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Biodistribution, excretion, and toxicity of inorganic nanoparticles.

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Table of contents.

Abstract.

The aim of this chapter is to provide a summary of the current knowledge of the full life cycle of inorganic nanoparticles in physiological environments related to medical applications. The response of nanoparticles inside organisms or released into the environment is complex and diverse, and a variety of parameters are involved: nanoparticles may be aggregated into microscopic particles or embedded in exposed materials; their surfaces, which determine their bioactivity, experience constant modifications; nanoparticles may corrode and dissolve; or they can suffer morphological modifications. Thus, in physiological environments and inside the body, the effects, biodistribution, potential toxicity and fate of nanoparticles depend not only on their composition and designed morphological and surface properties, but mainly on the modifications they undergo depending on the exposure media. All this is reviewed and discussed below.

Keywords.

Inorganic Nanoparticles, Nanomedicine, Nanosafety, Aggregation, Corrosion, Dissolution, Protein Corona.

1. Introduction: Inorganic nanoparticles and their interest in medicine.

With the advent of more increasingly complex nanostructures ensuing from the high demands posed by the requirement for more personalized treatments and precision medications, inorganic nanomaterials have emerged as flexible platforms for the development of theranostic nanomedicines. Owing to their inherent detectability by many biomedical imaging techniques combined with the robust inorganic structures providing shelter and/or stability to incorporated active molecules, some even exhibiting intrinsic therapeutic action (e.g. phototherapy, anti-inflammatory), inorganic nanomaterials are perfectly suited for performing combined diagnostics and therapy[1]. In addition, inorganic nanomaterials and nanoparticles (NPs) represent a vast array of different materials, including for instance metals and metal oxides, non-oxide ceramics, semiconductor nanocrystals (quantum dots, QDs), magnetic NPs, upconverting phosphors (UCPs) or nanoparticles (UCNPs) and carbon nanostructures (e.g. nanotubes, fullerenes, carbon dots, graphene, nanodiamonds) that can, in a single platform, perform the different activities mentioned[2].

These advantages have spurred the substantial growth of studies exploring new possibilities for medicine. Thus, among the pool of newly proposed materials, synthesized inorganic nanomaterials are being increasingly studied as alternative tools in medical applications. Main advantages of these materials include their successful use as robust drug carriers, as antennas that can be excited in biologically transparent environments, and being able to adjust the activity of conjugated biomolecules. Furthermore, their unique physicochemical signatures allow their tracking and easy detection in biological environments. All this is being translated into a new generation of diagnostics, imaging agents and therapies for detecting and treating disease in its earliest stages[3]. Moreover, their interest is expanded towards the possibility to combine these advances to enable the creation of multimodal/multifunctional nanosized particles that may combine diagnosis together with different therapies (chemo-, thermo-, radio-, immuno-therapies and so on) with synergistic effects, superior to any currently used treatment[1, 4]. This is especially important for instance for cancer treatment, since survival rates critically depend on the stage where cancer is diagnosed and single modality treatments usually cannot overcome associated drug resistance. Thus, the advantages of inorganic materials to create advanced functional NP-based platforms for detection and multimodal treatments of different diseases make them ideal candidates to be used as tools for the future of medical progress (**Fig. 1**).

[Insert Fig. 1 here]

Some of the main advantages of inorganic NPs for medicine can be summarized as follows:

i) Size considerations: possibilities for drug delivery. Inorganic NPs are small, and can therefore interact with molecular biological structures in a unique manner. Of course, NP size control is a paradigmatic feature for their claimed potential in nanomedicine. Size influences key biological interactions such as association with proteins, biodistribution and clearance rates. It is accepted that the final fate of NPs is strongly influenced by their size. For stable, low interacting and non-immunogenic NPs, it is considered that the smallest ones (<6 nm, core + surfactant) are rapidly cleared by the kidneys and the largest ones (>100 nm) are also easily removed from blood circulation by the cells of the Mononuclear Phagocyte System (MPS)[5] (**Fig. 2**). The variety of sizes and the narrow size distributions that can be nowadays easily produced[6] has enabled a better

understanding of the role played by this property in parameters that are important for different medical applications such as accumulation and penetration in targeted organs or tumors[7].

Importantly, their small size, similar to those of proteins, allow long lifetimes in blood, a better use of the enhanced permeation and retention (EPR) effect observed in solid tumours and in atherosclerosis, which is associated with chronic inflammation of arterial blood vessels[8], and an increased tissue or tumour penetration (see e.g. reference of Barua et al[9]). Here, it is widely accepted that a compromise between accumulation and penetration of drugs in the targeted area has to be reached. For instance, in cancer treatment, a cocktail of NP sizes can strike the tumor in different areas: larger NPs are more readily accumulated in the tumour, but they are restricted to regions on the periphery, close to blood capillaries, while smaller NPs are able to penetrate deeper into tumours[1, 9].

Therefore, due to the NP morphology control that the scientific community is achieving, inorganic NPs are being widely studied as drug delivery devices. They can be used as efficient drug carriers, allowing a high dose of drug to arrive at more delayed and intermittent times to specific targets, while protecting the drug and promoting endocytosis[10]. Also, they can modify the biodistribution of the drug in the body, tissues and cells[11]. Remarkably, here, the versatility in the preparation of different inorganic NPs allows the possibility of modifying pharmacokinetic aspects such as drug solubility, systemic distribution, metabolism and elimination. All these are important factors that not only can increase the amount of drug reaching the targeted area but also lower the toxicity to normal tissues[12]. Spherical NPs are usually the chosen option for use as carriers due to their simple synthesis and easy functionalization. However, exotic shapes such as nanorods, nanostars, nanocages and nanoshells, amongst others, can be finely synthesized in research laboratories and have also been proposed for biomedical applications, especially in imaging but also for therapy[13]. For example, the extravasation rate of rod-like NPs was reported to be higher than that for equivalent spheres due to tumbling effects[14] and cellular uptake and bacterial killing efficiencies have been reported to be higher for rod-shaped silica NPs as opposed to their spherical counterparts[15].

[Insert Fig. 2 here]

ii) Surface considerations: possibilities enabled by surface functionalization. Inorganic NPs can be tuned and adjusted with a proper and controlled functionalization with specific biomolecules. The modification of NP surface properties (functionalization with stealth agents, controlling surface charge) will enable to adjust one a case by case basis the desired behaviour inside the body. Here, the most paradigmatic case is targeted therapies. Since the discovery of the trans-activating transcriptional activator (TAT) from human immunodeficiency virus 1 (HIV-1) that can be efficiently taken up from the surrounding media by different cell types in culture[16], numerous peptides and small molecules have been developed to target specific therapies to different cell types and subcellular structures. The flexibility of the NP surface for functionalization using different chemistries makes them ideal tools for these targeted therapies[17].

In addition, the possibilities of the rational control on the functionalization of inorganic NPs with biomolecules is particularly important for immunotherapy, the training of the immune system to attack specifically cancer cells or to boost own immune system in a very general way, similarly as vaccines can do[18]. In this regard, inorganic NPs are excellent antigen presenters (e.g. monoclonal

antibodies designed to recognize and attack very specific target of tumoral cells[19] or well-tolerated adjuvants to enhance immunogenicity, as has been observed in different studies (see e.g. reference from Hubbel et al.,[20] and references therein). Again, the use of NP conjugates as adjuvants may present some natural advantages: rational design, low toxicity, low cost and modified and modifiable biodistribution.

Furthermore, packing molecules onto NP surfaces is known since decades ago to protect them against degradation[21] and the protection of the surface of the nanostructures with biological molecules such as albumin can improve their biocompatibility[22]. This is the case of Abraxane, considered the first formulation of chemotherapeutic drugs combined with the nanoparticle albumin bound (nab) technology to deliver and reduce side effects of paclitaxel for the treatment of different types of cancer[23].

iii) Inorganic NPs can also be used as *therapeutic agents by themselves.* First, it is possible because inorganic NPs can interact with photons of different wavelengths and trigger a variety of physical processes[12a]. Probably, the case that has attracted more attention in the scientific community is surface plasmon resonance (SPR) in the near infrared (NIR) region of metallic NPs[24]. The region of the spectra in which this SPR absorbs is strongly dependent on the shape of the NPs, and they can be designed to absorb at specific desired wavelengths. This is commonly achieved by using anisotropic NPs (e.g. Au nanorods[25], nanostars[26] and nanocages[27]). NIR is a region of the light spectrum where there is the window of optical transparency (also known as therapeutic window). Simplifying, it is a window of transparency for biological tissues from the overlapping light absorption of water, haemoglobin and melanin, basically. Thus, the possibility to excite in the NIR region rather than ultraviolet radiation allows for both minimization of photo-damage of biological specimens and maximization of the penetration depth into the tissue of the excitation light. Beyond imaging, an interesting application of inorganic NPs is in photothermal therapy, where NIR light absorbed by NPs can increase temperature up to levels for cellular death in the vicinity of the NPs, but not in unlabelled tissue[12a, 28]. At those wavelengths, photons can penetrate deep into tissue enabling the tumours to be reached. Here, UCNPs, which exhibit photon upconversion (two or more incident photons within the NIR region are absorbed by the UCNPs and converted into one emitted photon with higher energy[29] are especially suited as well to combine both molecular imaging and selective photothermal therapy[30]. Other examples of the use of NPs as therapy *per se* are the use magnetic NPs to treat cancer by inducing hyperthermia[1, 31] as the FDA-approved Nanotherm (based on superparamagnetic iron oxide NPs), and the antioxidant activity of CeO_2NPs to treat conditions related to oxidative stress and chronic inflammation[32].

iv) Inorganic NPs possess unique physicochemical properties, different from cells and tissues: possibilites for tracking their evolution and biodistribution. Here*,* it is worth mentioning that research and clinics have much broader tradition in using organic NPs and they arrived in the clinic before inorganic NPs, as in the case of Doxil, a liposomal formulation (hundreds of nanometres in size, biocompatible and biodegradable) of doxorubicin that increases the solubility of the active ingredient and modifies the dosage by sustaining it over time[33]. However, although organic NPs are generally simple to make and normally readily biodegradable, they are difficult to characterize and it is hard to monitor and trace their evolution and biodistribution inside the body. On the contrary, the unique physicochemical different signatures of inorganic NPs allows their easier detection in biological media and a more accurate monitoring of their

evolution and distribution in physiological environments and the body[3a]. To this, many inorganic materials possess intrinsic properties that can be exploited for different advanced imaging techniques, including super-resolution microscopy (e.g. nanodiamonds[34]), two-, three- or multiphoton microscopy (e.g. UCNPs[35], ZnO NPs[36] and other non-linear optical techniques[37]). Being electron-dense materials, inherently photoluminecent inorganic NPs may further be suitable for correlative light and electron microscopy (CLEM)[38], rendering them suitable as intracellular dual-contrast markers for studying intracellular processes and trafficking of biomolecules. Owing to the robust inorganic matrix, incorporated molecular imaging agents can also be readily photostabilized for long-term *in vivo* imaging applications not attainable by the molecular imaging agent itself[39].

Despite all these interesting advantages and promising results obtained in the research using nanomaterials for medicine, only few of them have reached the bedside[40]. They mainly include liposomes or organic particles but also FeOx, Au, or SiO_2 NPs are already approved; e.g. by the American Food and Drug Administration for clinical trials. A review from 2016 identified 51 FDA-approved nanomedicines and around 77 products in clinical trials[41]. There are some shortcomings in the application of nanoparticles in clinics that may be responsible for hindering a faster progress of nanomedicine. One of them, and similar to any drug development enterprise, is the enormous financial needs for new drugs and medical technologies to reach the market and the patients. As economic aspects are out of the scope of this chapter, we focus on the gaps in the knowledge and technical aspects to take into account to increase the number of nanomaterial systems to reach clinical applications. A major one is that, still, the safety of nanomaterials is a subject of a wide debate. Here, it is key to consider that the response of nanomaterials inside organisms or when released to the environment is complex and diverse and a variety of parameters are involved. Nanomaterials may be unstable and agglomerate, yielding microscopic particles, or they may end up embedded in exposed materials. Indeed, this aggregation may entail toxic effects as the lung toxicity described in section 2.1. From this same instability, nanomaterials may corrode and dissolve into molecular or ionic species, or they can suffer morphological modifications. Release of toxic ions may happen and thus, nanoparticles may act as a reservoir of them (see section 2.3). Importantly, the surface of the nanomaterials, which determine their bioactivity, experience constant modifications, particularly the adsorption of macromolecules from the media where they are exposed. This protein absorption in the bloodstream not only modify NP surface properties but also may result into protein changes and alter their metabolization (see section 2.2).

Thus, in physiological environments and inside the body, biodistribution and fate of nanomaterials will mainly depend on those parameters modified, processes that, in turn, depend on the characteristics of the exposure media[42]. And moreover, the characterization of the evolution of NPs in complex matrices such as biological environments and inside the body (intracellular, tissue and organ) is still a challenge and limited reliable data is available, adding more confusion and hampering nanomedicine development. A better knowledge of how they interact with cells, tissues, and organs is still required; including their subsequent release and/or intracellular degradation, transfer to other cells, and/or translocation across tissue barriers, the biodistribution and kinetics and their modes of clearance.. All ths will be reviewed and discussed below.

2. Physicochemical modifications of inorganic nanoparticles in physiological environments determine their effects: Safety and toxicity considerations.

The safe and effective use of promising NP based solutions for medical problems needs a proper evaluation and assessment of their behavior (ADME profiles) in the physiological media and possible unwanted effects. In this sense, despite the vast range of publications that addresses toxicity and safety aspects of nanomaterials, the potential benefits and risks that their use in medicine entails are still being debated. The poor knowledge of the nanomaterials responses and evolution inside biological media is recognized as one of the key points underpinning these controversies[43]. A paradigmatic case is the use of CeO_2 NPs in medicine. While it is reported many times to be beneficial protecting against oxidative stress[32], other studies, mainly related to the toxicity of the CeO_2 nanopowders employed in industry, show toxicity *in vitro* and *in vivo*[44]. Additionally, while some studies show anti-inflammatory effects and that CeO_2NPs are taken up by hepatocytes[32d, 45], others report macrophage (Kupffer cells) uptake by the liver[46]. Similarly, in the case of Iron Oxide NPs (IONPs, including superparamagnetic SPIONs and ultrasmall USPIOs), one of the first inorganic nanomaterial employed in medical research[47], reports of potential medical benefits and others about toxic effects keep being simultaneously published. While some reports show promising nerve cell regeneration activity[48], others find toxicity to neuronal cells[49].

As said, the modifications nanomaterials undergo in physiological media lay in the root of these controversies. Inorganic NPs employed in nanomedicine research are unstable once out of their synthesis media and inserted in physiological environments and in the bloodstream, thus partially modifying their as-synthesized characteristics. It is known that once NPs are produced they tend to minimize the high surface energy following what is called the Gibbs–Thomson effect. This effect refers to the observation that small crystals of a liquid melt at a lower temperature than the bulk. This is explained since as the size decreases, the surface tension increases. That is why NPs are systems in a metastable phase, and to reduce the surface area exposed, their fate is aggregation or dissolution towards more stable phases[50]. The most significant alterations the NPs undergo that affect their biological fate and effects are depicted in **Fig. 3** and they can be summarized as: i) the formation of the NP-Protein Corona (PC) as a result of the adsorption of proteins onto the inorganic surface, ii) NPs corrosion and/or dissolution into ionic species, and iii) the agglomeration and aggregation of the NPs, since this determines their proper interaction with biological entities.

[Insert Fig. 3 here]

2.1. Effects of the agglomeration and aggregation of nanoparticles.

It is known that the NP tendency to aggregate in biological fluids depends on parameters such as their surface charge or coating, as well as the characteristics of the medium in which they are dispersed (ionic strength, pHs, presence of macromolecules, proteins, etc). For example, Cho et al.,[51] and Casals et al.,[52] reported how NPs showed a dramatic change in their state of aggregation, dispersibility, and charge upon transfer from a buffered aqueous solution to commonly used cell culture media. When NPs are destabilized in biological media, the special physico–chemical properties that arise at the nanoscale (quantum confinement, superparamagnetism, extreme catalytic activity, etc.) are progressive/partially lost. Nor the

properties, nor the dynamics are longer the same. Agglomeration entails modifications in terms of specific surface area, concentrations, mobility, and so forth; which can be very different from the ones of the as-synthesized NPs[50]. Needless to say, this can modify the NP behaviour and effects. For instance, in *in vivo* conditions, highly agglomerated NPs will be less mobile than stable ones in the bloodstream, and thus, it can affect the NP concentration in different parts of the body, they can be accumulated or trapped in specific organs and/or they could not reach the targeted organ[53].

Following this reasoning, when assessing NPs effects, their aggregation state has been many times source of misleading conclusions. Since ancient times, we are aware that the toxicity of a substance is related to its dose (Paracelsus's *dosis sola facit venenum*), in such a way that an accurate determination of the dose -the number of NPs and their surface area are considered the dosing determining parameters[54]- is critical to properly assess the potential toxicity of a material. As a consequence, in some reports the onset of toxicity in viability experiments might be related to the onset of agglomeration: *in vitro*, NP agglomerates "rain" on top of cells due to the density, thus changing the object to test (no longer NPs) and increasing the dose on the cell[51, 55]. These large particles are indeed more difficult to be processed by the cells, as in the cases of frustrated phagocytosis of long (>5 micron) CNTs that caused chronic inflammation in the study of Poland et al.,[56] and intracellularly they could be a (too large) stone in the cell machinery (e.g. average mesh size enabled by cytoskeletal filaments is around 100 nm[57]).

Thus, even if NPs are not toxic by themselves, they may entail a potential risk as a source of aggregates when considerations about their colloidal stability have not been taken into account. In this context, an example that deserve special attention are NPs prepared as powders. These type of materials, often called *nanopowders*, are massively produced by the industry and represent a broad range of the materials to be used specially in low tech applications, from textile to cosmetics, but also sometimes in research. However, also this type of materials could enter in contact with biological entities, e.g. through the skin, by inhalation or even accidental ingestion[53-54]. To assess the biological impact of these type of commercial nanomaterials, the dry material has to be dispersed in physiological buffers prior to their exposure to cells or animal models. Here, the choice of the appropriate resuspension protocol is key to understand the obtained results.

2.2. Effects of the adsorption of (macro)molecules.

In order to analyze the NP behavior in physiological media, it is key to understand how NP and biological systems relate to each other. NPs interact with their environment through the surface, which is subject to continuous changes, while living cells communicates to the exterior mainly through their membrane proteins. It is well known that among the building blocks of life, proteins are of fundamental importance. In addition to its structural properties, almost all the interactions by which a cell recognize and relate to what is around it (recognition of signals to induce a response, immune recognition, etc.) are mediated by proteins. The two main characteristics that allow this interfacial role of proteins are their amphipathic character (combination of polar and nonpolar residues which enable proteins to have a three-dimensional structure and to be in contact with different environments) and the large number of hydrogen bonds and hydrophobic interactions that one single protein can perform[58].

Already in the first half of the 20th century, when the adsorption of proteins to implants started to be a concern in the medical community, hydrophobicity and surface charge were identified as the main factors to take into account to explain the protein adsorption to inorganic surfaces[58a]. The interfacial chemistry between blood serum proteins and inorganic surfaces is a dynamic process governed by the Vroman effect[59]. In 1962, Leo Vroman reported how the exposure of hydrophobic inorganic powders to blood plasma resulted in the removal of coagulation factors, and the inorganic surface became more hydrophilic[60]. Further, he showed this protein adsorption follows a competitive hierarchy: the highest mobility proteins arrived first and were later replaced by less motile proteins that had a higher affinity for the surface, mainly factor V and fibrinogen, in a process that takes up to few hours[59b]. This process is recognized as the general phenomenon governing the competitive adsorption of a complex mixture of proteins (as serum) to surfaces, as pointed out by Slack and Horbett[61]. Furthermore, since the 1950's, studies of other interface phenomena involving proteins have identified the adsorption of proteins to inorganic surfaces as a process that evolves to an irreversible state. Initially, the strongest argument for this irreversibility was that proteins are provided with multiple, although weak, anchor points. However, detailed studies suggest other mechanisms. The work of Alaeddine and Nygren[62] pointed out that the possibility that protein distribution on surfaces follows a crowding process where once the first proteins are attached, an initial cluster of proteins forms around these, thereby stabilizing them, and this mechanism is repeated until the entire surface is filled. Thus, not only affinity protein-surface but also mechanisms such as molecular relaxation time or spreading, depending on the time that proteins remain on the surface, have been identified as determining factor in making the adsorption as definitive[22, 58a, 63].

Accordingly, all these interfacial processes take place when NPs are dispersed in biological media, e.g. when incubated with cells in *in vitro* studies or after i.v. injection. However, some specificities in the case of inorganic NPs must be considered: NPs are not a fixed substrate but they move in solution, they have similar dimensions to proteins, and they possess a high curvature radii thus changing accessibility to their inorganic surfaces. All this effects modify the kinetics of encounter between the NP surface and proteins, the mechanisms of attachment, and the biological outcome[64]. Grouped under the name of the Protein Corona (PC) formation, these processes are key to understand NPs behaviour in biological systems. It is recognized that the proteins forming the "corona" remain associated with the particles under normal conditions of *in vivo* and *in vitro* exposure, thereby conferring their biological identity to the NP-PC composite and determining the interactions between NPs and the host in living systems. In other words, this corona of proteins “expressed” at the surface of the particle is what is “read” by cells[65]. There are plenty of references about PC formation studies in the case of inorganic NPs, to name a few using metal NPs such as AuNPs[22, 52, 66] and AgNPs[22]; hybrid metallic (FePt) NPs[67]; metal oxide NPs such as SiO_2[65], Fe_3O_4, CoO and CeO_2[22], TiO_2, and ZnO NPs[68]; CdSe QDs[69] and CdSe/ZnS QDs[67], among many others.

Many of these studies combine the mechanistic aspects of the corona formation together with their biological effects. Regarding biological implications, the aassociation with proteins may indeed biocompatibilize foreign matter as NPs, which could result in detoxification of problematic particles, as in the case of albuminization of drugs with severe side effects, the already mentioned Abraxane case[23]. Also important here is that the PC determine the NP surface charge displayed in biological media. Surface charge has been recognized as a key parameter

that strongly influences the approaching of NPs to negatively charged biological membranes, and therefore determine internalization, immune response and toxicity. Since decades ago it is known how positively charged macromolecules display incremented toxicity profile than their neutral and negative counterparts (see e.g. references of Ebbesen et al.[70], and Hoet et al.[71]). Inspired from this, several studies have been carried out involving nanoparticles of the same composition arranged with different surface characteristics. Hoshino et al.,[72] evaluated the toxicity ZnS-coated CdSe QDs, modified with carboxylic acids (negative), polyalcohols (neutral), and amines (positive). Results showed that, consistent with the case of polymers, the more positively charged the NPs, the higher the cytotoxicity. In another study, Goodman et al.,[73] found similar results using 2 nm core AuNPs with different surface characteristics. In addition, surface charge not only the interaction with cell membranes but also affect their biodistribution and residence time in the organism. Balogh et al.,[74] encapsulated AuNPs of differents sizes into dendrimers, providing negative and positive charges to the different composites. Results showed that the particles selectively accumulate in different organs depending either on size or charge alone. In this report, for instance, comparing particles of the same size, the positive ones persisted in the kidneys for up to four days (and finally excreted mostly by urine), whereas the negative and neutral particles remained in the liver and spleen over the analysis period. All in all, these results show that effects of NPs in contact with biological systems have to be analyzed together with the data about their PC formation process.

2.3. Effects of the corrosion and degradation of nanoparticles.

Corrosion, dissolution or disintegration processes of different materials have been widely studied for bulk materials in different areas of human activities, such as the iron corrosion (rust) associated with degradation of iron-based tools and structures like bridges. Potential risks and hazards derived from the introduction of metal ions in biological environments and ecosystems have been also studied since long. The ability of metallic particles to release metal ions and their induced toxicity has been focus of many safety studies, as it happens with any metallic implant where wear–corrosion greatly contributes to the release of ions responsible of health related problems[75]. Importantly, in biological environments, ions released may end up as different defined chemical species (speciation), and the different chemical species may induce different biological impacts[76].

Inorganic NPs are also subjected to these processes. Due to their reduced size, NPs have a high curvature and surface-to-mass ratio and corresponding low coordination atoms at the surface, which could enhance dissolution. However, there are many other factors to take into account, such as the metal solubility within a given environment, NP stability and aggregation states, functionalization of NPs with protective shells or coatings other properties of the exposure media such as pH, ionic strength and/or presence of adsorbing species. Thus, NPs may be subjected to a process of disintegration due to chemical reactions with its surroundings - merely from exposure to oxygen atmosphere or to certain substances as chlorine or even enzymes, or simply because the process itself is thermodinamically/kinetically favorable.

For the biological context, it is important to note that *corrosion* is the general term for the natural process by which a material is converted to a more chemically stable form, while *biodegradation* is recommended by the IUPAC to be limited to the degradation caused by enzymatic process resulting from the action of cells[77]. In general, organic nanomaterials are

more prone to be subjected to biodegradation[78]. The biodegradation of carbon nanotubes (CNTs) through enzymatic catalysis has also been described[79]. On the contrary, inorganic nanomaterials are more commonly corroded in biological environments by chemical reactions with oxidants such as oxygen or sulfur, or by some kind of other hydrolytic processes. For instance, it is known that cysteine dissolves gold[80] and chlorine dissolves gold and silver[81], among others. However, reports hypothesizing disintegration of metal NPs due to enzymatic activity in the lysozomes have appeared recently. In 2015, Jiang et al.,[82] compared the dissolution of AgNPs in acidic media and after endocytosis by epithelial cells. In model media 7.5% of total Ag was dissolved into ions while 80% was dissolved after endocytosis. Similar effects have been observed in the case of Au, Zn and FeOx NPs[83].

One important consequence of NP degradation or corrosion, given the importance of size in the nanometric regime, is that corroded particles are not in the same size range anymore when compared to the original materials, the size distribution of the NPs broadens and it may also can affect their morphology **(Fig. 4)**. Another important consequence is the toxicity of the associated metal cations. Nowadays, there is an increase of reports establishing relationships between observed effects after NP exposure and NP disintegration[69a, 84]. Probably, the most paradigmatic example is the case of nanosilver where the bactericidal effect of AgNPs have found to be correlated to the amount of Ag^{+} released ions[85]. Another famous case is the Quantum Dots. Kirchner et al.,[84c] and Derfus et al.,[69a] showed in the early 2000's how release of Cd ions caused the intracellular oxidation and toxicity of CdSe QDs. Cd binds to sulfhydryl groups of key mitochondrial proteins leading to cell death. Physiological levels of metallothionein, a protein found in the cytoplasm of hepatocytes which detoxifies Cd by sequestering it into an inert complex, were not sufficient to cells exposed to the high levels of Cd^{2+} ions released from the QDs. Finally, it is worth mentioning the feromuxytol case[86], an FDA-approved iron oxide NP suspension for anaemia treatment, which shows that, in the same way as the NP corrosion phenomenon could result in biological or environmental hazard in some cases, this process could be also harnessed for different applications, as the delivery of specifically desired compounds (in this case iron ions) for therapy.

[Insert Fig. 4 here]

3. Physicochemical modifications of inorganic nanoparticles determine their biodistribution and fate.

Once NPs have been exposed to organisms, the response of the bodies are diverse. As described in the previous section, the pharmacokinetics –and biological effects– of NPs depend on different parameters: size, shape, surface chemistry and surface properties (area, porosity, charge, coatings), agglomeration state, biopersistence, and dose. These parameters are likely to modify NPs fate, such as translocation across epithelia to other organs, binding to proteins and receptors, possible localization in different cellular organelles, induction of oxidative stress, etc. It is worth noting here that, in general, most of the research on NPs *in vivo* has been carried out in mammalian systems. For nanomedicine, oral and gastro-intestinal (GI) tract and intravenous injection have been considered as the most common administration routes; but others such as dermal, nasal or subcutaneous injection have been also addressed. In the case of nanosafety,

inhalation (respiratory system) has been also widely studied as a more likely route of unintentional exposure to nanomaterials.

Also as a general consideration, on exposure to the body, particles of different surface characteristics, size and morphology attract different arrays of serum proteins and opsonins forming the so-called NP-Protein Corona. Considerations about NP-Protein Corona have been extensively reviewed[64, 87], and also addressed in this chapter. *In vivo*, it is reported that complement proteins are likely to bind the NP-Protein-Corona in a process called opsonization, becoming the particles more susceptible to their removal by the action of phagocytes of the immune system[88]. After this, endocytosis/phagocytosis of the particles, generally by the circulating monocytes or the fixed macrophages, leads to their elimination from circulation towards organs with high phagocytic activity.

3.1. Biodistribution: Nnanoparticles entering the body.

As said, the liver is the major receptor site followed by the spleen, kidneys and other organs of the reticulum endothelial system (RES). These NPs collectors are regenerated within days or few weeks, and then NPs are excreted and disappear from the body. One of the first studies employing nanomaterials that showed these results was made by Nemmar and co-workers in 2002[89]. They used an aerosol consisting mainly carbon particles of 100 nm radiolabelled with 99mTechnetium (Technegass). They observed that the particles passed through the lung barrier in less than a minute, and reached the liver in less than one hour being accumulated there prior to their final elimination. In another work the same year, Brown et al.[90], reported similar behaviour.

After these studies and many others, it seems clear that after translocation form the respiratory system, the GI tract, the skin or i.v. injection, NPs are cleared up rapidly (within minutes) from the bloodstream, being liver and spleen (≈90 %) and the kidneys (≈9%) their typical final biodistribution[53]. In general, the larger ones are retained first in the spleen and liver and the smaller ones that pass this filter end up in the kidneys. From there NPs are expulsed with the faeces or the urine. As a guide, usually is considered that continuous capillaries found in most tissues, such as muscle, lung, and skin have a cut-off about 6 nm and fenestrated capillaries in kidney, intestine, and some endocrine and exocrine glands have a cut-off about 50-60 nm. Once the particles are excreted from the body, their final fate, as explained in the previous section is either dissolution, agglomeration or absorption onto sediments to be finally immobilized[91].

To overcome these limitations, nanomaterials can be designed so as to circumvent the first-pass effect in the liver. Surface modifications, as coating with polyethylene glycol (PEG), cell-penetrating peptides and other targeting molecules may prevent hepatic and spleen accumulation, opening the possibility to reach other organs. The possibilities that NP functionalization opens for targeted nanomedicine has been also discussed above. In addition, subcutaneous or intratumoral injection is proposed as the most promising route for successful implementation in patients, and they are currently the most frequently used route in animal experimentation[92]. This route of administration overcomes the limitations arising from systemic circulation, and in the case of oncology, targeting can also be optimized by delivering the nanomaterial directly to the interstitium of the cancerous tissue[93], since the interstitial pressure

in tumors seems to be higher than in healthy tissues, enabling higher leakage of the drugs[94]. Again, the fate of the nanomaterials after subcutaneous injection heavily depends on physicochemical properties of the nanomaterials in the conditions of the interstitial lymphatic flow. Note that despite its advantages and successes, intratumoral injection is only applicable for easily accessible tumors[92].

3.2. Subcellular localization: Nanoparticles entering the cells.

Once NPs have entered the body, and have been distributed, the next step is to investigate their penetration into cells after crossing the cytoplasmic membrane. This membrane controls entries into the cell and has a crucial role in development, up-take of nutrients, the immune response, neurotransmission, intercellular communication, signal transduction, and cellular and organism homeostasis. For this, it has been widely studied the different uptake mechanisms of different substances. These mechanisms mostly depend on the size of the object to be internalized. Small molecules, such as ions, amino acids or sugars cross the cell through pinocytosis, via membrane protein pumps or channels, while larger molecular entities enter the cell via endocytosis, in membrane-bound vesicles formed by invagination of the plasma membrane. Endocytosis can be active (receptor mediated) or passive (by adhesive interaction and invagination of the membrane). Whichever the endocytic routes of up-take (receptor-mediated, macropinocytosis, micropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (see e.g. reference of Conner et al.[95]), the material initially remains into a sub-cellular compartment, the endosome, which is still separated from the cytoplasm of the cell by a membrane.

It is very difficult to draw a map of the mechanism of NP internalization into cells since the endocytosis process for nanomaterials and other foreign materials is very diverse. The uptake mechanism activated will depend on the characteristics of the material (size, shape, composition, surface coating) the cell type (e.g. references of Kuhn et al.[96], and Mahmoudi et al.[87], showing different uptake mechanism for different nanomaterials) and the physical interaction of the material with the cellular membrane[97]. In addition, the chemical and physical properties of the cellular membrane responsible for the translocation of nanomaterials into cells, the nucleus and organelles are still unkown[92]. As a general consideration, it is important to note that the vast majority of studies showed NPs presence inside vesicular structures and not the cytosol (**Fig. 5**). Release from endosomes and reaching the cytosol is difficult. Some of the strategies performed to obtain cytoplasmatic release from the endosomes are the the use of disrupting peptides sucha as the mentioned Tat-petide[98], or proton sponge mechanism[99].

What is known, is that most of these endocytic routes end up in the lysosome and/or exosomes. Lysosomes are the degradative compartment of the cell where materials are exposed to high concentrations of a wide variety of hydrolytic enzymes and acidic pH, as discussed in section 2. In this sense, NPs do not remain for a long time inside those vesicles, either NPs are dissolved or are expulsed again in an exosome. In the case that NPs would remain inside the cell, their fate will be bonded to that of the cell. And since cellular recycling keeps in the body continuously regenerating, NP permanence in the body will be limited and disperse in time. The cells turnover rate varies from weeks in tissues such as the skin and gastro-intestinal tract, to years in bone or neurons. In any case, so far, reported cases of extended permanence of inorganic matter in the body are granulomatosis (as silicosis, asbestosis, etc.), where macrophages are not able to

internalize and degrade micrometric particles which ultimately leads to chronic inflammation (and cancer) as also it was shown for the mentioned case of >10nm CNTs[56]. Thus, these events are not likely to apply in the case of well-stabilized inorganic NPs, since they are much smaller and can be easily internalized by macrophages.

[Insert Fig. 5 here]

3.4 Long term effects.

There is still not extensive literature on the fate of NPs intended for medical applications over more than a few days and data is scarce. This could be most likely due to the limited characterization possibilities for tracking nanomaterials over long periods of time and the maintenance of the animal models[92]. It is important since in the case some NPs stay for long periods of time inside cells or organisms, even at non-toxic concentrations, they may have side effects. Thus, long term chronic and repetitive exposure should be considered[53]. It should be carefully investigated how cells respond to treatments with nanomaterials below the doses causing high percentage of cell death since even small changes may cause profound effects on the integrity and viability of the cells over multiple cellular divisions.

Probably due to those difficulties, data on long term consequences of exposure of inorganic NPs in the body is not only scarce but sometimes also contradictory. In a recent study carried out by Wang et al.,[100] using 3.5 nm Au NPs, presence of Au in kidneys and accumulation in tumor tissue in mice is reported for a period of 90 days. On the contrary, Naz et al.,[101] using 2, 5 and 10 nm Au NPs also for a period of 90 days found complete elimination for all sizes. Of course, for larger sizes, data is more concise and indicate that, although with some rates of clearance, there is still detectable presence of NPs or NP debris at least after months. For instance, Sadauskas et al.,[102] using 40 nm Au NPs found Au in the liver of mice after 6 months. Similarly, Goel et al.,[103] using 27 nm PEG coated AuNPs found Au in the tumor interstitium in mice after 120 d as well as in the spleen, liver and kidneys. Other inorganic NPs of interest in medicine may be more prone to dissolution and clearance through kidney after few weeks, as some small size (<10 nm) metal oxides[32d, 104], but still some reports found persistence of NPs in the case of large (>30 nm) FeOx[105] and SiO_2[106], among others. Both these results and the still lack of enough data of long term exposure calls for the need of studies of the chronic implications of the use of NPs in medicine.

4. Outlook and Conclusion.

NPs are unique tools for the successful application of novel nanotechnologies to health. These applications are growing progressively since the last two decades and they are raising high expectations for better, more efficient and affordable healthcare. On the one hand, in this chapter, we have pointed out how the firsts nanotechnology-based medical solutions are already on the market, many are in clinical trials, but the most of the future promising applications are still under development. The specificity of functionalized NPs for targetting at tissue and cellular level, in both diagnostic imaging and drug-based therapies is pushing the research forward to create novel theranostic nanoplatforms that will, in turn, prevent non-specific cell binding in healthy tissues (personalized medicine with less/no side effects). For instance, in diagnosis, hand-held devices with highly accurate, highly sensitive, multiplexed and inexpensive testings are already in the pipeline of many biotech companies. In addition,

molecularly targeted NPs with different labels offers many advantages over conventional molecular imaging probes. Also, a combination of labels for different imaging modalities can be attached to a single NP, and at the same time, the same NP can contain different targeting ligands which provide enhanced receptor binding affinity or specificity. In therapy, for instance, targeted medical applications in the focus of the European Technology Platform - Nanomedicine (ETPN) where nanomedicine will have higher impact are Alzheimer Disease, Cancer, Ophtalmology, AntiMicrobial-Resistance, Diabetes, Infectious Diseases, Atherosclerosis, Arthritis and Tissue Engineering.

On the other hand, in this chapter we have reviewed different studies of biodistribution and fate of inorganic NPs for nanomedicine and nanosafety, that show how the differently observed biological fate and effects are mainly related to its evolution (agglomeration, dissolution, protein adsorption) in the physiological media, making this a needed area of research that will allow for an efficient implementation of nanomedicine. It is accepted that NPs may be destabilized when travelling through different parts of the body. Their high surface energy tend to aggregate them homogeneously (forming polycrystalline particles) or heterogeneously (with molecules and structures of the surroundings)[63], both altering and modifying biodistribution. Similarly, during their time inside the body, NPs are affected by the presence of different redox states (from rather reducing to clearly oxidizing), pH (the late endosome and lysozomes can go down to 5) and the presence of nucleophilic species and ionic scavengers. Inside the body, the protein absorption onto NP surface not only modify NP surface properties but also may result into protein changes and alter their metabolization as preliminary results showed[107]. The consequences of these modifications in the protein conformation and metabolization in, for example, the immune response, it is still rather unknown. Importantly, all these modifications depend to a large extent on the characteristics of the biological media in which the NPs are dispersed and they are underappreciated parameters that need to be carefully addressed to better understand the NP effects. The development of reproducible and reliable analytical methods for the intracellular, tissue, and organ specific characterization of nanomaterials evolution is still a challenge, and limited data is available. But, ultimately, the knowledge of these pharmacokinetic and biodistribution aspects that drive NP behavior and effects will allow to take further advantage of NPs potential clinical benefits.

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Figure legends.

Figure 1. Overview of the versatility of inorganic nanoplatforms for medical applications.

Figure 2. The size of NPs determine the interaction with the immune system and hence the residence time in blood and associated effects.

Figure 3. Schema of the physicochemical modifications that NPs can undergo in biological environments.

Figure 4. Morphological modifications of NPs over time. TEM images of 8 nm Fe_3O_4NPs and 15 nm AgNPs as synthesized and after 100 days in cell culture medium.

Figure. 5. CeO_2 NPs internalized by human hepatocytes (HepG2 cells). TEM images of hepatocytes revealing internalization of the NPs and localization in the citoplasm. Left, bright field images; Right, dark field image of the same area allowing NPs to be easily distinguished.