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Bio-Nano interface and environment: a critical review

Gerardo Pulido-Reyes^{a,b}, Francisco Leganes^a, Francisca Fernández-Piñas^a, Roberto Rosal^b.

^aDepartamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, E-28049, Spain.

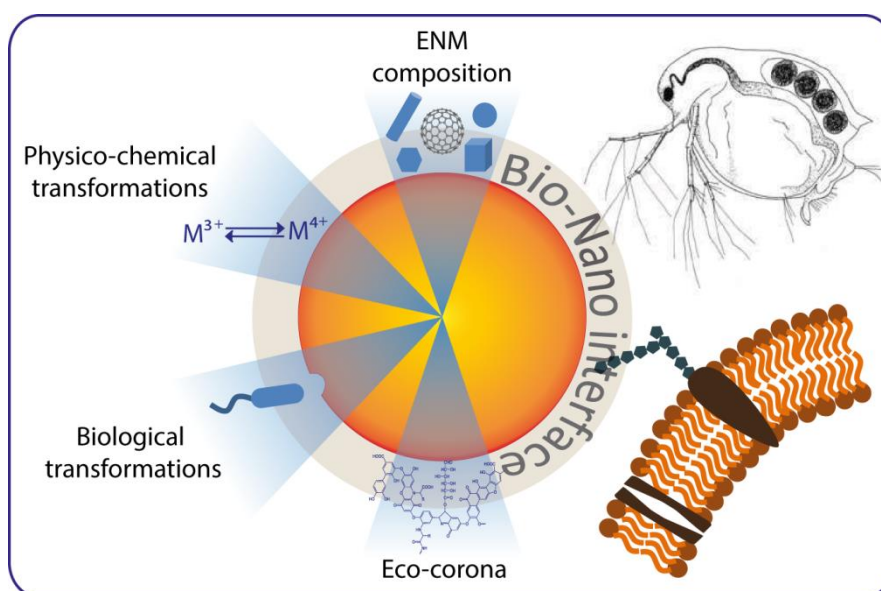
^bDepartamento de Ingeniería Química, Universidad de Alcalá, E-28871, Alcalá de Henares, Madrid, Spain.

ABSTRACT

The Bio-Nano interface is the boundary where the Engineered NanoMaterials (ENM) meet the biological system, exerting the biological function for what they have been designed or inducing adverse effects to other cells or organisms when they reach non-target scenarios, i.e: the natural environment. Research has been performed to determine the fate, transport, and toxic properties of ENM, but much of it focused on pristine or “as manufactured” ENM or where modifications of the materials were not assessed. This article reviews the most recent progresses regarding the Bio-Nano interface and the transformations that ENM suffer in the environment, paying special attention to the adsorption of environmental biomolecules on the surface of ENM. Whereas the protein corona (PC) has received considerable attention in biomedical field and human toxicology, its environmental analogue (the eco-corona) has been much less studied. A section dedicated to the analytical methods for studying and characterizing the eco-corona is also presented. We conclude with a Research Needs section where the key problems and knowledge gaps that need to be resolved in the near future regarding the Bio-Nano interface and eco-corona are presented and discussed.

Keywords: Bio-Nano interface, eco-corona, nanomaterials, nanoecotoxicology, environmental transformations, environmental chemistry.

GRAPHICAL ABSTRACT:



INTRODUCTION

Nanotechnology is considered as a new technology that will significantly improve, or even revolutionize, many areas and industrial sectors (Lahmani, 2016). The forecast for nanotechnology estimates that the global value of nano-enabled products, nano-intermediates, and ENM will reach \$4.4 trillion by 2018 [1][see also [2, 3]]. Nanotechnology has become a vibrant area of research over the past 15 years. This enthusiasm derives from two main factors: (1) the industrial interest in ENM, leading to their increased use in consumer products (ENM are used as chemically inert or active

additives that impart desired qualities, such as increased hardness or surface area, antimicrobial behavior, UV protection, and coloring, among others[4] and (2) the increased possibility of human and environmental exposure, which emerged concerns about the harmful effects that these new objects could have in human and environmental safety.

The more money is expected that the technology generates, the more money is funded by international agencies to deal with nanotechnology risk-related research: In Europe, the expense was €261M between 2006 and 2013, with a further €71M injection through Horizon 2020. Between 2006 and 2015, the US federal government invested US\$830M in nanotechnology environment, health and safety research [5]. In 2008, in particular, the US federal government published its first comprehensive research plan for nano-safety research [6] and, in 2012, the US National Academy of Sciences published an independent nanotechnology safety research strategy [7]. In Europe, the 7th Framework Programme included continuing thematic calls on nano-safety topics as an integral part of its Nanotechnologies, Materials and Production Technologies (NMP) programme. Additionally, since 2007, the Organization for Economic Cooperation and Development (OECD) also headed international efforts on validating toxicity test methods for ENM [8].

The potential benefits of nanotechnology depend on mastering a specific fraction, the ENM surface and its interaction with the surrounding environment. A complete understanding of the Physical Chemistry of ENM when its surface approaches macromolecular entities will help to improve the development of these materials. This interface, known as the Bio-Nano interface, hosts “the dynamic physicochemical interactions, kinetics and thermodynamic exchanges between nanomaterial surfaces and the surfaces of biological components” [9].

The aim of this critical review is to summarize the more recent research contributions to the Bio-Nano interface field from the last 5 years, although previous relevant bibliography is also included. The literature from January 2011 to December 2016 (and part of 2017) was searched using Scopus for studies related with the Bio-Nano-Eco interface field, focusing on how environmental variables and non-target organisms influence it. A description of the current techniques that are being used to study this fraction is included. Finally, a critical point of view and a summary of research needs are included as well.

UNDERSTANDING THE BIO-NANO INTERFACE

To fully understand the processes that happen at this interface, it is necessary to describe several elements: a) the nanomaterial surface; b) the medium where the ENM is suspended; and c) the properties and influence of the biological entities. The first factor influencing the Bio-Nano interface is the nanomaterial surface itself (Figure 1). Prominently, its characteristics are determined by its physicochemical composition, e.g.: elemental composition, size, shape, surface area, porosity, functional groups, ligands, etc., [10, 11]. Recently, it has been shown that size and surface structure have a crucial effect on the interaction with biological components like proteins. In this sense, silica particles of 200 nm (and bigger) induced conformational changes in myoglobin and Bovine Serum Albumin protein (BSA) upon adsorption [12]. Huang et al. [13] also showed that the NP surface characteristics are key parameters during the protein adsorption, since they might be able to module the protein conformation on NP surface. Surface oxidation state is a key factor that modulates the toxicity of cerium oxide nanoparticle. It has been determined that only the NP with high surface % of Ce^{3+} exerted toxicity in a study with an ecologically relevant aquatic organism [14].

The second factor, external to the ENM, but with a deep influence on nanoparticle fate and properties, is the characteristics of the surrounding medium (Figure 1). Not dependent, nevertheless, does not mean that they do not have important implications on the ENM. This parameter has a great impact on several measurable properties of ENM such as hydrodynamic size, state of aggregation, effective charge, dissolution or surface valences, which are all influenced by basic factors like ionic strength, pH and temperature, among others. Over the last years, it has been extendedly reported how the NP aggregation state and colloidal stability strongly depend on the medium where they are suspended [15-18]. Moreover, the dissolution of metal oxide nanoparticles extremely varies depending on the characteristics of the surrounding media. For example, it has been shown for CuO NP that the presence of amino acid rich environment can lead to nearly complete NP dissolution, while the presence of NaCl did not have any significant effects on the solubility of these nanoparticles [19].

The third main factor is derived from the biological entities themselves and determined by their surface composition and their ability of influencing their surrounding environment. This factor is greatly mediated by the presence of large organic molecules, which include natural organic matter (NOM), proteins and other biomolecules. For instance, it is established that the presence of NOM can control the nanoparticles aggregation state by creating an adsorbed layer on nanoparticle surface. The interaction may improve the steric stability of the system and its colloidal stability, hence letting the ENM remain suspended in the media [20, 21]. Alternatively, NOM can destabilize ENM, inducing aggregation by charge neutralization [16]. Louie et al. [22] have recently pointed out that NOM-weight-averaged molecular weight was the best indicator of ENM aggregation in the presence of NOM. The authors used Suwannee River NOM and five additional NOM isolates covering a range of sources (terrestrial, freshwater, and marine)

and preparations (fulvic and humic acids) and showed that there was a trend in how the ENM and NOM interact as biomolecules with molecular weight higher than 100 kg/mol provided better stability than lower molecular weight components for each type of NOM [22].

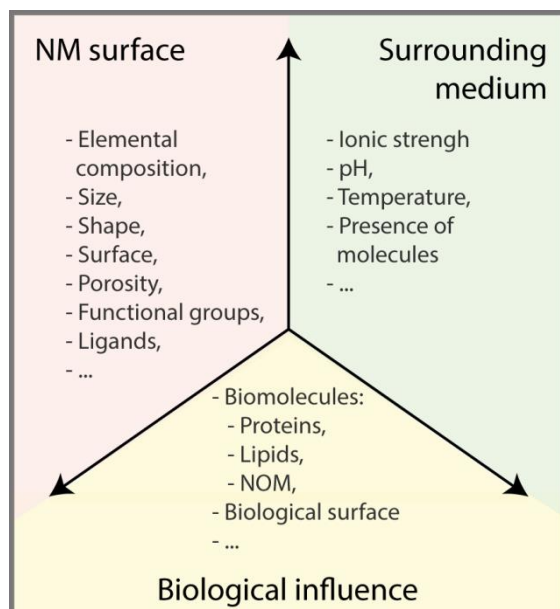


Figure 1.- The three sides of the Bio-Nano interface triangle. The main parameters governing the interface are surface, medium characteristics and biological factors.

These three general parameters are important in the fate, transport, behavior and bioavailability of ENM in the environment, but the last one, the adsorption of organic molecules on their surfaces, is critical when ENM approach biological surfaces. The formation of an external ‘biolayer’ in the extracellular environment has been shown to alter nanoparticle size, shape, and surface properties, creating a “biological identity” that is distinct from its initial “synthetic identity” [23]. Therefore, how ENM interact with different cells and organisms depends on the substances attached to their surface. Recently, the interaction between ENM and biomolecules has been extensively studied

in biomedicine [24, 25], due to the enormous applications of nanotechnology in this field [26-29]. The conclusions created a know-how about the behavior of ENM in complex matrices where they are surrounded by multiples 'bioelements' like proteins. The phenomenon describing the assembly of ENM and adsorbed proteins is called the ENM-protein corona (PC). The PC has been recognized as a dynamic entity that "evolves" as proteins continuously adsorb on the nanoparticle surface, desorb, and are replaced by other proteins. It is well-known that the surface of ENM is covered by a layer of tightly adsorbed proteins, the so-called hard corona (Figure 2) [30]. Strong binding affinity, long residence time, slow exchange time and high conformational changes are some of the most important characteristics of this layer. Some models suggest that on top of this 'hard corona' a 'soft corona' may exist, which consists of a more loosely associated and rapidly exchanging layer of biomolecules with low degree of conformational changes (Figure 2) [31].

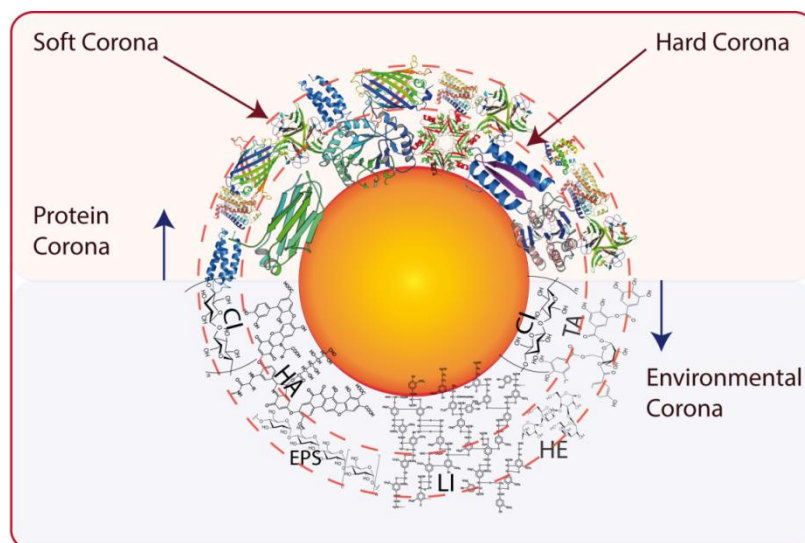


Figure 2.- ENM–protein (top) and environmental (down) corona. HE: hemicellulose; Cl: cellulose; TA: tannic acid; LI: lignin; EPS: exopolymeric substances; HA: humic acid.

Similarly, a NP-adsorbate association might also exist when ENM enter to the environment, ending up in the formation of an eco-corona. Lynch et al. [32] described that ENM could adsorb ‘ecomolecules’, which are macromolecules acquired by the ENM from the environment (Figure 2), mostly the already mentioned NOM or secreted biomolecules like Extracellular Polymeric Substances (EPS), among others. EPS are complex mixtures composed of proteins, polysaccharides, fats, nucleic acids, and inorganic substances released from different microorganisms [33]. Grunér et al. [34] have recently shown that hydrophobins, highly adhesive proteins secreted in large quantities by fungi, could strongly bind to polystyrene NP of different sizes and surface groups, increasing their stability when exposed to complex medium compared to pristine NP. Therefore, the adsorption of environmental molecules onto the ENM surface may

strongly modify NP behavior with respect to uncoated particles. In this regard, a number of studies address the effect of EPS interaction on the toxicity of ENM. Generally, EPS have protective effects for bacteria [35] and algae [36]. Su et al. [35] found that EPS-poor *Escherichia coli* cells were more vulnerable to Ag-doped multi-walled carbon nanotubes than EPS-rich cells. Recently, Zhou et al. [36] discovered that the amino and aromatic carboxylic groups in the EPS were involved in the interaction between EPS and different capped Ag nanoparticles (AgNPs). The authors showed that EPS could alleviate the algal toxicity of AgNPs not only by reducing the concentration of released free Ag^+ in the culture medium and inhibiting the cell internalization of Ag^+ , but also by restraining the toxic pathway of “Trojan-horse” mechanism by limiting the internalization of AgNPs. All those works highlight the importance of the formation of an eco-corona onto the ENM surface. However, much research is still needed to fully understand which types of environmental biomolecules are potentially able to adsorb on ENM surface and to determine the toxicological consequences of the formation of Bio-Nano interfaces.

TRANSFORMATION OF ENM IN THE ENVIRONMENT AND CONSEQUENCES FOR THE BIO-NANO INTERFACE

There are several routes by which ENM can enter the environment. They can be released by direct discharges of consumer products, accidental release during transport or production or by the intentional distribution of ENM for remediation purposes. Due to the high surface to volume ratio and reactivity of ENM, they are prone to suffer alterations in the highly dynamic environmental compartments. These changes can modify the original material yielding a different one. The resulting transformations of the ENM may

affect the Bio-Nano interface as well as their fate, transport and toxic properties. These transformations include chemical, physical and biological transformations.

Natural oxidation-reduction reactions, photooxidation/photoreduction, dissolution, sulfidation and aggregation are amongst the most studied physicochemical transformations (Figure 3). Depending on the particle redox potential and the prevailing conditions in an environmental compartment, ENM may be susceptible to oxidation or reduction. Zero-valent iron nanoparticles (nZVI) are being used for groundwater remediation [37], however, nZVI are easily oxidizable and could rapidly transform into particles without redox chemistry. It is known that nZVI will oxidize once into the environment, either completely or partially from Fe^0 to various Fe oxides and hydroxides. Aged/oxidized nZVI particles have lower redox activity and presumably lower reaction potential to remediate contaminants in groundwater. Reinsch et al. [38] examined the aging of commercially nZVI in solutions containing common groundwater anions (Cl^- , NO_3^- , HCO_3^- , SO_4^{2-} , and HPO_4^{2-}). The authors showed that inorganic anions do not inhibit the oxidation of nZVI with the exception of nitrate, which passivates the surface, thereby encapsulating the Fe^0 and decreasing particle reactivity. The decrease of redox activity also correlates with a decrease in the cytotoxicity of nZVI to *Escherichia coli* as demonstrated by Auffan et al. [39].

In other cases, the oxidation of metal ENM leads to the dissolution and release of the ENM elemental ions [40]. In this context, silver NPs (AgNP) are the most studied case among the metal NPs [41], due to the toxic effect of Ag^+ ions through various environmentally relevant species [40]. Mitrano et al. [42] elegantly studied the dissolution of AgNP at environmentally relevant concentrations (ng L^{-1}) in laboratory, natural, and processed waters using single particle ICP-MS (spICP-MS), proving that while the available techniques currently used are generally not capable of measuring AgNP-

transformation at these concentrations, spICP-MS can measure it even in complex matrices. One interesting result is that the effect of AgNP coating may be irrelevant in natural waters, as the authors did not find differences in the dissolution rates among coatings either in the 60 or 100 nm AgNP.

Moreover, nanoparticles and their ligands could undertake light-induced transformations via direct light absorption and reaction. Yin et al. [43] have shown that sunlight could accelerate the morphology change, aggregation, and further sedimentation of AgNPs in eight typical environmental water samples. Similarly, Cheng et al. [44] found that AgNPs undergo aggregation under sunlight irradiation, but they also evaluated the biological effect of these phototransformed nanoparticles towards the wetland plant *Lolium multiflorum* and observed that the toxicity of the AgNPs was significantly reduced by sunlight in comparison with non-irradiated samples. It is important to note that light reactions are also present in carbon based ENM where aggregation, Reactive Oxygen Species (ROS) generation and surface modification have been observed [45, 46]. In addition to these transformations, it has been shown that under ambient environmental light conditions, Ag ions bound to NOM can be reduced to form AgNP in river water or synthetic natural water samples [47], showing a new route for AgNP synthesis in the environment. Interestingly, Glover et al. [48] showed that, under environmental relevant conditions (relative humidity greater than 50%), new silver and copper NP could form in the vicinity of the parent particles or even in the proximity of metallic-non-nanoscale objects.

Another important environmental process which could affect ENM is sulfidation (Figure 3). This phenomenon may take place during waste water treatment [49] or in freshwater wetland [50]. It is already well-known that the sulfidation of metallic ENM in the environment reduces the release of ions and, thus, their toxicity to diverse organisms such

as *Caenorhabditis elegans* (*C. elegans* hereinafter, a model soil organism; [51]), *Danio rerio* (*D. rerio*, a model fish; [52]) and the aquatic plant *Lemna minuta* (duckweed; [53]), among other [53]. Interestingly, Starnes et al. [54] analyzed the transcriptomic profiling of nematodes which were exposed to pristine AgNP or sulfidized AgNP and found that their toxicological mechanisms were completely different. The toxicity of AgNP was explained by dissolution, release of Ag ions and particle specific effects, while the processes most affected by sulfidized AgNP was related to molting and the cuticle envelope.

Apart from the chemical transformations that have been described previously, there are several physical transformations involving ENM. Mitrano et al. [55] classified these alterations in two main categories: abrasion/mechanical erosion and aggregation. The authors focused on how mechanical or abrasion processes could end up in the formation of a nano-object and its subsequent release from the original material. However, the most important physical process affecting ENM is agglomeration/aggregation (including homoagglomeration and homoaggregation and their hetero-forms), as this is something that can change the high reactivity of ENM, due to the increase of the overall size. As described by Gonzalo et al. [18], the aggregation state of nZVI resulted in a non-linear dose-response toxicological curve due to the different colloidal stability, which was identified as the main driver for nZVI bioactivity. nZVI resulted in adverse biological effects towards a model microalga when destabilized (higher sizes), but not when it was forming a stable suspension. Altogether, agglomeration relies on a myriad of physicochemical interactions between the particles and water chemistry and this is the reason why, here, both ENM-transformation factors (chemical and physical factors) are shown together in Figure 3, instead of illustrating both parameters independently like Lowry et al. [50] and Mitrano et al. [55] did.

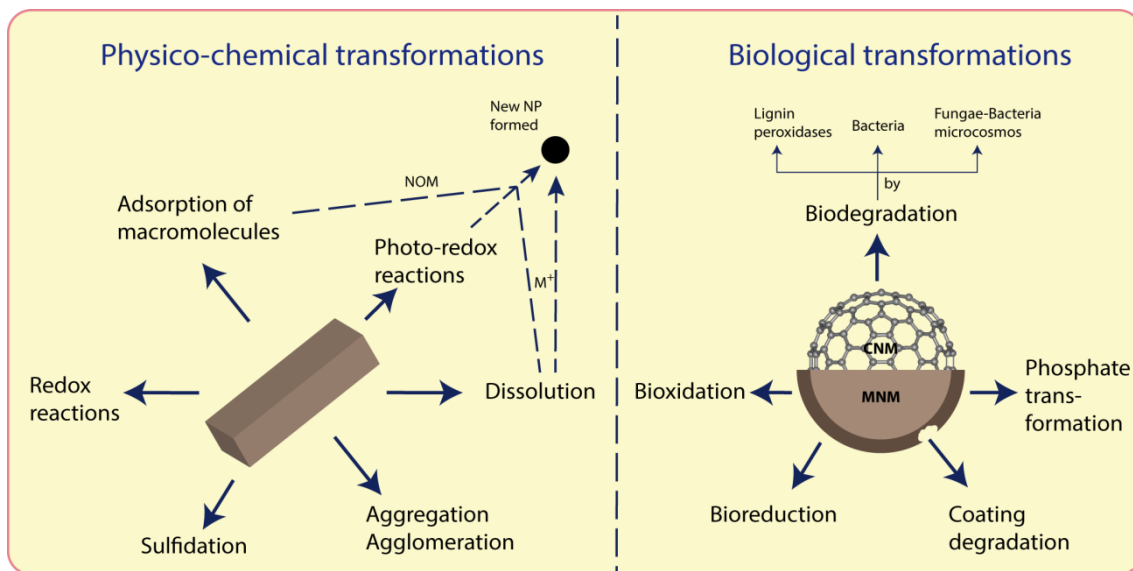


Figure 3.- Scheme of representative physicochemical (left panel) and biological (right panel) transformations of ENM in the environment. MNM: metallic ENM, CNM: carbon-based ENM, NOM: natural organic matter, M⁺ : metallic ion, NP: nanoparticle.

Biological entities, such as bacteria, fungi, microalgae, plants and other organisms, or their secreted enzymatic components could also have an effect on the coating and on the ENM itself. The process is known as biotransformation (Figure 2). Kirschling et al. [56] have demonstrated that polymer coatings covalently bound to nanomaterials are bioavailable and can be degraded by a community of different bacteria. Significant advances in ENM-biotransformation have been done with different plant organisms [57-63]. Parsons et al. [57] reported for the first time the biotransformation of NPs by a plant system where, from nickel NPs, a Ni(II)-organic acid complex was found in shoots and leaves. Furthermore, different processes of NP oxidation [58] and reduction [59] have been observed for plants exposed to Ag or CuO NPs, respectively. Recently, more studies have focused on Rare Earth Oxide NPs, due to their great potential in a wide range of

applications. The biotransformation of La_2O_3 , Yb_2O_3 and CeO_2 NPs was reported to follow a common transformation route with dissolution promoted by reducing substances and re-precipitation forming phosphates and other compounds [60-62]. Hernandez-Viezcas et al. [63] also reported a limited dissolution of CeO_2 NPs and surface bio-reduction from Ce (IV) to Ce (III) in soybean plants.

Several research groups have reported in recent years that carbon-based ENM (CNM) are susceptible to biodegradation as well [64-66]. Much work has been performed with human cells lines in view of the biomedical uses of CNM. Bhattacharya et al. [67] recently reviewed this topic, so the readers are encouraged to follow that reference (and references therein) for more information as the present work is mainly focused on the Bio-Nano interface from an environmental point of view. In this regard, it has been proved that lignin peroxidase (a ligninolytic enzyme) released from white rot fungi can induce the oxidative biodegradation of both oxidized and reduced graphene oxide nanoribbons [68]. Similarly, several studies have proved the biodegradation of different CNM by bacterial communities [69, 70]. Liu et al. [69] suggested that the direct contact between bacterial cells and materials promotes the oxidation of carbonaceous material. Interestingly, a fungi-bacteria soil microcosm rapidly mineralized a CNM. Carbon could also be incorporated into the biomass of a range of microorganisms, particularly Gram-negative bacteria and fungi [71]. However, more research is needed in this field to further confirm all these results; Parks et al. [72] showed that pristine and minimally-oxidized CNM are not easily biodegraded under environmental conditions such as exposure to the fungi or bacteria present in contaminated sediments and aerated sludge, indicating that this CNM might be likely persistent in environmental media.

Biological transformations of ENM that have been taken up by microorganisms have been observed in vivo using mussels [73] or worms [74]. Montes et al. [73] showed that CeO_2

and ZnO NPs were taken up by mussels, but, while CeO₂ NP remained unchanged in the pseudofeces, the ZnO NPs were completely transformed and excreted in Zn dissolved species. However, although CeO₂NP seemed to be unaltered, it is interesting to note that the biotransformation of CeO₂ could make them more bioavailable for other organisms such as deposit feeders (e.g., polychaete worms, amphipods, crabs) or grazers (sea urchins), among others. Several authors also reported the biotransformation of superparamagnetic iron oxide NP (SPION) in the nematode *C. elegans* [74, 75]. Gonzalez-Moragas et al. [74] observed a size decrease in SPION coated with citrate during digestion in the intestinal microenvironment of *C. elegans* while SPION coated with BSA proteins did not suffer any changes. Interestingly, the uptake of BSA-SPION was higher than citrate-SPION in larval population, indicating that different result could be obtained using different growth stages of the same organism or different coatings. On the other hand, extracellular biotransformation has been observed for a fungal organism (*Humicola sp.*). This species could transform 150–200 nm TiO₂ (with an anatase structure) to 5–28 nm TiO₂ (with a brookite structure) [76]. Unfortunately, the biotransformation mechanism was not proposed, so there are no clues regarding how the process was performed.

These results show that the biotransformation can produce new nanomaterials with a different toxic profile. Clearly, it is information of critical importance for assessing the biological impacts of nanoparticles and the kinetics of such impacts. Considerable research effort is still needed to clarify and fully understand the implications of the environmentally transformed ENM and their behavior within the environment. As shown earlier, it has been hypothesized that different ENM, once inside the environment, might be transformed into the same material [42]. This can be due, for example, to strong aggregation processes or the ENM surface adsorption of NOM, so imparting the same

environmental identity to different ENM. Lombi et al. [77] showed that neither surface coatings, at least for three different types, nor core composition (Ag or AgCl) of AgNP prevented the formation of Ag₂S, indicating that whatever the original AgNP is, the outcome of wastewater treatment in terms of speciation would be the same. The new environmental identity derived from such transformations could result in a complete loss of the individual nano-properties. Conversely, the release and subsequent transformation of NP may result in an increase of ENM diversity [42], due to the diversity of aging/transformation reactions that could end up in different transformed ENM. Besides, the coverage of ENM surface by an eco-corona could facilitate the grouping and read-across of different ENM based on their environmental fate, making it easier to deal with the huge variety of existing and future ENM.

BIOLOGICAL IDENTITY OF ENM

Once ENM interact with the environment and get in contact with living organisms, they become exposed to a huge variety of transformations. In particular, active biomolecules may form a corona around them, transforming the bare or pristine ENM into a modified ENM potentially bearing a biological component. The biological identity of an ENM depends on the composition of the surrounding biological environment and could determine the subsequent interactions with cells: It is what the organism ‘sees’ and interacts with [32]. From now on, we will focus on how these transformations and, specifically, the acquisition of an environmental corona, will impact the interaction with

biological systems, membranes and cell envelopes, paying special attention to ENM-cellular uptake and endocytosis.

The ENM specific interaction with biological surface (cell membranes or cell walls) and the way they enter cells is a complicated issue. Many efforts have been done using modelling programs and artificial phospholipid bilayers to try to understand the parameters and processes that drive the bio-NP interaction in a simplified scenario [78-80]. Nevertheless, in vivo studies with whole organisms are also being performed. Jacobson et al. [81] recently studied the interaction between gold nanoparticles and the Gram-negative bacteria *Shewanella oneidensis*. The authors demonstrated an electrostatic association of cationic NP with the negatively charged polysaccharide portions of lipopolysaccharides in the cell envelope. However, bacteria constitute a large domain of prokaryotic microorganisms with a huge diversity on their cell envelope and content of lipopolysaccharides, so the mode of contact and outcomes of the NP-biological interaction may be completely different depending on the composition of the bacterial surface.

Whether ENM might pass through the biological barriers and enter into cells (or not) has been a matter of intense research during last years [82-84]. Zhu et al. [85] suggested that the internalization of NPs is mainly a size-dependent process in eukaryotic cells, due to the well-designed endocytic machinery involved (Figure 4). It has been noted, however, that NP-hydrophobicity could be an important parameter due to the lipophilic nature of cytoplasmic membranes [80]. In this regard, there are five endocytic pathways that can be used for nanoparticle endocytosis: phagocytosis, macropinocytosis, clathrin-mediated, caveolin-mediated, and clathrin/caveolin-independent endocytosis (Figure 4). In addition to them, there is another one which is independent of endocytosis: ENM direct penetration, suggested from particles with very low sizes (<10 nm) [86].

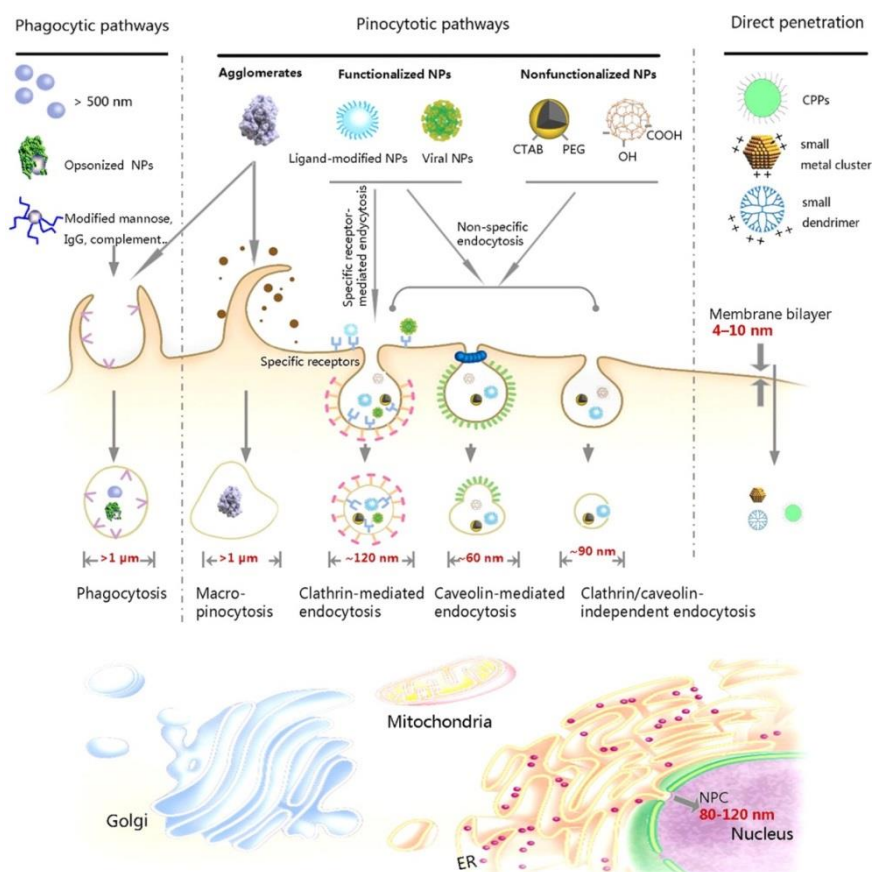


Figure 4. Routes of ENM entry, depending on the size of the initial material. Phagocytosis could be the way used for large particles or aggregates. Other ways include pinocytosis (mediated by specific receptors or not) and direct penetration. Reprinted with permission from (Zhu, M., Nie, G., Meng, H., Xia, T., Nel, A., & Zhao, Y. (2012). Physicochemical properties determine nanomaterial cellular uptake, transport, and fate. *Accounts of chemical research*, 46(3), 622-631.). Copyright (2012) American Chemical Society).

The same routes of entry outlined in Fig. 4 should explain the internalization of ENM in several environmentally relevant eukaryotic organisms [75, 87-89]. In many microorganisms (such as bacteria and algae) the cell wall protects cells with pores in the 5-20 nm range [90]. Accordingly, it has been found that stabilized CdTe Quantum Dots (QD) could enter the freshwater alga *Ochromonas danica* directly through

macropinocytosis [91]. Interestingly, the CdTe quantum dots suffered further modifications inside the algal cells as the photoluminescence pattern decayed sharply due to the adsorption of different biomolecules to the surface of QD. Moreover, Hoepflinger et al. [92] recently reported that clathrin pathway played an important role in the endocytosis process in a multicellular green algae (*Chara australis*). *Chlamydomonas reinhardtii*, a model unicellular microalga, has also been shown to possess a clathrin-mediated endocytosis pathway which is a probable way for ENM internalization [93, 94]. NP internalization has been recently described in *C. reinhardtii* for QdTe/CdS QD [95], CeO₂ [96], Ag [97], PAMAM-dendrimers [98] and CuO NPs [99]. Further research is needed for determining the effect of coatings in ENM uptake; some results pointing towards an increased internalization of ENM having organic coatings [96, 97, 99]. However, how the ENM cross the cell wall and use these pathways to enter inside cells is not fully understood yet. Ma et al. [89] extensively reviewed the scientific literature on this topic and provided a thorough classification of the nano-bio interactions between ENM and different organisms: Bacteria, algae, invertebrates and fish. The readers are encouraged to follow this article and their referred studies.

The specific interactions at the Bio-Nano interface, the localization of ENM, inside organisms and cells, and the potential toxicity of ENM may vary depending on their biological identity. There are several factors influencing the composition and evolution of the biological identity, namely exposure temperature, exposure time, nanoparticle hydrophobicity, size/surface curvature, surface charge, surface functionalization, physiological media/nanoparticle concentration ratio and topology [25]. Once the biological identity is formed, the effect of the ENM with their biological identity could be different, depending on which type of biomolecule is adsorbed or the medium where they are suspended. It has been shown that the formation of the ENM-corona decreased

ENM-induced toxicity because of a reduced cellular uptake [100, 101] or the inhibition of ROS formation [102]. Additional environmental examples can be found for AgNPs [21, 103-105] or fullerenes [106] for which the adsorption of NOM decreased the toxicological effects towards several model organisms. Conversely, the adsorption of biomolecules on the ENM surface can induce protein denaturation and cell damage [107-109] and it has also been reported an increased accumulation of NP in biofilms of *Pseudomonas putida* [110]. It is therefore not surprising that the corona effect is contradictory in various biological systems considering that different cell types and environment may generate different corona composition and also favor the uptake of specific surface biomolecules.

Clearly, it is the biomolecular corona that primarily interacts with biological systems and thereby constitutes a major element of the ENM biological identity. The molecular corona may be conveyed when the ENM moves from one biological environment to another [25]. Lundqvist et al. [111] simulated the passage of a ENM from one biological fluid (plasma) into another (cytosolic fluid) and concluded that there was a significant evolution of the PC from the first to the second biological solution, but the NP retained a “fingerprint” of its history in the final corona. The time evolution of PC under realistic in vivo conditions was recently considered by Hadjidemetriou et al. [112]. Their results showed that a complex PC formed in only 10 min and, although the total amount of protein adsorbed did not significantly vary, the abundance of each protein identified fluctuated over time indicating a competitive exchange processes. As cells constantly excrete to their microenvironment several proteins, nutrients, small solutes, ions,..., it is also interesting to know how conditioning of the cell culture medium influences the biological identity of nanoparticles. Albanese et al. [113] demonstrated that the secretion of different molecules alters the extracellular environment and can lead to nanoparticle aggregation and changes

in the PC, which affect the rate and mechanism of cellular uptake. The same process could be observed in the natural environment. Aquatic plants (*Potamogeton diversifolius* and *Egeria densa*) exposed to AgNPs produced exudates (mainly dissolved organic matter) that altered their aggregation state and ability to release Ag^+ [114, 115]. Hayashi et al [116] explored the importance of corona composition for ENM recognition by coelomocytes (leukocyte cells) of the earthworm *Eisenia fetida*. The authors showed that the earthworm was able to recognize AgNPs covered with a corona of native *E. fetida* coelomic proteins, compared with the same particles coated with a non-native corona made from fetal bovine serum.

Table 1 lists the main articles characterizing the interaction between ENM and biomolecules (mainly proteins) in studies using environmentally relevant organisms. Hayashi et al. [117] and Canesi et al. [118] used different biological fluids from the model fish *D. rerio* and the marine bivalve *Mytilus galloprovincialis*, to study the spontaneous macromolecular adsorption forming a corona of biomolecules onto ENM. In order to detach the adsorbed biomolecules to the ENM surface, several steps of centrifugation and washing were conducted. Interestingly, NP could adsorb particular biomolecules from a species specific [116, 118] or gender specific environment [117] and this corona was recognized and even accumulated more easily than other coronas in blood or immunocyte cells, indicating that ENM can acquire a completely different corona, and thus a completely different biological identity, depending of the biomolecular environment where they are suspended. Furthermore, that corona might evolve when the ENM move into a new environment with different biomolecules which have higher affinity than those previously adsorbed as it has been already mentioned [111-113].

Besides the aquatic plant assays stated above, other works assume the hypothetical crossing of ENM through the different biological barriers and entry into the biological

fluids in their studies about the effects of specific biomolecular coronas. There is an interesting example of a complete characterization of an eco-corona in an environmentally realistic scenario [119]. The authors elegantly assessed the toxicity and the interaction of two different polystyrene NP (PSNP) with the biomolecules secreted by *Daphnia magna* and the impact of these interactions on the ratio of NP uptake/removal. They observed that the eco-corona increased the uptake and toxicity of PSNP. Interestingly, the removal of eco-corona modified polystyrene nanoparticles was less efficient than uncoated polystyrene in the gastrointestinal tract of *Daphnia magna*.

Table 1. Studying the eco-corona from its composition to its biological effect using environmentally relevant organisms.

Organism	ENM	Method of Separation	Method of characterization	Ref.
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<i>Daphnia magna</i>	PSNP	Sedimentation, centrifugation and washing	Gel Electrophoresis (PAGE)	Nasser et al. (2016) [119]
<i>Mytilus galloprovincialis</i> (hemocytes)	PSNP	Centrifugation	Gel Electrophoresis and nano-HPLC-MS/MS	Canesi et al. (2016) [118]
<i>Danio rerio</i> (blood cells)	SiO ₂ NP	Centrifugation	TEM, DLS, NTA, Gel Electrophoresis (PAGE)	Hayashi et al. (2016) [117]
<i>Eisenia fetida</i> (coelomocytes)	AgNPs	Centrifugation	TEM, DLS, NTA, Gel Electrophoresis (PAGE)	Hayashi et al. (2013) [116]

PSNP: Polystyrene based-NP. PAGE: polyacrylamide gel electrophoresis. HPLC-MS: High-performance liquid chromatography coupled with a mass spectrometer.

Evolving or changing the environment where the eco- or PC has formed and how it changes through time adds, thus, novel additional levels of complexity in the study of bio-nano interactions. The vast majority of works dealing with PC refer to biomedical applications, with little attention to environmental scenarios. The evolution of eco-corona through different conditions and time, the biological consequences of eco-corona towards different trofic levels are important issues to be addressed. Further studies are needed to cover those research gaps.

STUDYING AND CHARACTERIZING THE BIO-NANO INTERFACE

To fully understand the behavior of ENM in contact with biological entities, the ENM need to be characterized both in their pristine state and in complex environments, which include their eco or protein corona. This task requires the use of several techniques from diverse disciplines, including physical, chemical and biological sciences. Characterizing the surface adsorption of molecules on ENM is problematic due to several reasons. For example, discriminating between species adsorbed to the nanoparticle surface and those in solution can be challenging because of their similar chemical signatures and the high chemical complexity of biologically relevant media (which may contain many different biomolecules together with a complex background of colloidal organic matter). This difficulty can be avoided by isolating the ENM from other colloidal species in the sample matrix prior to analysis and characterization. Currently available separation methods include column chromatography, field flow fractionation and their derivatives.

A liquid chromatography method has been recently used to separate AgNP and Ag⁺ in environmental water samples such as lake water and wastewater treatment plant influent and effluent [120]. The authors developed an approach for rapid and baseline separation of soluble Ag(I) from AgNP covering the 1 to 100 nm range. Proulx et al. [121] have also used chromatography coupled to single particle ICP-MS method for distinguishing Ag, Au and polystyrene NP spiked into naturally sampled river water. The hydrodynamic chromatography was able to remove much of the background signal due to environmental colloids and natural organic matter, allowing for a reasonable separation of the NP.

Although chromatography has been used to separate NP, the most widely used method to separate NP from complex matrixes is field-flow fractionation (FFF). AgNP with different coating agents could be separated from sandy and clay soils by FFF with in-line UV-vis spectroscopy used to detect the concentration of eluted particles and DLS to determine nanoparticle size [122]. Poda et al. [123] developed and applied an FFF-

ICP-MS method for the separation and characterization of AgNP mixtures. The technique was also applied for biological media to characterize silver nanoparticles before and after exposure to the freshwater oligochaete, *Lumbriculus variegatus*. After exposure, the tissues were extracted and analyzed by FFF–ICP-MS. The size of the extracted AgNP increased from approximately 31 to 46 nm, indicating a significant change in the NP characteristics during exposure. The ability to discern particle size along with its composition further demonstrated the utility of this method for environmental applications.

The above mentioned techniques separate ENM from complex samples, but do not deeply characterize the biomolecules adsorbed onto their surface. While the assessment of the biomolecule interactions with ENM is becoming routine in medical and human toxicological analyses, much work is still needed in the environmental or ecological context. Some methods coming from biomedicine are normally used (see Table 1), demonstrating their suitability for characterizing environmental coronas. As a non-exhaustive list, Table 2 shows the most used techniques for PC isolation, separation, and identification, which, as proved by the articles reviewed before, are also suitable for eco-corona studies.

Table 2: Techniques currently in use to study ENM corona and the Bio-Nano interface.

Analytical Methods for Corona Evaluation	
	▪ Centrifugation
	▪ Field-Flow Fractionation (FFF)

▪ Column Chromatography
▪ Nanoparticle tracking analysis (NTA)
▪ Circular Dichroism
▪ Isothermal Titration Calorimetry
▪ SDS-PAGE
- Capillary Electrophoresis
- One-Dimensional Gel Electrophoresis
- Two-Dimensional Gel Electrophoresis
▪ UV–Visible Spectroscopy
▪ Fluorescence Spectroscopy
▪ Mass Spectrometry
▪ Fourier Transform Infrared and Raman Spectroscopies
▪ Nuclear Magnetic Resonance (NMR)
▪ Differential Centrifugation Sedimentation
▪ X-ray Photoelectron Spectroscopy (XPS).
▪ Electron and Atomic Force Microscopies.

In many cases, a direct observation of the Bio-Nano interface is required. Electron microscopy techniques such as Transmission electron microscopy (TEM) and Scanning electron microscope (SEM) provide very high resolution, making them useful for observing the Bio-Nano interface. Numerous groups used TEM [14, 124, 125] (see Figure 5) or SEM [125-127] for studying the interaction of ENM on environmentally relevant organisms. Atomic Force Microscopy (AFM) has also been used to study ENM interactions with whole cells. Dorobantu et al. [128] successfully used AFM and acquired images of several prokaryotic and eukaryotic cells following exposure to AgNP. TEM, SEM and AFM images generally identify adsorbed nanoparticles on the cell surface and

prove useful for demonstrating changes in cell morphology and membrane integrity following ENM exposure (TEM coupled with an energy-dispersive X-ray spectroscopy could determine potential metallic ENM internalization as well). Studying Bio-Nano interfaces is not a trivial task. The effort of researchers from different disciplines is required to deal with the numerous analytical problems that appear in the evaluation of real environmental samples. The application of the multiple analytical methodologies required for fully characterizing the Bio-Nano interface is frequently beyond the capabilities of individual laboratories, making collaborative research a need to understand this complex interface.

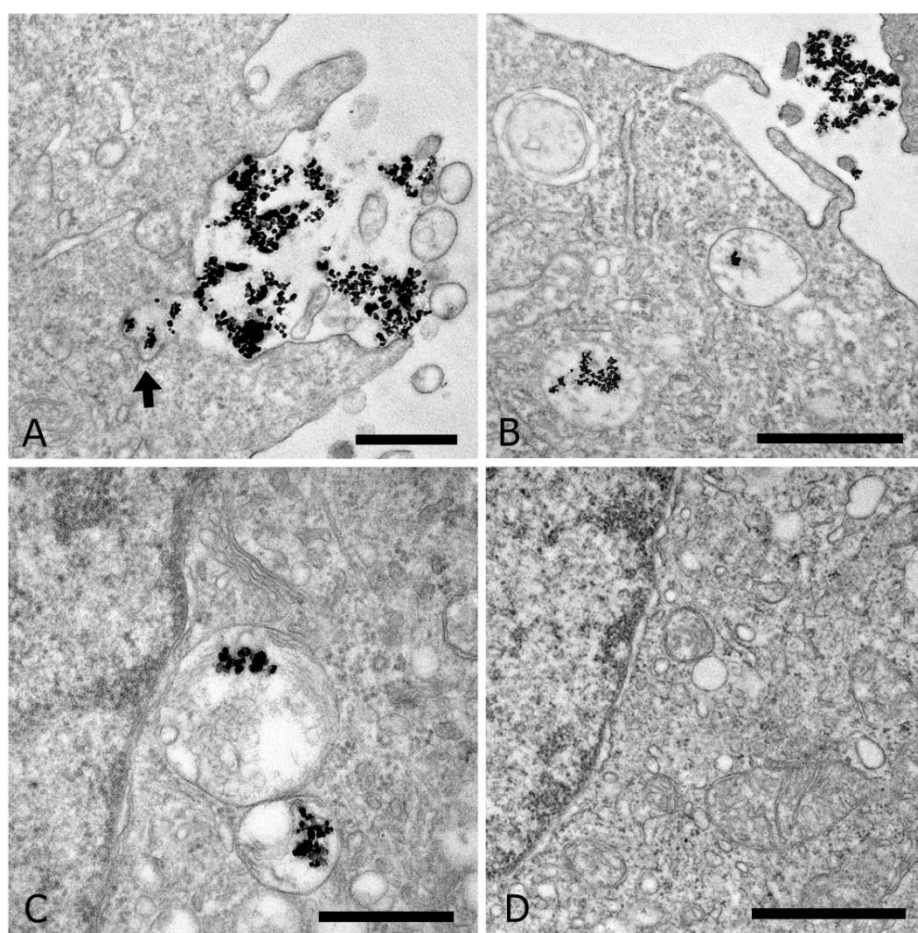


Figure 5.- Internalization of TiO_2 NP in a embryonic cell line of sea bass observed by TEM. Reprinted with permission from Picchietti, S., Bernini, C., Stocchi, V., Taddei, A. R., Meschini,

R., Fausto, A. M., ... & Scapigliati, G. (2017). Engineered nanoparticles of titanium dioxide (TiO₂): Uptake and biological effects in a sea bass cell line. *Fish & Shellfish Immunology*, 63, 53-67.

CRITICAL OPINION & RESEARCH NEEDED IN THE BIO-NANO INTERFACE FIELD

The traditional assessment of the biological effects of ENM is not valid anymore. As discussed along this article, the ENM are affected by many physicochemical and biological transformations which endow ENM with new identities from the pristine or “as manufactured” materials. Therefore, a case-by-case approach to assess nanotoxicity is questionable due to the high volume of work required and the continuous advances of nanotechnology. It is necessary to consider not only the current diversity of ENM and the enormous variety of transformed ENM, but all the new ENM that are expected to come to market. High-throughput based-methods could rise as key strategies to fill current gaps in ENM knowledge. The work of Kaweeteerawat et al. [129] is a good example of what can be done and obtained using this approach. The authors analyzed a data set consisting of the toxicological response of *Escherichia coli* to a library of 24 metal oxide nanoparticles and identified the physicochemical drivers (ENM conduction band energy and metal ion hydration enthalpy) for the observed biological effect. An enormous ENM library as Chen et al. [130] developed, where a broad range of single and multimetallic

NP made of different combinations of five different metals were synthesized, could be also used as reference materials for future toxicity and ecotoxicity studies to withdraw general toxicological descriptors.

The environmental transformation of ENM in the environment and the biological effects of transformed ENM is a research field requiring particular attention. Although some processes have been outlined during the last years, as covered in the ‘Transformation of ENM in the environment’ section, additional studies using realistic environmental conditions, which reflect the complexity of actual environmental systems, should be implemented. In this sense, microcosm and mesocosm experiments, which mimic the environmental conditions in a given compartment, could help to study the evolution of pristine to transformed ENM and the resulting biological consequences. Recently, some recommendations and considerations regarding the exposure and design of mesocosm studies in the assessment of ENM environmental hazards have been listed [131]. Among them, controlling the aging of ENM along the mesocosm experiment is of vital importance. The aging process for which ENM could undergo further transformations is somewhat overlooked in the scientific literature and their effects might be underestimated. There is a need for establishing standard methods for aging ENM that provide useful information and also reproducibility among laboratories. Gubicza et al. [132] showed that coated gold nanoparticles grew during a long-time experiment from 2-5 nm to about 25 nm exhibiting drastic morphology changes from initial spherical type to different regular shapes such as bipyramid, decahedron, deca-tetrahedron, triangular plate and rod. Ellis et al. [133] studied the environmental fate and migration of AgNP by using static water ‘microcosms’ and highlighted the relevance of surface coating as well as water chemistry on the fate of AgNP. This basic microcosm approach, associated with the addition of different trophic organisms to the system, will help to study the behavior

and fate of AgNP in a more realistic scenario. The huge variety of physicochemical variation that ENM face in the environment (namely tides and seasons) may enhance or even accelerate all these morphological and transformational changes described above.

More realistic scenarios imply the study of complex matrices. Though multiple ENM may exist jointly in product formulations, different ENM are rarely studied together [134]. Neither the mixture of NP and water pollutants has been frequently addressed [124, 135]. An interesting example has been provided by Fries et al. [136] who studied the interaction of the antibiotic ciprofloxacin, TiO₂NP and NOM in an aqueous environment. Regarding the study of corona, an ideal scenario involved the understanding of how it is formed in the suspending medium, how it is modified in the vicinity of the cell surface (uptake surface), in blood or other biological liquids, and inside the tissues and cells (intracellular environment) [137]. More research is still needed to cover the existing knowledge gaps regarding the eco-corona formation and evolution in the environment as only a few articles addressed this topic (Table 1). Moreover, it is necessary to enlighten whether or not the eco-corona governs the interaction between ENM and cells and also to develop methods that allow characterizing ENM as a function of the eco-corona. Although this strategy was implemented for biomedical applications, the vision of grouping ENM [32] by their eco-corona might also be applied to environmental samples. A thorough characterizing of ENM eco-coronas would provide a mechanism of grouping and reading-across of ENM that could allow a further insight on predicting the fate and behavior of ENM in the environment. The characterization of samples containing very low concentration of NP with heterogeneous size distribution, as well as those susceptible to undergo dissolution or other transformations, is a challenging task for current analytical instruments. The implementation of improved methods to separate ENM from interfering colloidal species would facilitate a more accurate ENM characterization in complex

matrices. Current methods also need to be adapted and new ones developed to generate consistent results for separation, detection and quantification of ENM at environmentally relevant concentrations. Moreover, it is necessary to implement more studies which use techniques that could characterize the corona as formed, given the fact that the current techniques to study the adsorbed biomolecules could greatly alter the corona and induce some artifacts. Instruments for tracking ENM in situ or in vivo are lacking. Besides, the bioaccumulation of ENM through the trophic chain has seldom been examined due to the lack of suitable techniques. In this sense, methods such as the one proposed by Yang et al. [138] might open a new door in the in vivo quantification of ENM in single cells. The authors proposed a single-cell mass cytometry, a method coupling time-of-flight ICP-MS with flow cytometry, for the quantitative determination of ENM combined with multivariate phenotypic analysis. It would be interesting to know whether this technique may be applied with common single cell organisms used in toxicological studies such as *C. reinhardtii* or other unicellular entities.

Due to the high amount of money spent by national and international research agencies to study the impact of nanotechnology at all levels, the promotion of international collaboration should be a priority. In this regard, the discussions on global Environment, Health and Safety (EHS) research needs and challenges led to a joint initiative to create the EU-USA Communities of Research [137], which include voluntary activities of EU and US scientists to facilitate collaboration in nanosafety research. This could be a good scenario for collaborative research among groups from different disciplines as required by the study of Bio-Nano interface phenomena. Cooperative efforts involving different areas of expertise and background (physical, chemical, and biological sciences), are needed to provide fruitful interdisciplinarity and cross-fertilization.

Competing interests

The authors declare that they have no competing interests.

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