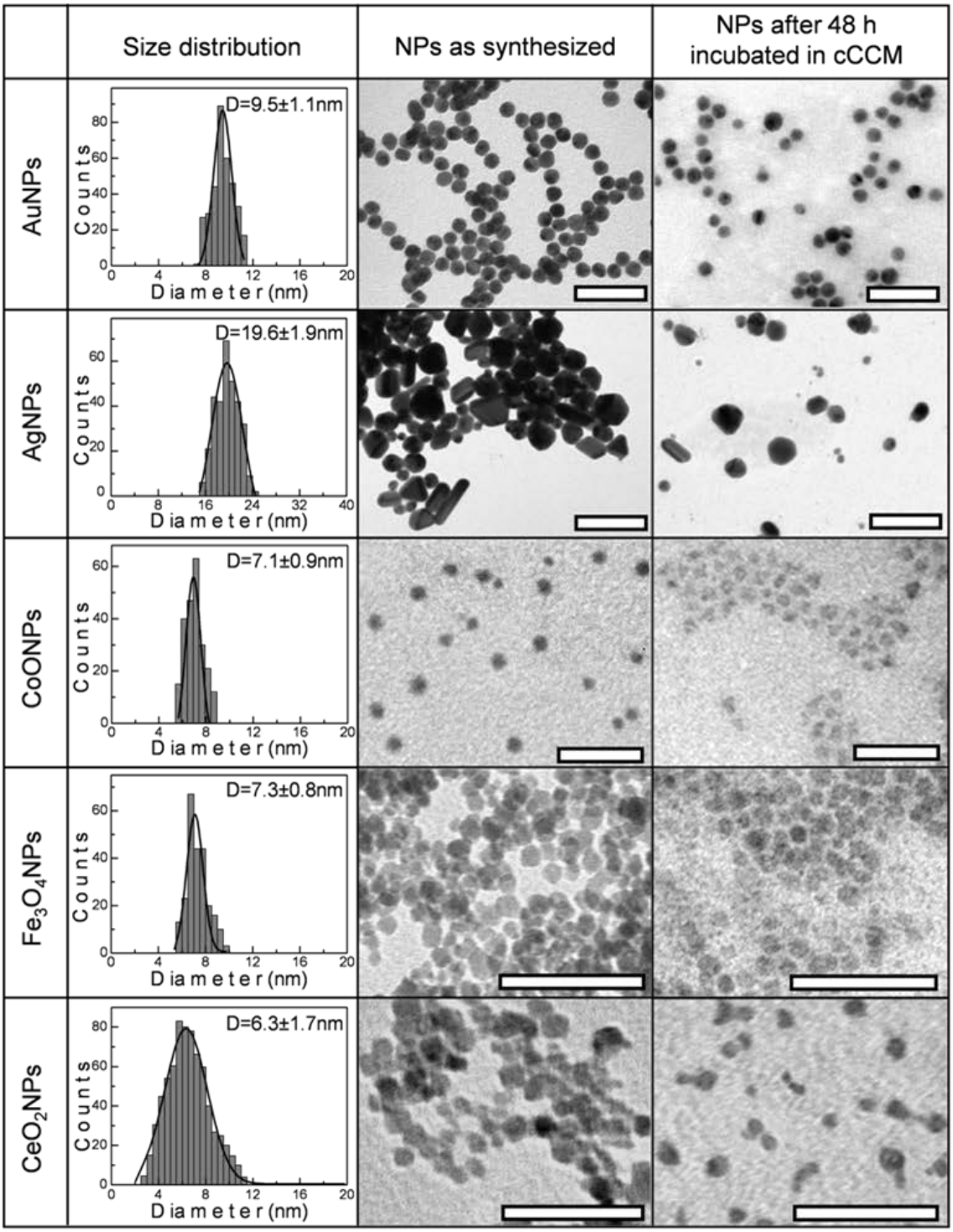

Figure 5. Inorganic NPs in complete cell culture medium: Left and middle colums: Size distribution and TEM images of the NPs as synthesized used in this study. **Right column:** TEM images of the same NPs after 48 hours in cCCM. NP morphology and size distribution in cCCM experiment modifications that could be attributed not only to protein adsorption but also on NP corrosion as shown in section 6. Scale bars are 50 nm.

Figure 6.

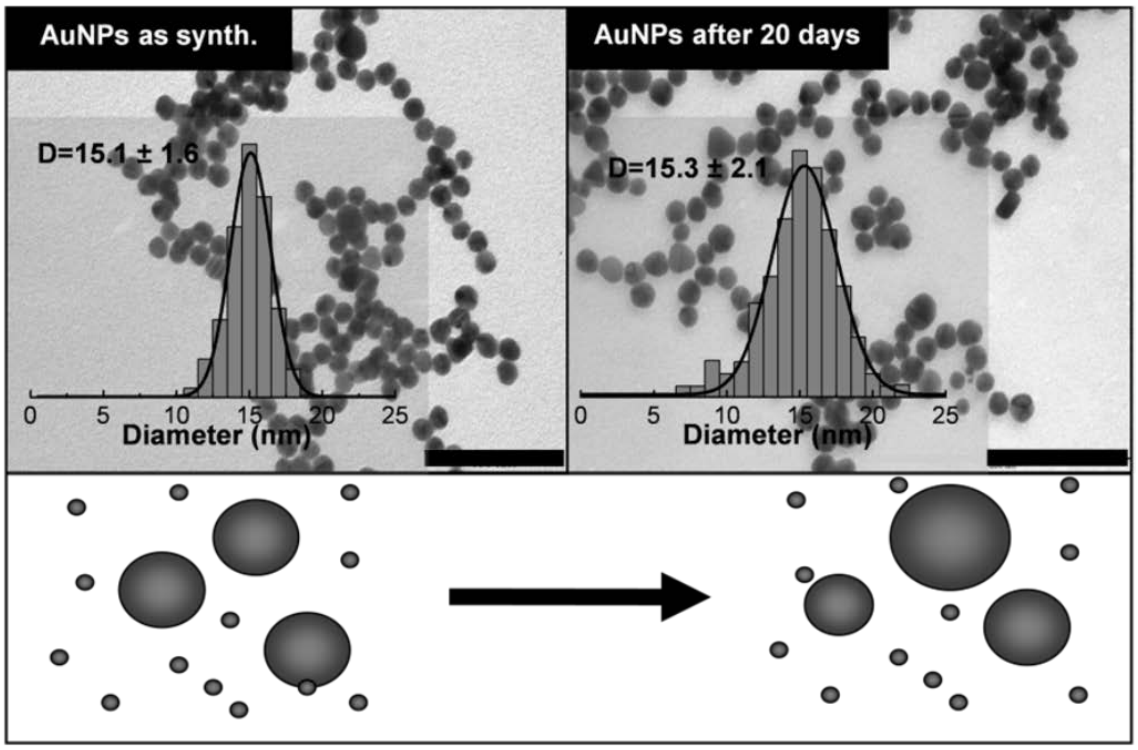

Figure 6. AuNPs degradation over time: Up: TEM images of 15 nms AuNPs as synthesized (left) and the same AuNPs after 20 days on the bench (right) which reflects the modifications on morphology and size distribution. Scale bars are 100 nm. **Down:** Schema of the rippening process.

Figure 7.

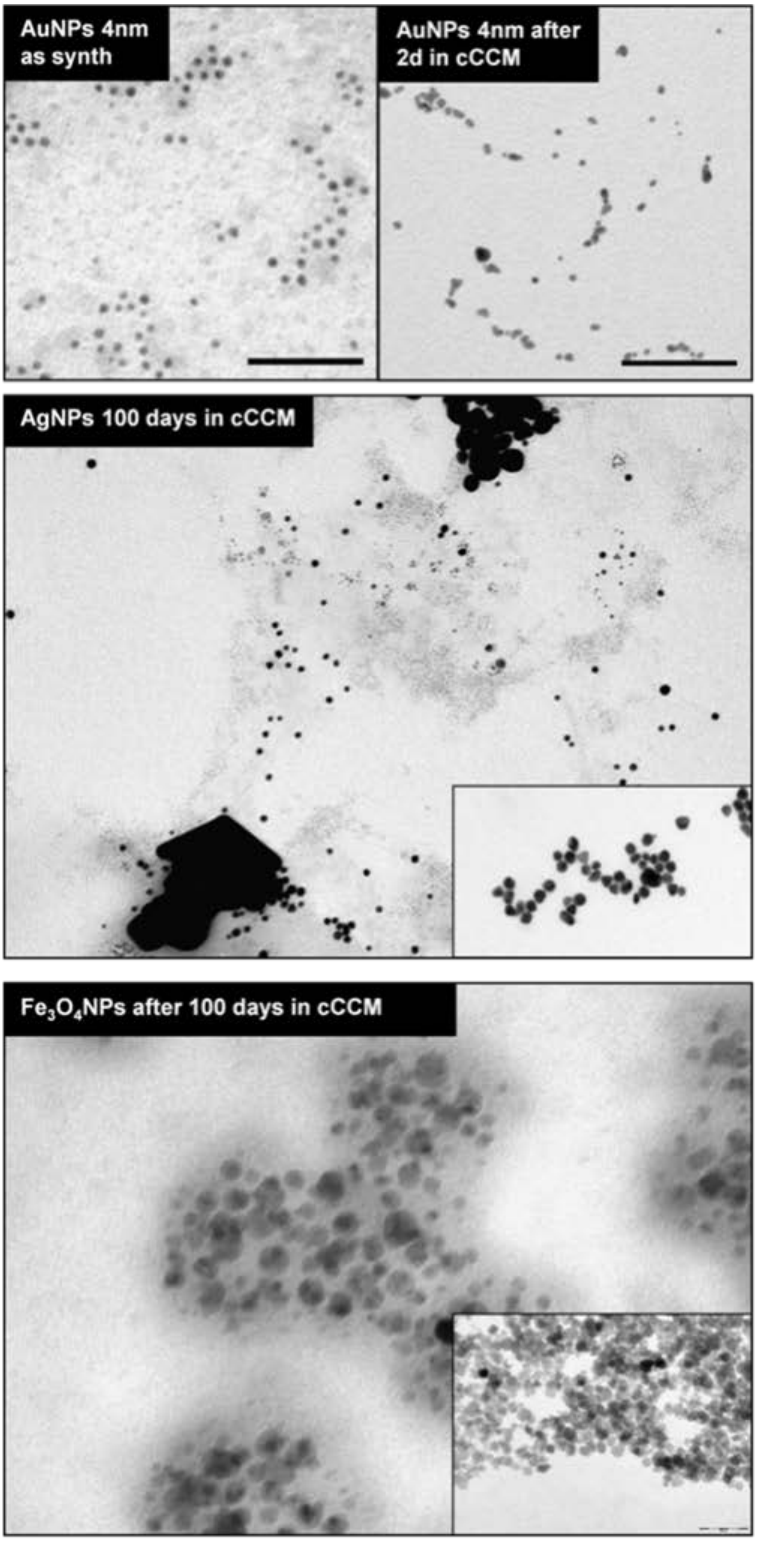

Figure 7. Morphological modifications of NPs over time. Up: TEM images of 4nm AuNPs as synthesized and after 2 days in cCCM. **Middle and Down:** TEM images of AgNPs and Fe3O4NPs after 100 days in cCCM. **Insets** show these NPs as synthesized.

Supporting Information.

Resuspension of fine powders (nanopowders) for nanotoxicity studies.

1. Dissolve the nanopowder in PBS at 1 mg/ml ratio. This will yield about 100 □g/ml in cCCM.
2. Sonicate this solution for 30 minutes.
3. Wait for 1 hour to settle the newly formed agglomerates, and discard them recovering the non-precipitated fraction.
4. Sonicate this other solution for 30 minutes.
5.- Wait for 1 hour. At this point is likely that other precipitate appears, which must be discarded again. Keep this precipitates to weight them and to known how much sample have been lost from the solid to the colloid. Our estimations are about a 10% lost in the process, but the size distribution is now truly nanometric.
6. Expose to the cells right after the procedure. Even though this procedure, stability is not very strong thus cells will experiment some “mild persistent rain” of NPs. To avoid that, concentrations and sizes have to be smaller.

Figure S1.

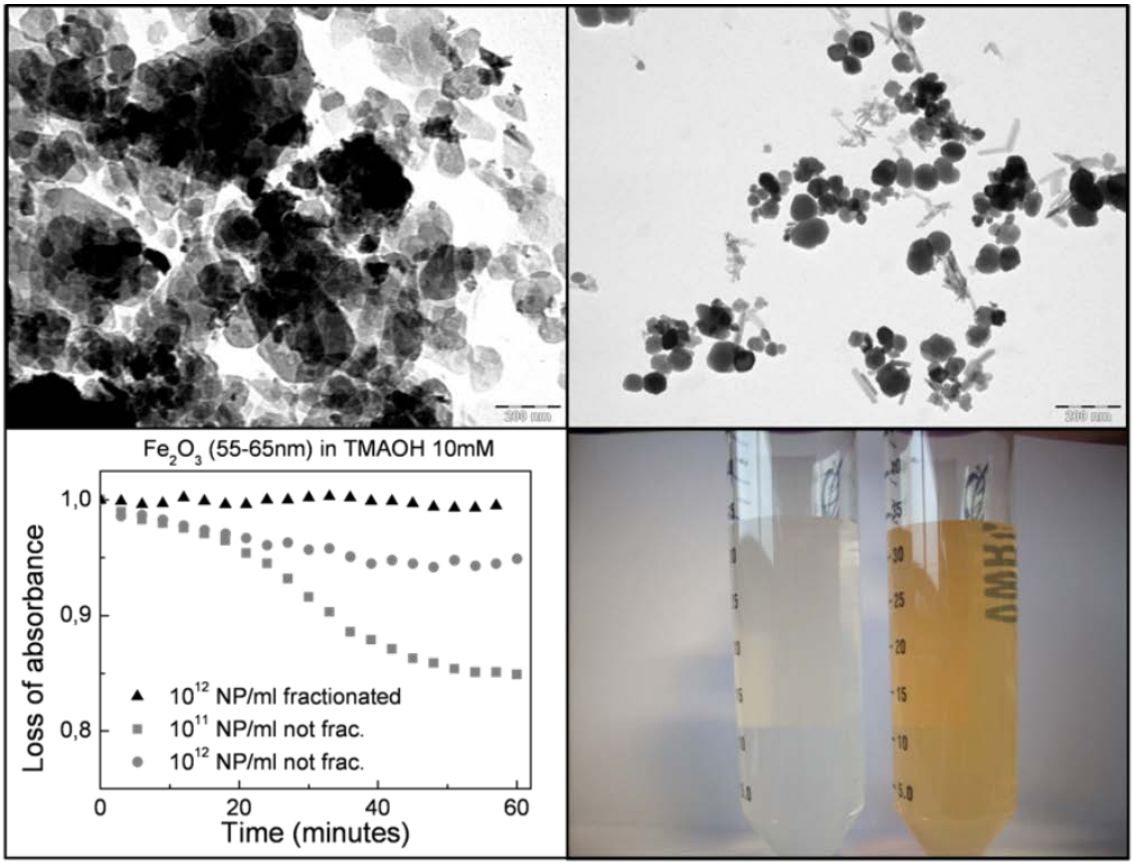

Figure S1. Size fractionation of commercial nanopowders. Up: TEM images of γ–Fe_2O_3 nanopowder before fractionation process (left) and after fractioning (right). For fractionantion process details see supporting information. Scale bars are 200 nm. **Down left**: Loss of absorbance of γ–Fe_2O_3 nanopowders not fractionanted at 10^{11}NP/mL (circles) and 10^{12} NP/mL (squares) and fractioned nanopowders at 10^{12} NP/mL (triangles). As an example, measures of absorbance were taken during the first hour after resuspension or after fractionation. The improved stability of the fractionated powder is clearly seen. **Down right**: Liquid fraction after 1 month on the bench of fractionated and not fractionated γ–Fe_2O_3 nanopowders resuspended at 10^{12}NP/mL in TMAOH 10mM. Clear liquid correspond to the not fractionated sample and characteristic orange liquid of iron oxide colloids is fractionated sample.