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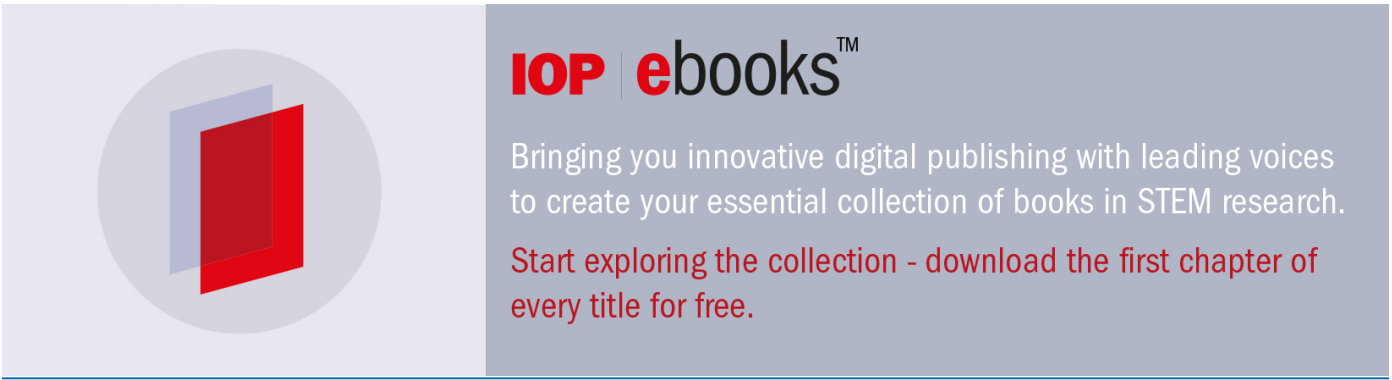

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Safer by design strategies

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Abstract. Throughout the EU funded FP7 project GUIDENano, we are trying to control and monitor the evolution of nano-enable products during their lifecycle. Small alterations of the nanoparticle (NP) state may have critical consequences on the NP behaviour and performance. For this reason it is important to highlight the importance of an extensive and proper characterization to define the NP physico-chemical characteristics under several environmental conditions. Furthermore, this characterization is necessary to ensure that obtained results are reproducible and allow understanding the behaviour of the NP on biological systems. In this paper different strategies reported in the literature regarding the safety-by-design concept are summarized. Several strategies from the synthetic point of view that help us to modulate the main factors which determine the safety of nanomaterials are proposed.

1. Introduction.

The growing interest in the field of nanotechnology leads to an increase in the number of nanoparticles (NPs) producers and users, making indispensable the promotion of safe strategies in the development and management of nanomaterials (NMs).[1–3] In fact, the rise of the production of NPs is estimated on thousands of tons by 2020,[4] accompanied by the increase of congresses, publications and research projects related to nanosafety in the last 10 years,[5] and consequently reflected in the increasing number of nano-enabled products that reach the market. However, some concerns have been arisen over current uncertainties on the safety of such products and they need to be carefully addressed to avoid human and environmental damage which could hinder the development of the nanotechnology field. Consequently, the exposure to nano-sized materials (NM) and their potential to cause harm to humans and other biological organisms have focused the attention of several recent studies.[6–9] In these studies, it is agreed that there is a need to understand the NP behavior during its full lifecycle in order to design materials with a high yield and quality at the same time along with well controlled properties. In fact,

if there are not more nano-enabled products in the market is partly due to the risks associated with nano enabled-products by consumers, regulators and insurance communities. It is in this context, that safety by design strategies, are translated into *nanosafety by design* strategies, which needs of a precise knowledge of NP behavior in order to properly design safe products.

The need to develop commercial products that are at the same time useful and safe, starts at the very beginning of their conception. The objective is establishing safer by design selection rules and synthetic approaches that can be used for the reduction of nanotechnology associated risks. Taking into consideration all stages of the life cycle of these products, these rules should protect the safety of workers, users and consumers and product end of life. In detail, safer-by-design approaches aim at the reduction of NMs hazard and exposure, the reduction of NMs migration and release, and the controlled degradation of these once they are released from their matrices. Traditionally, several strategies have been employed towards these aims:

1. **Reducing toxicity** of the employed materials. For instance, **i)** avoiding the use of intrinsically toxic elements or substances where possible, e.g. Cd,[10] **ii)** modifications in the size and shape to reduce the toxicity of the NMs in a specific biological system;[11] **iii)** increase in hydrophilicity to decrease the potential to cross biological membranes; or **iv)** changing the oxidation state to mitigate NP reactivity. Regarding size, an important point is reported in the case of non biodegradable inorganic fibers longer than 10 μm where macrophages cannot engulf them, setting then a defense action well known as frustrated phagocytosis.[12] In these cases, the NPs cannot be completely phagocytosed inducing thus chronic inflammation and consequent carcinogenicity (*asbestos-like* effect).[13]
2. **Reducing release** of NMs from the matrix during their life cycle. Normally, NPs are components embedded in a solid or liquid matrix forming part of different products. It is possible to reduce the release of NM from the matrix by controlling van der Waals, ionic, coordination and covalent bondings between NP and matrix using ligands and compatibilizers.[14] In the case of inorganic NPs, not only the release of the NP should be controlled but also the release of its constituent ions, since toxicity of NPs have been often attributed to their leached ions rather than the NPs themselves.[15,16] In this case, encapsulating the NMs makes possible to preserve the properties of the core material along with protecting it from dissolution, as in the case of ZnO NPs coated with a *nanothin* amorphous silica.[17]
3. **Reducing the persistence** of NMs. If NPs are used massively in consumer products they will inevitably end up in the environment. Thus developing strategies which control the end of life of the NPs are needed. As NPs have a high surface energy, they are prone to both aggregation and dissolution.[18] Thus, NPs can be irreversibly aggregated (and easily sintered) until they reach bulk sizes of well-known materials that we know how to deal with safely, or otherwise, NPs can be corroded into ionic species where our knowledge to safely deal with them is broad.

In this work, the aggregation of TiO_2 NPs and corrosion of silver NPs (AgNPs) was monitored in order to establish procedures where the NP is not only defined by its composition but its physical (colloidal) and chemical stability, so they can be properly and safely handled.

2. Experimental procedures: synthesis of nanoparticles

The nanoparticles used in the studies were synthesized as follows.

2.1. TiO_2 nanoparticles

TiO_2 nanoparticles were prepared following a procedure previously described on the literature by precipitation of Titanium tetrachloride ($TiCl_4$) in aqueous medium.[19] The stock solution of Ti^{4+} (0.7 mol/L) was prepared by dissolving the $TiCl_4$ precursor in HCl (3 mol/L) solution. For the production of TiO_2, an aqueous Ti^{4+} stock solution (20 mL) was diluted in Milli-Q water (270 mL) at room temperature. The pH of the mixture was fixed at 5 by the addition of NaOH (3 mol/L). Suspensions

were aged at 60 °C for 24 hours and the solid was collected by centrifugation. Samples were further purified by three centrifuge cycles with water of the same pH as used for the synthesis and finally re-suspended in an aqueous solution of TMAOH.

2.2. Silver nanoparticles (AgNPs)

The typical procedure for the synthesis of AgNPs have been previously described on the literature.[20] Briefly, 100 mL volume of aqueous solution containing sodium citrate (SC) (5 mM) and tannic acid (TA) was prepared and heated with a heating mantle in a three-neck round bottomed flask for 15 min under vigorous stirring. A condenser was used to prevent the evaporation of the solvent. After boiling had commenced, 1 mL of $AgNO_3$ (25 mM) was injected into this solution. Then, the reaction was cooled until the temperature of the solution reached 90 °C. Then, 100 μL of SC (25 mM), 250 μL of TA (2.5 mM), and 250 μL of $AgNO_3$ were sequentially injected (time delay 1 min). By repeating this process and adjusting the amount of Ag precursor injected, different AgNP generations of progressively larger sizes were grown. Aliquots were purified by centrifugation at 18000 g for 20 min in order to remove the excess of TA and further redispersed in Milli-Q-water or SC 2.2 mM before sample characterization.

3. Results. NPs Evolution.

A comprehensive knowledge of the NPs evolution during their intended use but also storage and disposal, and knowledge on their most common transformations, opens the possibility of making safer NMs.[21] The importance of a proper and extensive characterization to understand and control possible variations on the starting material is crucial in the development of improved NMs.[22] The use of reliable equipments and commonly present at laboratories such as UV-Vis Spectrophotometer, can provide useful information about physico-chemical properties of NMs. Furthermore, it allows us to characterize variations on NMs properties such as sizes, morphologies or the effectiveness of binding a coating layer to the NP surface quite univocally. Other techniques are needed to fully characterize NMs, as electron microscopy and X ray diffraction, and others spectroscopic techniques are also fast and sensitive to the NMs state, as Dynamic Light Scattering or Nanoparticle Tracking Analysis, however, the former are complex and the latter are simple but difficult to interpret, making UV-Vis a good tool for the rapid assessment of NM sample transformation.

It is well-known that NMs possess unique and different physico-chemical properties compared with their corresponding bulk materials.[3,15] These properties lead to biological effects which could be completely different to their reference materials **(Fig. 1)**.[23] In fact, initially, the European Commission Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has concluded that NMs might have different toxicological properties compared to bulk substance and thus the risks of the NMs should be assessed case by case.[15,24] However this would be extremely expensive and cumbersome, so general it is expected that as more NMs are analyzed and studied, behavioral trends of different families of NMs will arise allowing for class grouping and simplify tracking and monitoring of NMs. This has been done previously with pharmaceuticals, nutriceuticals and chemicals and there is no reason why this could not happen with NMs. Thus, classification of NPs in families depending on their biological effects, and identification of the specific parameters that controls NP toxicity, will be a more realistic approach to NP safety and it is being already addressed by the scientific community.[25]

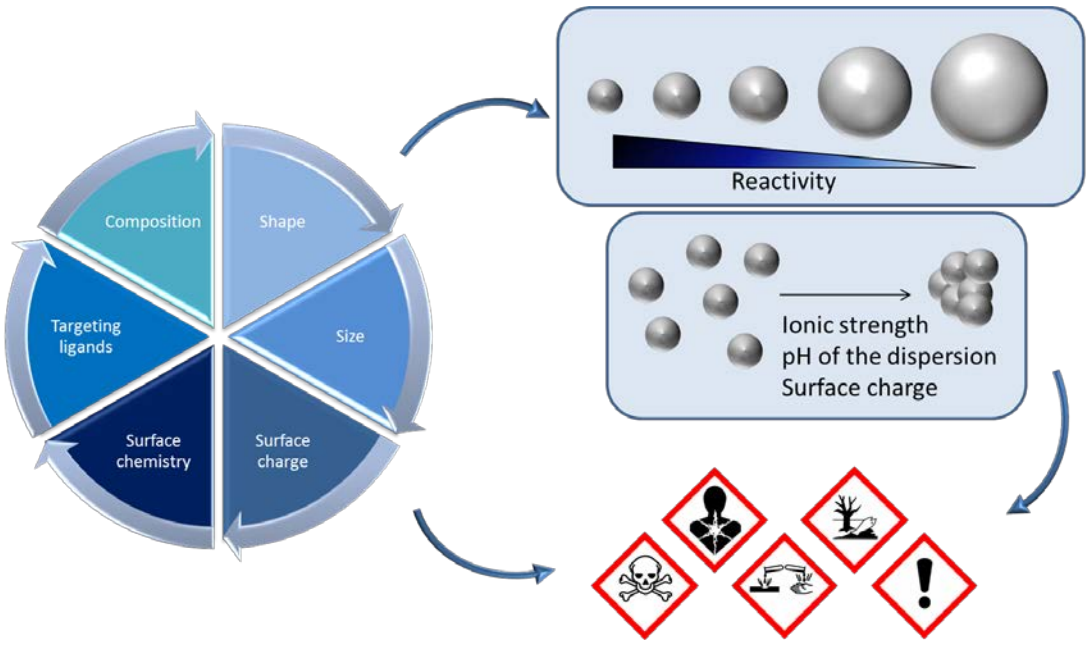

Figure 1. Illustration of physico-chemical characteristics of nanoparticles associated with their possible toxic properties.

Two of the most common transformations that NPs can undergo when dispersed in biological media, are aggregation and chemical degradation (oxidation, corrosion, dissolution). Controlled colloidal stability (aggregation, sedimentation and stickiness) or controlled chemical reactivity and integrity of the material are needed to study and to monitor the evolution of the NPs during their lifecycle. Note that the loss of colloidal stability, aggregation and sedimentation as much as the corrosion and dissolution may influence biological assays and knowledge of these processes are therefore necessary to make a correct interpretation of them.[26]

3.1. Aggregation

Aggregation of NPs is a very common phenomenon in biological media. Parameters such as concentration of NPs and chemical nature and the ionic strength of the dispersing medium can cause the cross-linking and aggregation of colloidal NPs.[27] As NPs tend to agglomerate/aggregate after relatively short incubation times in different buffers and biological media, coating them with stabilizers, such as the well known biocompatible FDA approved PVP or PEG, to provide colloidal stability in physiological media, is a very common strategy.[28] Despite that, the stability of NPs is not yet well understood and controlled and it is therefore important to study the aggregation behaviour of NPs to predict their fate in different aqueous media and environments in order to analyze their biological effects.

In the experiments performed with TiO_2 NPs, the state of aggregation can be fine-tuned in the presence of different TMAOH concentrations during the synthesis process. The addition of TMAOH can modulate the state of aggregation by stabilization of TiO_2 at different degrees of aggregation.[29]

The stability of the NPs was studied over a period of 48 h in the absence of light. This is because illumination of TiO_2 NPs may promote catalytic TMAOH degradation and consequently favour aggregation. Aggregation has a direct effect in buoyancy and therefore sedimentation profiles can be employed as a measure of the degree of aggregation. Thus, analysis of samples colloidal stability was obtained by performing a straightforward sedimentation assay by UV-Vis spectroscopy. Note that the sample was left unperturbed between analysis inside the UV-Vis Spectrophotometer. **Fig. 2** shows

how the sedimentation of large aggregates is faster compared with the other two samples. In the specific case of small aggregates the sedimentation is negligible. It is interesting that from 20 h onwards the sedimentation is almost imperceptible even in the case of large aggregates, since the effective concentration of NPs has decreased indicating how aggregation is directly related to concentration (NPs have to meet before they aggregate).

The sedimentation study for AgNPs was performed following the same methodology for a period of 48h **(Fig. 2)**. A similar behavior in AgNPs can be observed compared to the previous ones, since the sedimentation process of small AgNPs is almost imperceptible during the complete assay **(inset figures)**. However, in the case of AgNPs the sedimentation process is more evident compared with TiO_2 NPs, because the higher density of silver. It is important to highlight that in both cases, the sedimentation process is reversible, after a hand shake the NPs restore their initial spectra, which indicates that NPs are not irreversibly aggregated.

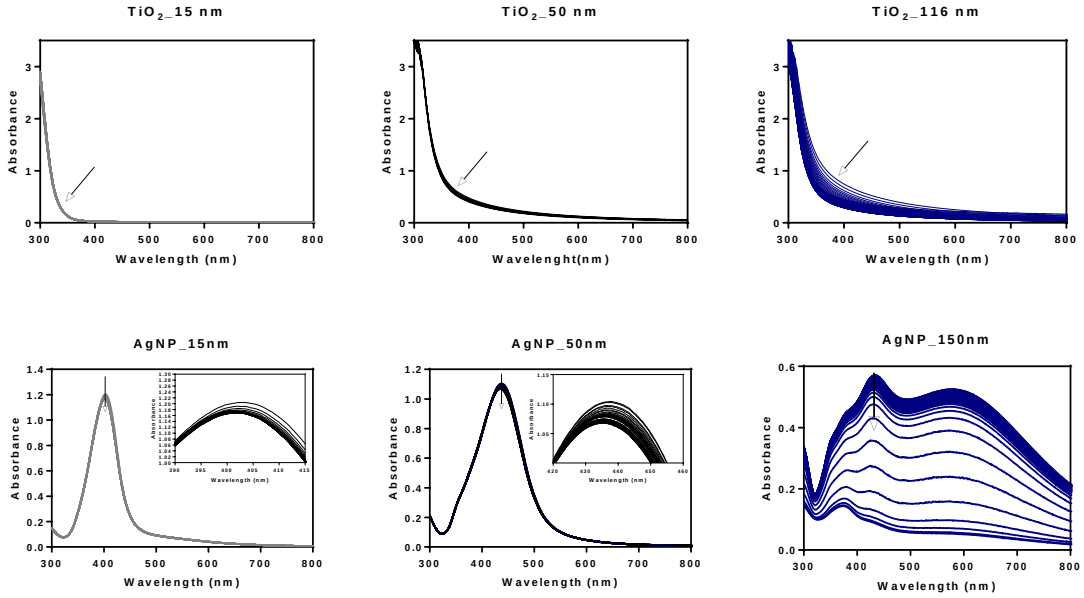

Figure 2. UV-Vis spectra profiles of sedimentation process of TiO_2 nanoparticles and AgNP during 48 h recorded at time intervals of 1h (from t_0 to t_{48} from top to bottom).

3.2. Dissolution

It is important to analyze the processes and mechanisms of dissolution in order to define strategies to control it, because it is well known that the release of cadmium from quantum dots or silver ions from AgNP contribute to the toxicity of these materials.[15,30] Not only because the released atoms, but also the redox process itself alters the metabolic status of cells.[31] Corrosion critically depends on the presence of dissolved oxygen and ions (nucleophiles) in solution. In this experimental case the behaviour of three different sizes of AgNPs when exposed to different environmental conditions were analyzed as it is shown in **Fig. 3**.

AgNP were exposed for 48 h to: **(1)** nitrogen atmosphere (AgNP as synthesized but preserved under inert atmosphere used as a control of non-oxidation), **(2)** Dulbecco´s Modifed Eagle Medium (DMEM) and **(3)** hydrogen peroxide (H_2O_2) at 1 mM concentration. During the process the samples were studied by UV-Vis spectroscopy as it is shown in **Fig. 3**. To ensure that the results observed are only due to the dissolution process the samples were always shaken before each UV-Vis acquisition.

As it is shown in the different graphs **(Fig. 3)** it can be concluded that the AgNP reactivity depends strongly on size and environment. The smaller the size, the more reactive the nanoparticle. We can observe this effect more notably in the presence of H_2O_2, where the oxidation occurs much faster in the AgNP_15 nm than in the other two samples. In the presence of DMEM it can be noted that oxidation and aggregation occurs simultaneously, due to the high ionic strength of this media. It is important to highlight that both processes can occur simultaneously and that one can promote or inhibit the other, since corrosion increases the ion concentration in solution which may lead to aggregation,[32] which in turn decreases dissolution by decreasing surface to volume ratio. In order to decouple the aggregation and dissolution process in our assay and observe a proper corrosion study, the samples were stabilized with PVP (polyvinylpirrolidone) at 0.1 mM, obtaining the spectra profiles shown in **Fig. 3**.

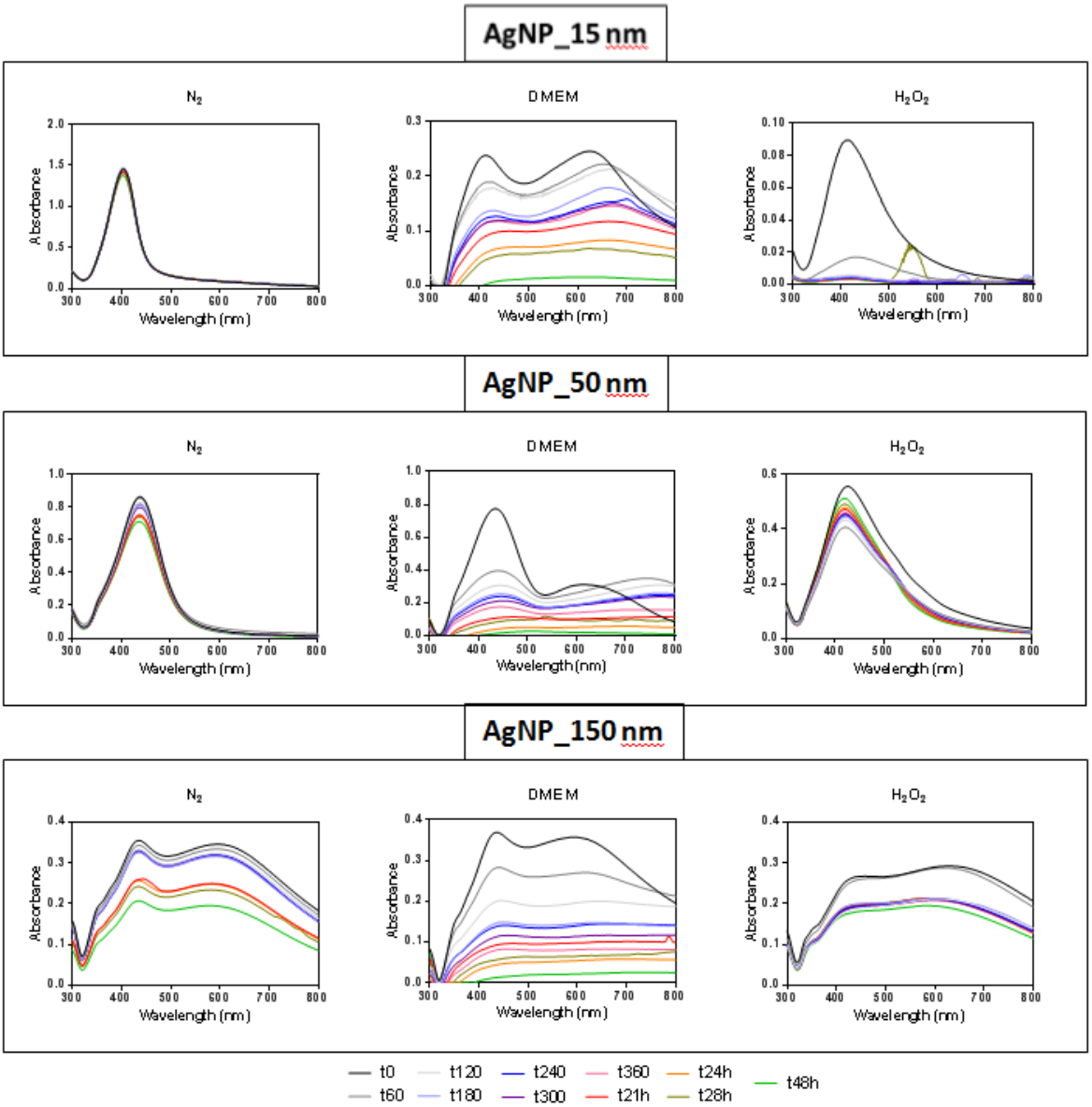

Figure 3. UV-Vis spectra profiles of corrosion process of AgNP 15, 50 and 150 nm under inert atmosphere, DMEM and H_2O_2 during 48 h.

4. Conclusions.

Regarding the **experimental data**, it has been shown that aggregation and corrosion process of TiO_2 and Ag NPs with different diameters can be presented by monitoring sedimentation and dissolution studies using an UV-Vis spectrometer. We would like to highlight that among all approaches available to study aggregation and sedimentation processes, in this paper it was presented an affordable, easy and accurate method that provide useful information to study both processes thanks to the optical properties of colloids.

It is also demonstrated that the reactivity depends on the size of the NPs. The smaller is the size, the more reactive is the nanoparticle. Furthermore, it can be concluded that as NP size increases the NP becomes more stable chemically but is more difficult to maintain it in suspension. Besides, it was observed that aggregation/sedimentation and corrosion are interlinked. Understanding the aggregation process of the NPs or controlling the state of aggregation due to the presence of surfactants, it is possible to understand the corrosion process of the material.

The behavior of NPs in biological media or its release to the environment is complex and depends on several factors in which aggregation and corrosion are included. For this reason, it is important to carry out a proper and extensive characterization of NPs to obtain an optimal and safe design. Three parameters have to be taken into account in the characterization of NPs: **i)** controlling and monitoring our NPs "from cradle to grave", changes in their size, shape or oxidation states during lifecycle; **ii)** controlling the release of the NPs from the solid or the ions from the NPs; **iii)** controlling the persistence of NPs, including possible transformations and corrosion.

Finally, it is important to highlight that the concept of safety by design starts in the inception point and it has to be considered during all lifecycle of the NPs and bearing in mind that sometimes is not possible to make something completely safe but safer is a more realistic goal.

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