Potential Nanoparticles Toxicity

Today, the majority of innovation in nanotechnology is restricted to spin-offs generated in the academic environment where funding agencies set research efforts towards common goods, in medicine, energy and environment.

From the societal point of view, and for similar reasons (small companies cannot financially support worldwide patents), patents are only presented in *rich* countries where benefits are more secured, allowing its free implementation in the rest of the world. Therefore, in these cases, the most critical point regarding responsible development is safety, and safety comprises human and environmental safety. Therefore, the question we need all to pose is: *does the nanoform of a substance entail an increased risk?*

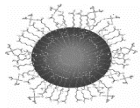
This question is fundamental to us. The potential negative impact of nanotechnology in health and the environment has worried society, and even if this is often overlooked by scientists, technologies need to get introduced into social environments and therefore the applications that develop are shaped by a mix of social and technological forces. If society embraces and finds uses for a technology, then it survives. Otherwise, not matter how good the technology is, it will die. Thus, effective communication is mandatory, which should include, in addition to state-of-the-art performance, safety studies, and a nanosafety-by-design approach contributing to full life cycle assessments and viability studies. Because of this, we take the advantage to describe our position regarding potential nanoparticles risk in the frame of our work.

The surprising properties of NPs are fundamentally due to their high surface to volume ratio, finite size effects, collective behaviour and interaction with light of any wavelength (for hyperthermia, diagnosis and imaging purposes) This results in a broad spectrum of chemical, physical, catalyst, optical and magnetic behaviours which can be sized for many uses. Interestingly, their exuberance of degenerated states at the macromolecular level allows their use as versatile molecular sensors and actuators, as much as it makes them complicate to master. For similar reasons, nanoparticles are intrinsically unstable and may easily heterogeneously or homogeneously aggregate, chemically transform and corrode and disintegrate. To be exposed to biological systems, for a nanoparticle, it suffices to have few albumin proteins absorbed onto it and then they can be introduced in physiological environments where many are dissolved and metabolized. In principle, it has been observed up to now that cells deal easily with tiny particles, and no significant acute toxicity has been found in in vitro and in vivo studies at realistic doses, unless toxic components were present in the formulations.[1]

At the origin of nanotoxicity and nanosafety concerns, it was pointed at the well-known fact that cells have problems dealing with *micrometric* insoluble particles. Asbestos fibers, with dimensions greater than 20 micrometers, up to hundreds, induce frustrated phagocytosis[2], chronic inflammation, asbestosis, and years later, cancer. This is not the case for small, sub-micrometric particles. A concern then was if small NPs could accumulate and aggregate up to such dangerous sizes. In this regard, NP dose and persistency are key to determine this potential risk. If the NPs do not aggregate, they may dissolve. When they

[1] Harald F. Krug Angewandte Chimie 2014, 53, 12304–1231

[2] https://diamondenv.wordpress.com/2011/04/15/frustrated-phagocytes-and-the-fibre-paradigm/



dissolve they yield ions (metal cations) that may be toxic, as in the well-known case of cadmium or silver NPs. In parallel, the corrosion process is a redox active process that may stress the cell environment. However this effect has been observed to be transient and only significant at rather high doses. Therefore, regarding nanotoxicity, and the associated risks to work with NPs, current knowledge indicates that many NPs in their intended uses do not need special care beyond being treated like other chemical substances, even if some particularities may apply.

Nanotoxicity is a young field that can be considered to be about 10 years old. Despite this youth, much knowledge from metal toxicity, microparticle toxicity (sarcoidosis, asbestosis, silicosis), environmental pollution and other disciplines have contributed significantly to the rapid establishment of the nanosafety discipline. It is also important to be aware that simple nanoparticulate materials have been used in consumer products for a long time, as food additives (E-171 to E-175 have a nanometric portion of iron oxide, aluminium oxide, titanium dioxide, silver and gold, respectively), in cosmetics, as simple as talc, catalysts, paint pigments, coatings and others. Up to now, we have been mainly reproducing nanomaterials that already exist in nature or that somehow are already produced by man in a more imperfect and unaware manner. Small, (about 20 nm) iron oxide NPs have been found in natural unpolluted soils or inside bacterial magnetosomes, and nanometric TiO_2 has been used by the tonne in the cosmetic industry as sun screens and other formulations for decades now. When we get the next generations of nanoparticles, additional care will need to be considered. Before that, and as no acute effects have been observed or identified, more subtle effects will need to be investigated. Also, these results are related to healthy conditions and acute doses. Thus, despite the absence of signs of alarm, it is desirable to perform long term studies at chronic and subtoxic doses and in compromised states (when the body is weakened by disease). Alterations of the immune system and changes inbiodistribution in the case of inflammation might exacerbate or suppress acute effects and accumulate in organs (if the NPs succeed in entering the body, which is very unusual, even after dermal contact or ingestion). Thus, chronic exposure at subtoxic doses, long term effects, repeated doses, or co-exposure of different types of NPs and other toxins (such as LPS, allergens or chemical toxins), or exposure to NPs in the case of disease, e.g.: during cirrhosis may be more critical and need to be studied. Focus has to be put also on the immune system, which is responsible for detecting, categorizing and managing external invasion. The immune system has memory, so repeated exposure to NPs could alter immune response.

Nanoparticles may exist in different forms during their full life cycle, normally: pristine (as synthesized), functionalized (ready to be used and during use), disposed and degraded (after use). The exposure and biological effects depend on the state of the NP at each point of their life. Iron oxide nanoparticles have not been found toxic in any of these forms unless they were functionalized with toxic moieties.

While it has been observed that NPs do not penetrate the skin and are not up-taken after ingestion, concerns remain with respect to pulmonary exposure. It is the ability of small dry NPs to be aerosolized from dry powders and enter the lungs. Experimental studies in animals have shown that at equivalent mass doses, poorly soluble nanostructured metal oxides in the form of agglomerated or aggregated nanoparticles (e.g., titanium dioxide, aluminium oxide, and manganese dioxide) are more potent in animals than equivalent single well dispersed particles of similar composition in causing pulmonary inflammation and tissue damage. For these and other poorly soluble particles, a consistent dose-

response relationship is observed when dose is expressed as particle surface area. These animal studies suggest that for nanostructured materials and larger particles with similar chemical properties, the toxicity of a given mass dose will increase with decreasing particle size due to the increasing surface area.[3] Therefore, the breathing of solid nanoparticles, especially aggregates made of persistent materials, is highly unadvisable. However, even for poorly soluble particles of relatively low toxicity, animal studies have identified doses that were not associated with observed adverse responses.[4] For example, a recent animal study reported mass doses of either fine or nanostructured TiO_2 in rats at which the lung responses did not significantly differ from controls, while crystalline silica caused more severe lung responses at the same mass dose.[5] In addition to particle size and surface area, other physical and chemical properties of particles are known to influence biological interactions, including solubility, shape, surface reactive sites, charge, and crystal structure.[6] Note that this is not the case for BioGAS+ which is made of non-persistent NPs; they are not aggregated and they do not carry toxic moieties or toxic additives or toxic excipients.

In the following the main causes associated to NP induced toxicity are listed. In principle, at realistic doses in a controlled manner, inorganic NPs have basically shown toxicity due to aggregation or dissolution, or because they were carrying toxic moieties.

i.- Toxicity has been observed in the case of some cationic (positively charged) NPs.

This is well known for both biological (antimicrobial peptides) and micrometric (organic) particles where cationic charge at their surface makes them interact strongly with cell membranes, thus interfering with its normal functioning and inducing cell death. See for example Chitosan functionalization of gold nanoparticles.[7] This charge is carried by molecules attached to the surface or by the inorganic surface itself if it is at pHs lower than the NP isoelectric point, although toxicity has only been observed when the cationic charge is maintained in the physiological media. In the case of BioGAS+, it is prepared at basic pH displaying a negatively charged surface which becomes neutral when dispersed in the working environment. At acidic pH, where the BioGAS+ NPs would present positive surface charge, they dissolve.

ii.- Toxicity has been related to aggregation.

Aggregates caused direct acute toxicity when mice were intratracheally instilled with carbon-nanotubes, and they suffocated due to tracheal clogging, indicating the poor dispersability of hydrophobic nanostructures in biological systems.[8] Risks have also been observed in the case of penetration of non-biodegradable persistent micrometric particles (in principle bigger than 20 micrometers) in the lungs and related with frustrated phagocytosis and the onset of chronic inflammation, as in the case of silicosis, granulomatosis and asbestosis. When a strange object is detected by the immune system, and not categorized as danger, it is simply phagocytized and removed away from the biological machinery whether denaturalized protein aggregates or cell debris. This applies

[3] Oberdörster, G., Ferin, J., Lehnert, B. E., Correlation between particle-size, in-vivo part icle persistence, and lung injury, Environ. Health Perspect. 102 (S5), 173–179, 1994.

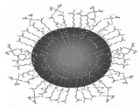
[4] Warheit, D. B., Webb, T. R., Sayes, C. M., Colvin, V. L., and Reed, K. L., Toxicol. Sci. 91(1), 227-236, 2006

[5] Warheit, D. B., Webb, T. R., Colvin, V. L., Reed, K. L., and Sayes, C. M., Toxicol. Sci. 95(1), 270–280, 2006.

[6] NANO TC 229 WG 3 072–2007__Revised Draft TR Health and Safety Practices 2007–10–19–1

[7] Chitosan functionalisation of gold nanoparticles encourages particle uptake and induces cytotoxicity and pro-inflammatory conditions in phagocytic cells, as well as enhancing particle interactions with serum components. *Journal of nanobiotechnology* 2015, 13 (1), 84.

[8] Warheit DB1, Laurence BR, Reed KL, Roach DH, Reynolds GA, Webb TR. Toxicol Sci. 2004 Jan;77(1):117–25



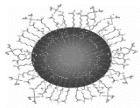
for nanoparticles (3 to 100 nm), viruses (20-400 nm), bacteria (≈1000 nm), and eukariota cells (≈10.000 nm). However, when the object is too big (beyond 10 micrometers),[9] the immune cells cannot engulf it and then triggers a chemical defence against the non-biodegradable material. This leads to tissue irritation and in the long run, may cause cancer. Needle-like microparticles such as asbestos 10 x 500 microns, are especially effective to induce this effect.

In determined conditions, NPs could aggregate to micrometric sizes. But as the size increases, the likeliness for exposure and particle penetration also decreases. There are many strategies to avoid aggregation developed for decades in different fields of material science and chemistry. There are two simple ways to avoid aggregation; to avoid high concentrations (if there are few NPs, it is difficult for NPs to meet to grow and form an aggregate), and to use anti-aggregation agents. Aggregation is a phenomenon thermodynamically favored, driven by the reduction of the high energy surface of the nanoparticles. Absorption of molecules which provide electrostatic charge or steric repulsion to the nanoparticle serve to maintain their isolation even at high concentrations. In complex media it is observed that nanoparticles are rapidly coated by molecules from the environment, their surface energy decreased and their tendency for aggregation cancelled. In the case of BioGAS+ only when the material is prepared is there is risk of aggregation, and none once they have been dispersed in the working environment. Likely, when inorganic NPs are dispersed in serum they are rapidly coated by proteins (forming the so-called protein corona) which avoids their further aggregation, which would always be a subject of concern. Also, aggregation can be programmed, for example as a way of disposal, producing aggregates which are larger than the micrometric critical size and easily operable as bulk materials.

iii.- Toxicity has been related to breathing dry (powdered) nanoparticles (and its aggregates).

Fortunately, NPs do not cross the skin and do not get inside the body from the intestinal tract (humans have been eating soil for millenia and naturally small NPs form and dissolve or aggregate constantly). The critical point here are clearly the lungs, even if the mucociliary escalatory system may be effective in removing foreign matter from the lungs (especially small NPs). Therefore, it is not recommended to be exposed to nanoparticle aerosols, and for that reason, it would be enough to avoid working in the dry phase. Wet NPs do not leave the solution, they stay in the liquid body and are not transferred to the atmosphere; if the drop is dried they aggregate and stick to the substrate. In a study of chemical contamination in the laboratory by electron microscopy and ICPMS, dispersion of the NPs from the liquid phase was not observed. The conclusions were that once the NPs have been somewhere, a tiny residue remains for ever even after washing (similarly with ions) but that there was no cross-contamination, even at extreme proximity from the vessels and vials that contained the solution. The ambient filters and air purifiers were empty of observable NPs (other than the micrometric particles of dust), concluding that the NPs cannot leave from the wet phase. At the same time, it has been observed that ultrafine powders of nanoparticles are easily aerosolized and transported long distances.

[9] Kostas Kostarelos Nature Biotechnology 2008, 26, 774 – 776.



iv.- Toxicity has been observed when the NP act as a reservoir of toxic ions that are delivered during corrosion.

The paradigmatic case is CdSe nanoparticles which *become more toxic* with time, as they corrode and yield Cd ions. Indeed, to dissipate surface energy, if the nanoparticles do not aggregate or associate with coating molecules, many of them will disintegrate. This is a common phenomenon in nature and widely studied by geochemistry where a nanoparticle is an intermediate state between the micrometric particle and the dissolved ions. Or, like in microbiology, where bacteria synthesize small inorganic nanoparticles of toxic ions to detoxify the environment. Changes in the surroundings when the nanoparticle leaves the synthesis environment lead many NPs to disintegration, by corrosion and other chemical transformation that dissolves it. In this process the NP yield ions, and also may yield electrons. Electrons are reactive and generate reactive oxygen species (ROS) which may be toxic if sustained for a long time (if the stress causing the response is maintained). Metallic cations are often bioactive, for example, cadmium, mercury and lead cations are very toxic to us, nickel is allergenic, cobalt is carcinogenic, silver cations are toxic to bacteria, and copper ions are toxic to fungi (and fungi are toxic to bacteria). Besides, iron is a common ion in biological systems at very high concentrations. Indeed, the slow dissolution of iron oxide NPs into iron ions has made iron NPs an active principle (feromuxytol) to treat ferropenic anaemia, controlling the dosing at the molecular level. Basically, the pattern of exposure, the dosing profile, is different when using the ionic species directly, or when these are provided by a dissolving NP (acute *vs* sustained dosing).

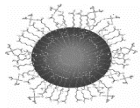
v.- Toxicity has been related to the capacity of NPs in presenting antigens or allergens.

NPs can be good aggregators and orientators of molecules to be presented to the immune system.[10] Indeed, nanoparticles are excellent molecular carriers and a whole scientific field is developing around it, which could be a cause of major concern if functionalizable nanoparticles were dispersed in the environment and unfortunately associated with antigens or allergens before homogeneous or heterogeneous aggregation. For example, when car combustion emission microparticles are coated by pollen grains, they become more allergenic. This is one of the reasons why allergies in urban areas are more intense than in the countryside. Therefore, the NP surface has to be passivated before being uncontrolledly dispersed. Fortunately, the concentration of toxins and allergens in the environment in comparison with the rest of the inert or tolerable molecules is very low and the promiscuity of the nanoparticle surface very high, so it would be unlikely that naked nanoparticles meet toxins, antigens or allergens before their surface is passivated by other molecules.

vi.- Toxicity has been associated also with catalysis.

Toxicity has been associated also with catalysis especially in the case of photocatalysis with NPs as TiO_2 that are able to generate toxic free radicals when illuminated. Catalysis is a surface phenomenon, and the high surface to volume ratio of small nanoparticles has been exploited for years in the chemical industry. Despite the natural suitability of inorganic NPs for catalysis, it is well known that it is unexpected that NPs will act as powerful catalysts unless they are designed to do so. Indeed, normally, nanoparticle surfaces are

[10] Homogeneous conjugation of peptides onto gold nanoparticles enhances macrophage response *ACS nano* 2009, 3 (6), 1335-1344



rapidly passivated with organic molecules that interface the inorganic core with the environment. Lacking that protecting layer, nanoparticle life is extremely brief and they absorb irreversibly or vanish. In this case, the protecting layer dumps the catalytic powers of the inorganic nanoparticle. This is the case of TiO_2 coated with a thin transparent layer of Al_2O_3 in sun screens. The TiO_2 is still able to absorb the high energy photon, but the electron thus generated is buried at the interlayer and rapidly recombines without generating the free radicals responsible for photocatalytic TiO_2 induced toxicity. In any case, iron oxide nanoparticles are not photocatalyst, nor considered effective catalyst with some exceptions for oxidation reactions.

vii.- Toxicity has been related to hydrophobicity.

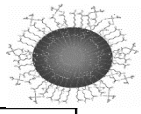
Toxicity has been related to hydrophobicity and since hydrophobic substances hardly disperse in biological environments, attention has to be paid to amphiphilic or detergent-like molecules that can be transported by NPs, such as in the well-known case of gold NPs coated with a cationic detergent like CTAB[11]. This detergent forms a double layer vesicle-like coating on the NP surface and can be dispersed in biological environments and then, in contact with cells, they may expose their hydrophobic core to the cell membrane (which has also a vesicle like structure with an inner hydrophobic core), perturbing it. These are similar effects to those observed with pure detergent molecules; however, it would happen at lower detergent doses in the case of association to NPs. Therefore the unintended mix of detergents and NPs it seems inadvisable. Note that detergents are already poorly biocompatible, although fortunately they are normally highly biodegradable.

viii.- Toxicity can be observed if tissue is irradiated when nanoparticles are present.

The only toxicity related to irradiation of a NP containing body is related to the increased dosing of the received radiation. Therefore one should not be exposed to radiation, magnetic hyperthermia in the case of superparamagnetic nanoparticles, or x-ray radiotherapy in the case of heavy metal nanoparticles. Note that for MRI imaging superparamagnetic nanoparticles are used as safe contrast agents.
All this knowledge allows us to work under nanosafety by design paradigms. Safety by design, from its definition, is a concept and movement that encourages construction or product designers to "design out" health and safety risks during design development. The concept supports the view that along with quality, program and cost; safety should be determined during the design stage in order to avoid development of technologies that result in being unsafe once they are already developed. Otherwise, we will suffer until the technology is forbidden, after problems have already been created, and the environment, polluted for decades.

Thus, risk mitigation methodologies can be developed and implemented taking into consideration the whole life cycle of a nano-enabled product. Innovative safer-by-design approaches beyond surface modifications can be designed, taking into account all the existing information on structural features that determine NPs' toxicity, release and degradation. In the case where hazards are found, NPs have to be re-designed so that detrimental specific NP characteristics are decreased while maintaining the desired unique intended parental properties. These strategies should not only focus on reducing NP hazards, but on reducing NP release from matrices or promoting their degradability after release.

[11] http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2988217/



To reduce toxicity of the NPs.
Shape and size modifications.
Increase in hydrophilicity to decrease the potential to cross biological membranes.
Increase in lipophilicity to promote aggregation and precipitation.
Modification of the intrinsic bulk composition of NPs or changing oxidation state to mitigate reactivity.
Design NPs that lose their catalytic activity when released from their embedding matrices
To minimize release of NPs from their matrices.
Induction of strong van der Waals and covalent bonding between the NPs and their matrices.
Development of barriers by multilayer approaches involving development of multilayer films, or multicoating approaches.
Self-healing pairs of NP / matrix by developing a suitable ionic approach to improve the formation of ionic bonding inside the host.
Induction of self-assembly of NMs in aqueous media or at high temperatures by introducing labile or different functionalities to increase the coalescent character of the NPs (sintering).
To reduce persistence of NPs
Development of new high biodegradable NPs under certain temperature or oxidative conditions.
Modify oxidation state of NPs.
Introduce impurities to increase the instability and degradability of NPs.

Table 1. Safety by design approximation extracted from GuideNANO (www.guidenano.eu)

In addition to safer-by-design approaches, best practices for handling / packaging, different levels of confinement, and use of general exposure control measures and personal protective equipment (PPE) have to be included. The protection factors towards NPs for existing PPEs have yet to be fully evaluated, but in principle, protection against chemical substances does work for protection against nanoparticulate matter. When necessary, technological solutions have to be developed for exposure reduction and PPE (e.g., selecting less permeable materials, introducing double layers, use of nonwoven fabrics, and ventilated/pressurized systems). Depending on the efficiency of different exposure reduction technologies available, technological improvements in water and air filtering (e.g., foam technology) and treatment (destruction of NPs) may need to be developed.