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IN VIVO DEGENERATION AND THE FATE OF INORGANIC NANOPARTICLES

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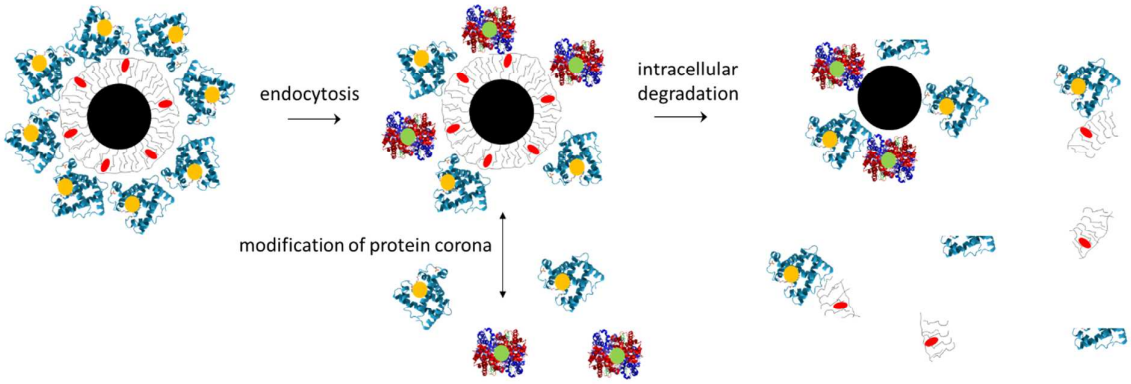
Nanoparticles comprise of a core, a surface coating, and a corona of adsorbed biomolecules, of which all parts can have a different fate.



Abstract

What happens to inorganic nanoparticles (NPs), such as plasmonic gold or silver, superparamagnetic iron oxide, or fluorescent quantum dot NPs after they have been administrated to a living being? This review discusses the integrity, biodistribution, and fate of NPs after *in vivo* administration. The hybrid nature of the NPs is described, conceptually divided into the inorganic core, an engineered surface coating comprising of the ligand shell and optionally also bio-conjugates, and the corona of adsorbed biological molecules. Empirical evidence shows that all of these three compounds may degrade individually *in vivo*, and that each of them can drastically modify the life cycle and biodistribution of the whole hetero-structure. Thus, the NPs may be decomposed into different parts, of which biodistribution and fate would need to be analyzed individually. Multiple labeling and quantification strategies for such a purpose will be discussed. All reviewed data indicate that NPs *in vivo* should no longer be considered as a homogeneous entity, but should be seen as inorganic/organic/biological nano-hybrids with complex and intricately linked distribution and degradation pathways.

Nanoparticles as tunable tools towards application in nanobiomedicine

The applications of engineered nanomaterials (NMs) are not only increasing in technical products, but are also more and more common in biotechnology and biomedicine [1-4]. The intersection of nanotechnology and biomedicine defines one of the most exciting and cross-disciplinary developments over the last decade [1, 2, 4, 5]. NMs and in particular, colloidal inorganic nanoparticles (NPs), are increasingly considered as novel, promising tools with improved therapeutic efficacy, biodistribution, and pharmacokinetics [3, 6-9]. Recent advancements in synthesis and the ability to rationally manipulate NMs' and NPs' features, such as their physical, chemical, and biological properties open up additional possibilities in designing a new generation of nanoprobes for theranostic applications [10-18]. For example, considerable progress in the development of magnetic NPs with engineered physicochemistry and tailored surfaces properties [19] has opened up a variety of clinically relevant applications, such as magnetic resonance imaging (MRI), drug delivery, magnetic hyperthermia, and low cost *in vitro* diagnostics [3, 6, 8, 9, 20-24]. Also, plasmonic NPs, in particular Au NPs, are used for similar purposes [25], ranging from plasmonic sensing, photoacoustic imaging, drug delivery, photothermal therapy (PTT), photodynamic therapy (PDT), and many others.[26-29] Fluorescent semiconductor NPs, so called quantum dots (QDs) [30], have been proposed as contrast agents for fluorescence imaging, guided surgery [31], PDT, *etc*., though clinical *in vivo* use is still

under debate due to their potential toxicity. Moreover, with the advent of the concept of so-called 'personalized medicine', the field of nanobiomedicine has started to grow, producing a huge variety of different multi-functional NMs. However, despite the increasing production of new nano-tools, to date only a few of them have reached the clinics [32]. Yet, some formulations based on gold and iron oxide NPs have already been approved or are in phase 3 of clinical trials. One of the most challenging technical difficulties that NMs are facing in biomedicine, is to successfully cross biological barriers and to specifically recognize their targets while they circulate through the body [5] (*cf.* Figure 1). Moreover, the use of NMs may pose unknown risks to patients, thus current enthusiasm for nanotechnology might shift towards safer approaches [5, 33-37]. This review focuses on NMs and NPs designed for biomedical application. However, as these materials are also used in industrial and technological sectors, from which they may be released into the biological environment at a certain period of their life cycle, the arguments on degradation of NPs given below also apply to materials involved in non-intentional exposure of living organisms.

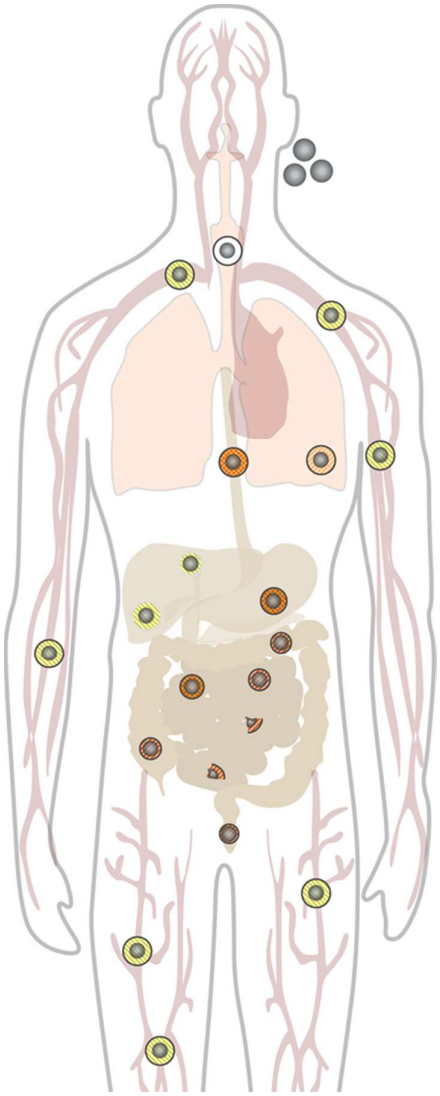


Figure 1: The *in vivo* pathway and fate of NPs through the human body. Whereas accidental exposure of humans to NPs and their entry into the body most likely occurs through the inhalation and oral route, biomedical applications mainly focus on intravenous injection or oral application routes. Upon inhalation, ingestion, or injection of pristine NPs, they come into contact with various complex physiological environments, leading to the covering of NPs with various biomolecules. Upon the formation of such a corona made of biomolecules, the *in vivo* transformation of pristine NPs is rapidly initiated. During their passage through different compartments of the body, the *in vivo* degeneration and fate of NPs differ significantly, which is indicated by the different symbols for the NPs. Whereas NP-biomolecule corona complexes are quite stable in plasma, their uptake into organs, such as the liver can trigger corona and NP degradation. Likewise, the conditions in the digestive tract, such as low pH and presence

of surfactants, may lead to similar degradation processes. Such *in vivo* transformation of nanomaterials should be considered in nanotoxicology and nanomedicine.

Nanoparticles in biological environment transform into composites

What happens to NPs once they have been administered *in vivo*? Though the *in vivo* biodistribution of inorganic NPs (*e.g.,* plasmonic NPs, superparamagnetic NPs, quantum dots) is relatively well investigated, the appraisal mainly concerns the inorganic NP cores. However, the plain inorganic cores would not be stable in biological environments. Without organic surface coating, either obtained by chemical design or due to adsorbed proteins, the NPs would agglomerate. Thus, NPs within the *in vivo* environment are complex hybrids with an inorganic core and an organic/biologic surface coating [38, 39], *cf.* Figure 2. Conceptually, we will describe each NP as a hybrid object composed of three different entities: the inorganic core, the engineered surface coating, and compounds adsorbed from the biological environment.

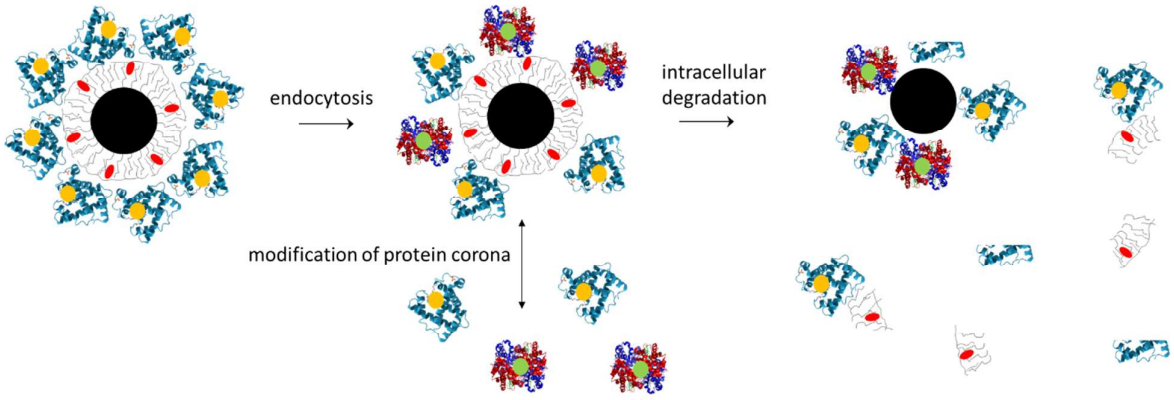


Figure 2: A typical inorganic NP within *in vivo* environment comprises of an inorganic core (drawn in black), a capping organic surface, which provides colloidal stability (drawn in grey) and a shell of adsorbed proteins (drawn in blue). The NPs can change their physicochemical properties under *in vivo* conditions. This may involve dynamic exchange of the protein corona, depending on the variations in the biological environment and/or the activity of "cellular degradation machineries". Degradation may decompose the NPs into individual parts. Depending on the NP core material, the inorganic cores may start to decompose, and thereby change their physical and morphological properties. The organic coating around the inorganic NP core could also be partly removed, as adsorbed proteins may be digested. A possible dissolution of the NP core is not depicted in the sketch.

The NP core defines the "physical identity" or, in other words, the basic functional physical properties of the NP, such as being plasmonic, superparamagnetic, or fluorescent [40]. In this way the core may provide the contrast for several imaging/detection modalities or create heat upon excitation for hyperthermia treatment, *etc*. The persistence of the core's physical properties in biological environment is critical for NPs' theranostic efficiency. However, biotransformation of the NPs (aggregation, dissolution, degradation) might jeopardize such properties over time, depending on the NPs' environment.

The engineered surface coating determines the intrinsic physico-chemical properties of the NPs, sometimes also termed "synthetic identity" [41]. Typical surface coatings for inorganic NPs involve short ligand molecules such as lipoic acid [42, 43] or peptides [44], silica shells [45, 46], polymer micelles [47], or lipid micelles [48, 49]. The resulting intrinsic physico-chemical properties, such as surface charge, hydrophilicity/hydrophobicity, *etc.,* play an important role in the colloidal stability of the NPs. Appropriate surface coatings prevent NPs from agglomeration and ensure dispersion of the NPs in complex environments. The coatings also determine how the NPs interact with biological environments, *i.e.*, how molecules from these environments adsorb to the NPs' surface. Distinct surface coatings were shown to have a profound impact on the NPs' biocompatibility and fate, including cell viability, or cell adhesion, the NPs' cellular uptake, lifetime in the blood system, and the biopersistence in tissues belonging to the mononuclear phagocyte system (MPS; formerly referred to as reticuloendothelial system, RES) [50-56]. In addition, coatings may involve functionalization via conjugation with targeting ligands and/or bioactive molecules for obtaining multifunctional 'intelligent' NPs [57]. In this way the engineered coating plays an important role in active targeting schemes.

It is often neglected that in complex physiological environments, such as blood, a certain degree of *in situ* biotransformation will most likely occur for all NPs. For the majority of *in vivo* applications, NPs will be intravenously injected, and will be immediately exposed to a highly complex biological environment. There, a plethora of ions [58, 59] and biomolecules, such as lipids, metabolites, sugars, and especially proteins, [60] will adsorb onto the surface of the NPs, mediated by van der Waals, electrostatic, hydrogen bonding, and hydrophilic/hydrophobic interactions [38, 61-66]. The sum of all adsorption processes will result in the formation of the so-called 'biomolecule corona', of which, so far, the protein corona (PC) has mostly been studied. It is now accepted, but far from being understood in detail, that

the formation of a protein/biomolecule corona critically affects not only the physico-chemical characteristics of NPs [67], but also the (patho-) physiological and biomedical identity, often simply referred to as "biological identity" [41, 68] of NPs in general [5, 69-71]. Hence, the properties of corona-covered NPs differ (in most cases significantly) from those of the intrinsic physico-chemical properties of the NPs, before their exposure to biological environments [5, 33, 64, 71]. In the area of corona research, the term 'hard corona' was coined to define a protein adsorption signature of a NP, but is sometimes also used to describe a NP's 'long-lived' equilibrium protein signature, *e.g.*, the plasma protein signature of a NP in the blood [4, 62, 64, 71-73]. On top of this 'hard corona', some models also suggested the existence of a 'soft corona', which can be conceptualized as a putative, loosely associated, and rapidly transient layer of biomolecules [62, 72, 74-78]. However, since such a 'soft corona' seems to desorb during current purification processes, its existence, (patho)biological, and medical relevance still remains vague [3, 5]. Here, a standardized definition would be very helpful. In the following, only the analytically accessible proteins associated as NP-protein complexes will be referred to as 'protein corona' (PC). Notably, the PC not only (co)defines interaction interfaces between NPs and biological environments, but may also additionally trigger the NPs' 'transformation' by altering their colloidal stability. The PC can either have a stabilizing effect by inducing steric stabilization, or have a destabilizing impact, caused by protein mediated bridging, charge compensation and/or by the introduction of charge inhomogeneity onto the NPs' surface [63, 79-82]. Upon aggregation, multiple interactions may result in stronger affinities compared to proteins binding to single NPs, which is likely to occur in a biological solution, in which NPs are highly diluted. Moreover, there could even be a trapping of proteins in such aggregates, with otherwise low or no affinity for single NPs. Depending on the type of NP, such aggregation may also require a certain time, and may thus additionally impact the evolution of the PC [79-82]. As protein adsorption is a dynamic process depending on affinities of individual components for each other, it also varies with the ratio of NPs over available proteins. An important consequence is that the PC differs in a medium with 10% serum, which is currently used in cell culture *in vitro,* in comparison to *in vivo* conditions, in which the protein ratio is much higher [68].

Taking together the statements from the last paragraphs, NPs have to be seen as complex hybrids formed *via* their interaction with biological environments. The interplay between molecular constituents of the biological medium, in which the NPs are dispersed, and the synthetic NP surface, determines the surface properties of the NPs and their colloidal stability.

Being colloids, the "synthetic identity" [41] of NPs as well as their physical properties need to be characterized in solution (*ad minima* in water), which defines their surface charge, hydrophilicity/hydrophobicity, and aggregation state. In the next step, the "biological identity" [41, 68] involves also molecules from biological media, which adsorb to the NP surface. In this way, for a full characterization, NPs dispersed in the actual biological medium are required. A final step of characterization is to follow the processing of NPs within an *in vivo* system.

Application routes and biodistribution of nanoparticles

Upon administration, NPs will interact with cells. It is well recognized from cell-culture studies that almost all mammalian cells can, in principle, incorporate NPs to some extent, due to a variety of non-specific uptake mechanisms [83]. Many studies have explored the NP properties which influence the efficiency of cell uptake and determine also their intracellular processing, resulting in complete or partial degradation, or storage of biopersistent NPs in unchanged form in the cell [84]. However, how relevant are these *in vitro* studies for the *in vivo* situation? Mammalian cells can clearly process and degrade larger molecules and NPs. Yet, this is not routinely necessary for all cells. Cells in culture are also either dividing rapidly (cancer lines) or are restricted in their metabolism (primary culture) and interactions. Contrariwise a close interaction between tissues exists *in vivo*, in which certain cell types, such as the professional macrophages in the mononuclear phagocyte system (MPS), are very potent in a fast clearance and processing of larger NPs from the blood flow, whereas other cells may be very limited in their capacity to uptake and process NPs. Importantly, *in vivo*, the NP clearance directly depends on the global status of the immune system [85]. This implicates that, besides the physico-chemical properties of the individual NP, the cell type, which is primarily exposed to the NPs, and the model system used, will also influence the biodistribution, intracellular transfer, and degradation of NPs *in vivo*.

Bolus injection into a peripheral vein seems to be the most recurrent application route for most nanomedical applications. Following intravenous *(i.v.)* injection, the blood flow would spread the NPs into the right heart, through the lung capillaries, back to the left heart and then into the arterial system supplying each organ with the respective NPs. Under this condition, a main blood fraction will enter the liver and spleen, which have a huge capacity to remove xenobiotic NPs from the blood stream. A few studies have quantified the distribution of labeled NPs [86, 87], and it can be expected that a large fraction of most protein-covered NPs will

be taken up by these organs within minutes after injection (*cf.* Figure 3). Another cell type, involved in the efficient uptake of NPs *in vivo* are endothelial cells, which line veins, arteries, and capillaries throughout the body, and thus come in direct contact with the injected NPs [4]. Evidence indicates that there could be a difference between endothelia in the liver (liver sinus endothelial cells (LSECs), and peripheral endothelial cells (PECs)), which differentially uptake NPs with respect to their surface charge, with anionic NPs taken up by the liver [4, 80, 88, 89] and cationic NPs preferentially binding to PECs [90].

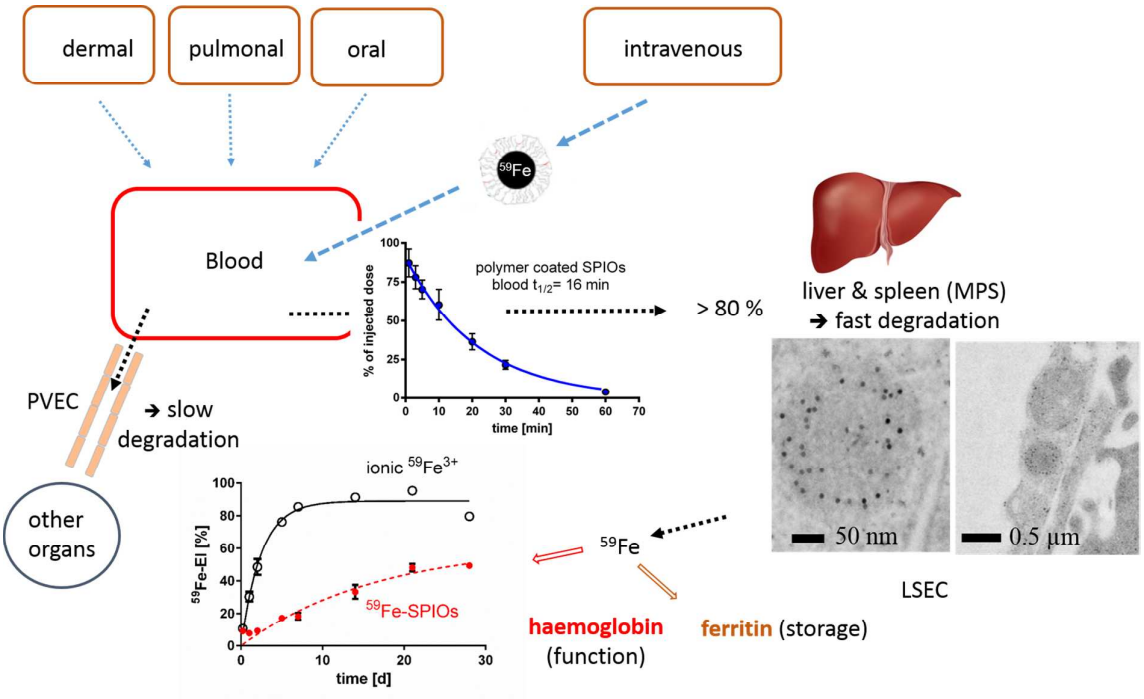


Figure 3: Distribution and degradation of an intravenously injected ^{59}Fe-labeled FeO_x NPs in mice. The FeO_x NPs consisted of a monodisperse iron oxide core (11 nm diameter) coated with an amphiphilic polymer, poly(maleic anhydride-alt-1-octadecene), resulting in a 25 nm negatively charged NP in aqueous solution. A large fraction of the NPs is taken up within minutes by liver cells (Kupffer cells and liver sinusoidal endothelial cells (LSECs)). The degradation of FeO_x core of the NPs can be monitored and quantified by measuring the amount of ^{59}Fe incorporated into the hemoglobin of newly formed erythrocytes. The FeO_x NPs were also incorporated in peripheral vascular endothelial cells (PVECs), which represent a large surface area in veins and capillaries. Differences in the degradation efficiency among different cells indicate that FeO_x NPs or NP remnants might remain in some cells, and thus elicit a cell-specific chronic toxicity, which is hard to measure *in vivo* with standard toxicity

tests. MPS = mononuclear phagocyte system (mainly liver and spleen). The figure represents a compilation of images from several publications [80, 86, 88, 89].

The situation is different after injection of NPs into the peritoneal cavity or directly into a tumoral lesion [91], which are also relevant for nanomedicines [92]. From the peritoneal cavity, the NPs have to pass the visceral peritoneum first, a monolayer of mesothelial cells, and then enter the interstitium, with its lymph or blood vessels, which can transport them into the blood pool. Likewise, also pulmonary [93], dermal [94], and oral uptake routes [95, 96] have to be taken into account, in particular in case of environmental NP exposure due to air pollution or spills. Very little quantitative information is available so far on cell processing efficiency after *in vivo* administration through abovementioned routes. Lademann *et al.* investigated the NP–skin interactions for silica, titanium dioxide and silver NPs. The vast amount of topically applied solid NPs stays on the skin's surface, deeper penetration of single NPs was seen by X-ray microscopy, Raman spectroscopy and flow cytometric studies, with hair follicles representing an important storage and putative entry site [97, 98]. Kreyling *et al.* found that inert gold NPs, administered intratracheally are phagocytosed mainly by lung macrophages and only a tiny fraction of gold NPs translocated into systemic circulation [99, 100]. Enterocytes in the gastrointestinal tract seem to represent an effective barrier against NP uptake. So far, experiments with gold NPs [95], carboxylate-functionalized polystyrene NPs [101], and iron oxide NPs [88] have been performed to elucidate the oral application route. Bargheer *et al.* studied the intestinal absorption of iron from ^{59}Fe or ^{51}Cr-labeled cores of FeO_x NPs. After oral application of labelled NPs in mice, a significant absorption of ^{59}Fe but not of ^{51}Cr was observed by sensitive whole-body-counting. As ionic Cr^{3+} is known to be almost not absorbed in rodents, the results show a partial degradation of the iron oxide cores in the acidic stomach, followed by the physiological absorption or released ionic Fe^{2+}, and almost exclude a relevant intestinal absorption of intact NPs. It should be emphasized that we know far too little about the accurate distribution of a given NP composite *in vivo* and that reliable techniques are urgently needed to document which cells do take up the respective NPs and to what extent.

Biological environments may impose hostile conditions to nanoparticles

As outlined in case of intended or accidental *in vivo* exposure, NPs take a complex route through the body. From administration until eventual intracellular long-term deposition or alternatively excretion, the NPs are exposed to a variety of different biological environments. The hostile conditions in those environments can vary significantly, for example in the pH or

in the local protein (*i.e.,* enzyme) species composition, *etc*. Highly acidic compartments can be either found on the organ level, inside the stomach or tumors, or on a cell organelle level, in endosomes / lysosomes. Low pH and protein adsorption can affect colloidal stability and favor NP aggregation inducing detrimental modifications of their magnetic or optical properties, when the NPs are clustered inside intra-cellular compartments [102-104]. Acidic media also facilitate the corrosion of inorganic NP cores [105], such as in the case of plasmonic Ag NPs [106-108], superparamagnetic FeO_x NPs [109, 110], or fluorescent CdSe NPs [111]. Silica NPs can be, for example, completely dissolved by hydrolysis [112]. Enzymes and reactive oxygen species on the other hand have been demonstrated to be able under certain conditions to digest carbonic parts of NPs, as demonstrated for example for carbonic, purely inorganic carbon nanotubes (CNTs) [113], the strongest and stiffest materials in terms of tensile strength and elastic modulus [114]. Thus it is likely that enzymes can also digest parts of organic surface coatings around inorganic NPs. Indeed, the first evidence has been reported [115-117]. Enzymes may also attack proteins adsorbed onto the NP surface [118], and the PC has been demonstrated to dynamically evolve [119].

Thus an important question to be addressed is the *in vivo* integrity of the NPs. Will the organic surface coating and the PC of internalized NPs remain unmodified, or will they be (partly) degraded *in vivo*? Continuing with this train of thought leads to many potential implications. If the organic NPs' ligand shell is degraded after internalization, how much can fancy surface chemistries performed on NPs direct their biodistribution? This would have an impact on the ongoing discussion of passive *versus* active tumor targeting. Can organic surface coatings possibly resist enzymatic degradation? On the contrary, while for some applications NP degradation is unwanted, other applications might require it. For example, can inorganic NPs be designed to be degradable into fragments that can be cleared from the body by renal excretion? Improved NP design could accordingly allow for a new generation of inorganic NPs tailored for medical applications with controlled degradability.

Since this kind of research is still in its infancy, this review aims at providing an overview about what is already known about the *in vivo* integrity of inorganic NPs, and on how their *in vivo* physico-chemical and biomedical properties evolve. As in this review NPs are considered as hybrids, including inorganic core, engineered surface coating, and adsorbed biological molecules (*cf.* Figure 2), the fate of all these compounds needs to be investigated for a detailed analysis. The fate of a given hybrid NP *in vivo* depends on various factors such

as entrance route, PC formation in blood, distribution in the cardiovascular system *etc.*, uptake into different cell types, intracellular degradation and release, and processing of inorganic core or organic materials, *etc*. Even if uniformity and full colloidal stability of the NPs is provided during intravenous injection, it cannot be expected that all NP components end up in the same functional pool *in vivo*. Therefore multiple labeling strategies are needed, with which the different components can be observed independently [120]. These would involve reliable quantification techniques for the inorganic core material as well as for attached organic molecules, in order to balance at least the main distribution paths for a given NP. Radioactive labeling is a historical tool with great value to monitor the transport and metabolism pathways of biomolecules. This technique has already been used to trace the kinetics of Au [121], Cd, and Fe-based NPs *in vivo* [86]. In addition, MRI and fluorescence imaging can be used to record biodistributions [87, 122, 123], but are incapable of detecting absolute amounts, as the fluorescence of molecules and magnetic resonance relaxivities may depend on NPs local environment and aggregation state. It is also of utmost importance to involve all the controls which are necessary to prove that the labels are attached permanently to the respective NP compound [124, 125]. Detachment of the label would lead to the determination of the biodistribution of the free label, and not of the labeled NPs. In the following text the *in vivo* fate of three different NP compounds, *i.e.* cores, engineered coating, and adsorbed biomolecules will be discussed individually. We will hereby start from the outside to the inner parts of the NPs, *i.e.,* from the PC, to engineered organic coatings, to the inorganic core.

Degradation / evolution of the corona of surface-adsorbed biomolecules

The first part of NPs, which is likely to interact with the environment, is the PC, being the outermost entity. Having pointed out that the PC clearly (co)defines the "biological identity" [41, 68] and fate of NPs' in biological environments, it is clear that understanding its evolution and exchange processes *in vivo* becomes crucial to produce nontoxic and effective NPs [3, 62, 71, 126]. *In vitro* experiments have shown that the formation of the PC influences cellular uptake of NPs, which appears to be dependent on the nature of the adsorbed proteins [3, 61, 62, 64, 71, 126]. Elucidating the fate of the PC during the delivery of the NPs *in vivo* is also a key determinant for developing efficient nanomedicines [3]. The complexity of the biological environment can strongly alter the composition of PCs during their systemic route through the body with possible biological implications (*cf.* Figure 3). The specific fate of NPs mostly depends on the

entry or chosen administration route (*cf.* Figure 3). For example, an intravenously injected NP formulation will incur the following biological processes: vascular transport, extravasation, interstitial passage (extra-cellular matrix), cellular uptake, and clearance. Thus, NPs and more realistically, cognate PCs, will need to overcome different biological barriers before reaching their final intended or unintended destination.

Numerous studies have shown that the physicochemical properties of pristine NPs, such as size, shape, and surface chemistry (termed the 3 'S') can influence the amount, composition and *in situ* evolution of the PC, which in turn can (co)determine the NPs' bioactivity [33, 63, 64, 72, 127-129]. For example, there is evidence that the PC is capable of regulating various cell-NP interactions [64, 126, 130-132], blood residence time [133, 134], (tumor) cell targeting activity and pharmacokinetic profiles [134], albeit the underlying molecular mechanisms are not yet fully resolved. A variety of *ex situ* and some *in situ* studies have been conducted to dissect and mechanistically understand the biomolecule corona on the nanoscale, its dependence on the NPs' physico-chemical properties and its impact on the biotransformation and fate of NMs in the human body and environmental systems [33, 63, 64, 72, 128, 129, 135]. Typically, corona profiles differ significantly from the protein composition of the investigated (biological) fluid [62-64, 82, 126]. Distinct proteins will either enrich or display only weak affinity for the NP surface. Despite significant work concerning the important relation between the original surface functionality of the NPs and the nature of the corona, it currently still remains impossible to predict or to simulate these interactions in complex physiological environments [5, 63, 64, 72, 126].

Despite the complexity and analytical challenges already occurring for *ex situ* characterization of the PC, additional challenges are faced during its *in situ* analysis. Particularly, when NPs move from one physiological (micro)environment of the body to another, *e.g.*, from the circulation *via* different cellular uptake mechanisms into cells and different organs, such as the liver or spleen, a key question is whether the original corona remains stable or is subjected to substantial changes, which again adds an additional level of complexity [62-64, 71, 72, 86, 92, 105, 125, 126, 129, 136, 137]. So far, it is assumed that even after passing through several "physiological (micro)environments", the final corona would still contain a fingerprint of its history and keep a memory of its prior passage through the body [63, 105, 138], which is in line with recent reports showing the stability of PC signatures *ex situ* [5, 64].

Though other studies suggest that PCs may be subject to modifications in their composition

while they reside in the body, when they encounter different biological environments, as it occurs for NPs conceived to target tumors [3, 63, 105, 139, 140]. A study on silica NPs indicated that PCs formed in serum and then transferred to cytosolic extracts experienced qualitative changes in the PCs' composition. Yet, these results should be carefully interpreted, as analytical methods may not detect low corona protein concentrations [138].

However, the majority of the studies conducted so far demonstrated a stable PC fingerprint, originating from the biological fluid the NPs encounter first, unless processing is performed by enzymatic cellular machineries [63, 125, 138, 141]. Here, dissolution processes have been recognized as being essential for the NPs' fate, biodistribution and also toxicity, particularly for metal and metal oxide NPs [126, 142-146]. Even when investigating the intracellular fate of silica-coated magnetite NPs by recovering NP-containing cellular organelles, employing magnetic separation techniques, studies demonstrated that PCs, associated with NPs extracted from different cellular compartments, still retained a cytoplasmic fingerprint, albeit additional proteins adsorbed to the "precoated" NPs [147]. Radioactive double-labeling was a convenient analytical technique for investigating the stability of a preformed protein corona *in vivo*. For example, adsorbed ^{121}I-labelled transferrin was transported efficiently into the liver [80]. Collectively, the conservation of specific protein signatures in the PCs from different physiological compartments encountered by the NPs may potentially allow reconstructing the NPs' history through the body. The extent of rearrangements/evolution in PC fingerprints are related to the NPs' 3 'S', and to additional factors such as exposure time [64], temperature [148], and the composition of the encountered physiological environments. As the PC remains an unpredictable complex factor, there are currently numerous attempts to chemically prevent and/or modulate protein adsorption [62, 63, 134, 149-151]. Such chemical strategies include hydrophilic oligomeric or polymeric PEGylation [152], zwitterionic low molecular weight and polymeric coatings, which reduce PC formation.

Again, there are many issues that have yet to be unraveled. Which role does enzymatic degradation play in addition to composition exchange for the PC? *In vitro* experiments have suggested that after intracellular incorporation, lysosomal enzymes may digest parts of the original PC [118]. Advanced techniques are required to detect the modifications occurring *in situ*. These techniques would complement classical methods, which allow detecting the composition of the PC, but require extractions steps, which might be responsible for the formation of a new equilibrium after each purification procedure, and might significantly

change the PC composition [153]. Standard *in situ* techniques such as dynamic light scattering (DLS) and fluorescence correlation spectroscopy (FCS), which can be conveniently applied to PC characterization in sample solutions of biological liquids, [148, 154, 155] are unlikely to be suitable for *in vivo* analysis. Instead, spectroscopy techniques in which the read-out signal depends on the adsorption of proteins, such as surface enhanced Raman scattering (SERS) may offer good possibilities [156-158].

Degradation of engineered surface coatings of nanoparticles

The engineered organic surface coating providing the "synthetic identity" [41] of the NP lies beneath the PC. However, concerning the *in vivo* stability of engineered surface coatings, there is not much work reported in literature. Clearly there is indication that *in vitro*, inside endosomes / lysosomes part of the surface coating may be released from the NP core. The endo/lysosomal enzyme cathepsin L, for example, potentially cleaves a third of the human proteome, and the degradation of peptides conjugated to the surface of NPs has been shown within endosomal compartments [115]. The enzyme α-glucosidase has also been demonstrated to degrade the carboxydextran shell around NP cores [116].

Degradation has been shown quantitatively by using a double labeling technique. A ^{14}C-labeled peptide or a ^{125}I-labeled protein was covalently bound to ^{59}Fe-labeled iron oxide cores, and the radioactivity of the labels was followed in the blood and in *ex vivo* samples of organs after two hours. From both NPs, no separation between core and shell molecules was found while in blood circulation (*cf.* Figure 4). A covalently bound 11-As myelin basic protein (MBP) was transported efficiently into LSECs, where the immune competent peptide must have been separated from the core and was presented on the surface of the cells, because the onset of experimental autoimmune encephalomyelitis was completely prevented in a mouse model of multiple sclerosis [89] (*cf.* Figure 4, upper lane). Likewise, covalently bound or *in vitro* adsorbed ^{125}I-labeled mouse-transferrin was not separated from the ^{59}Fe-label in blood, and was transported into the liver, where however, a complex metabolism and re-distribution of this physiologically relevant plasma protein occurred (*cf.* Figure 4, lower lane) [80, 88].

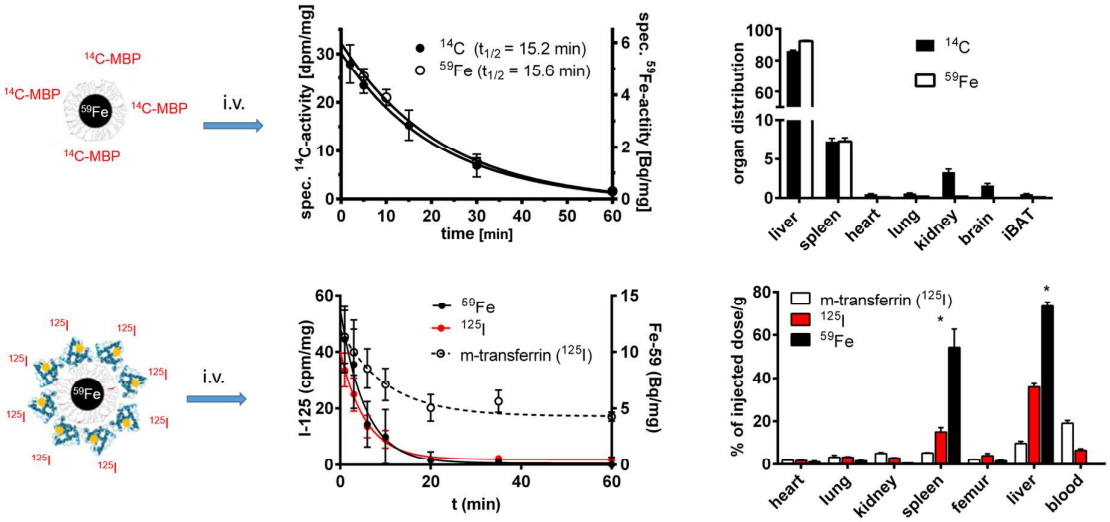


Figure 4: Fate of two double-labeled NPs *in vivo*. The upper lane displays polymer-coated ^{59}Fe-labeled FeO_x NPs. The carboxyl groups of the surface were covalently coupled to a ^{14}C-labeled peptide (11-As, myelin basic protein). After *i.v.* injection in groups of mice (n = 3-4), the ^{59}Fe and ^{14}C-label disappeared synchronously from blood following a first order kinetics due to the fast uptake mainly into liver and spleen [89]. In the bottom lane, polymer-coated ^{59}Fe-labeled FeO_x NPs were covalently labeled with ^{125}I-labeled mouse-transferrin. The blood half-life and the organ distribution were followed in groups of mice after *i.v.* injection. Again, a synchronous removal of both labels from the blood was found, probably indicating the uptake of intact NPs into the liver, without separation of the protein covered shell/polymer from the core already in the bloodstream [80].

The use of radiolabeled NPs thus clearly provides a powerful tool, especially for the sensitive and reliable quantification of the distribution of the different parts of NPs. However, this requires special equipment, and the selection of appropriate isotopes is critical. For each isotope the individual transport mechanisms must be carefully taken into account by including a control group, in which an ionic probe of the element under study is handled in the same way as the NPs.

In yet another study partial separation of the engineered polymer shell around Au cores was demonstrated [125]. Here the polymer shell was labeled with ^{111}In, and the Au NPs with ^{198}Au *via* neutron activation, *cf.* Figure 5. After intravenous application to rats, biodistributions of ^{111}In and ^{198}Au were recorded after one hour and 24 hour retention. Controls ensured that the labels were not lost and in fact corresponded to the locations of core and polymer shells. The data presented in Figure 5 indicate that in particular in urine, more polymer than NP cores

were found. Therefore, it was concluded that after intravenous injection, the polymer coated Au NPs are cleared by the immune system and are transported to the liver, where they are endocytosed. While in this way the majority of the NPs (comprising core and engineered polymer shell) are trapped in the cells of the liver, this also initiates their degradation. Proteases in the endosomes/lysosomes of the cells where the NPs are located may start cleaving the polymer shell. In the particular case, the polymer shell comprising peptide bonds could be readily cut by proteases present in endosomes/lysosomes. The liberated polymer fragments were small enough to be exocytosed leading to final renal excretion.

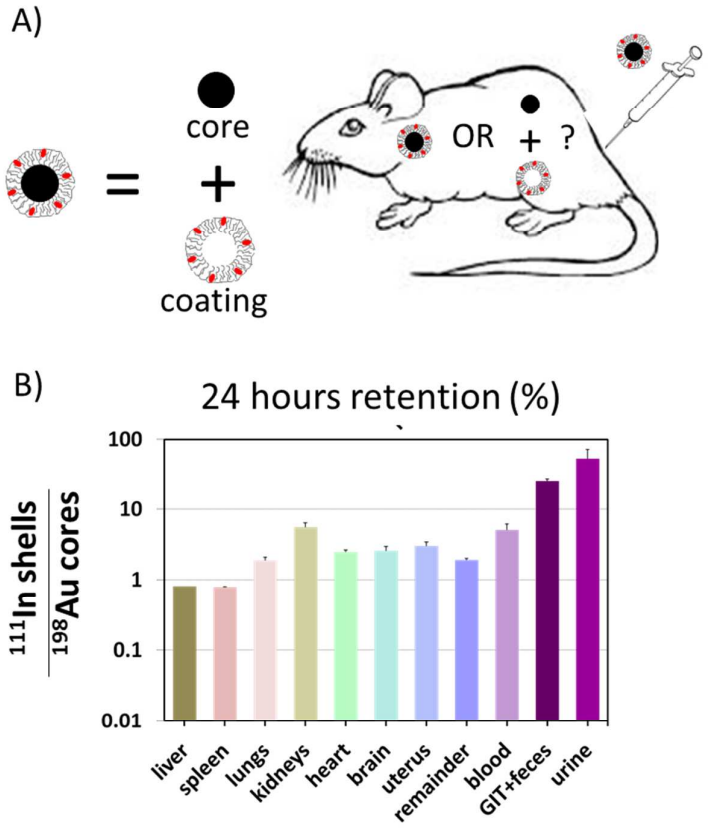


Figure 5: A) Au cores were coated with an amphiphilic polymer. Cores and polymer shells were labelled with ^{198}Au and ^{111}In, respectively. The question was whether *in vivo* the polymer shell would remain around the cores or whether it would separate. B) The ratio of radioactivities originating from ^{111}In and ^{198}Au is shown for different organs 24 hours after retention. In case this ratio is higher than one, polymer must have come off the NP cores. Figure adopted from Kreyling *et al* [125]. GIT = gastrointestinal tract.

While these findings offer a first indication that engineered surface coatings can be degraded, there is not much quantitative information to be found in literature. It is clear that in endosomes/lysosome enzymes exist, which can cleave organic surface coatings. These

involve proteases, designed to cut proteins or polypeptides into peptide fragments, which can cut peptide bonds (such as cathepsin L or trypsin), and enzymes, designed to cut polysaccharides into saccharides (such as α-glucosidase), which can cut sugar bonds. However, this does not exclude the existence of engineered NP coatings, which could withstand enzymatic degradation. In addition to intracellular enzymes, degradation may occur in blood by esterases [159], which could, for example, cut coatings containing ester bonds. In this way, for each engineered NP coating one would need to take into account which enzyme could degrade this coating, and what the biodistribution of this enzyme is. Double labeling of the core and the shell and double detection schemes, such as correlative microscopy (scanning electron microscopy / fluorescence microscopy), will surely be a useful tool for the investigation of particle and coating distribution.

Degradation of inorganic nanoparticle cores

Ultimately, the inorganic core, as the innermost part of NPs may also be degraded. Obviously the degradation of inorganic NP cores will be highly dependent of the composition of the NP material. While for example NPs made of Ag, ZnO, CdSe, and FeO_x are known to corrode, and thus release metal ions [105], other materials such as NPs made from Au are typically considered to be inert and thus stable against degradation. However, as will be discussed in this section, such NPs can also undergo structural alterations. This can be assumed from different points of view. First, ligands such as thiols can strongly bind to the Au surface, and under certain conditions, this may lead to pulling-out Au atoms *via* the ligand from the Au surface [160, 161]. Thiols are available in cells, as for example in the glutathione, an antioxidant produced by the cells. Thus, in principle, it is possible that Au NPs (very slowly) dissolve. Second, Au NPs can be made with different shapes. The surface A and volume V of a cubic NP with side lengths d is $A = 6d^2$, $V = d^3$, respectively, leading to a unity surface $A^* = 6$ for a NP with unity volume $V^* = 1$. In contrast, for the NP sphere with diameter d the surface and volume are $A = 4\pi(d/2)^2 = \pi d^2$, $V = (4\pi/3)(d/2)^3 = (\pi/6)d^3$, respectively, leading to a unity surface $A^* \approx 4.8$ for a NP with unity volume $V^* = 1$. Thus, for the same volume, a cubic NP has a significantly bigger surface than a spherical NP. The most stable and energetically favorable configuration is the one with the smallest surface and thus NPs with spherical geometries. Thermodynamic laws might therefore govern shape transitions of internalized NPs. In addition, some crystals' facets may be more stable than others, stabilizing different shapes or favoring degradation on preferential faces. Thus, even for "inert" materials such as Au, degradation or crystalline reorganization might occur to some extent.

The case of superparamagnetic iron oxide (FeO_x) NPs is discussed, which is known to be chemically reactive. In order to quantify the inorganic core of NPs and its degradation residues over time in the body, elemental analysis such as inductively coupled plasma mass spectrometry (ICP-MS) is often used. The difficulty is however to distinguish between the original NPs and their products of degradation. For FeO_x NPs, elemental analysis is inappropriate due to the high amount of endogenous iron forms. However, FeO_x can be quantified and distinguished from endogenous iron and non-magnetic residues by following their magnetic properties with nanomagnetism methods, such as electron paramagnetic resonance (EPR) or temperature-dependent susceptibility measurements [110, 162]. These magnetic characterizations give information about the biotransformation of the superparamagnetic iron core at short and long terms. Soon after engulfment of FeO_x NPs by macrophages, their intracellular confinement impacts their magnetic dynamics due to impaired rotational and translational mobility as well as magnetic interactions. The high local density of FeO_x NPs in lysosomes results on one hand in a decrease of magnetic susceptibility and, on the other hand, in an increase of the temperature of transition between superparamagnetic and ferromagnetic regime, affecting in turn the MRI relaxivity and heating capacity of the FeO_x NPs under alternating magnetic fields [102-104]. At longer term the evolution of superparamagnetic properties of NPs may reflect the degradation of their iron oxide core [109, 110, 163]. MRI provides a non-invasive mean to detect the distribution and integrity of FeO_x NPs over time in the same animal, although quantification remains challenging since MRI relaxivities significantly depend on the local environment and physical state of NPs. EPR was used to quantify the dissolution or elimination of superparamagnetic iron oxide from liver and spleen over one year after intravenous administration of FeO_x NPs at the relevant dose for MRI application (2.5 mg/kg body weight). This included 7-8 nm spherical FeO_x NPs with a hydrophilic glucose-derivative coating, proposed by Guerbet *et al.* as contrast agent for MRI [110], 20 nm FeO_x nanocubes, coated with polyethylene glycol (PEG) [91, 163], and 13 nm iron oxide/5 nm gold dimer hetero-structures, coated with an amphiphilic polymer or PEG [50]. As expected, the nature of the coating of the FeO_x NPs determined their initial uptake in the organs of the mononuclear phagocyte system [50], the PEG-coated NPs being less accumulated in spleen and liver than the NPs coated with amphiphilic polymer. More surprisingly, the difference of initial coating has a long-lasting effect on the degradation/elimination of FeO_x NPs, which persisted longer in spleen and liver (more than one year), when coated with the amphiphilic polymer. Regardless of the NPs, the elimination

of magnetic iron was almost complete in liver after a few months, while 10 to 30% of the initial amount of magnetic iron still persisted in spleen six months after administration. It is worth noting that the total uptake of NPs is much higher in liver than in spleen (15 to 80% of the injected dose in liver depending on NPs *versus* 2 to 6% in spleen), but the concentration per gram of organ is larger in the spleen. Thereby the degradative capacity of the organ could be saturated by high local concentration of the material in the spleen.

Apart from MRI, the *in vivo* degradation of FeO_x NPs can be also followed *via* radioactive labeling. The core material, *i.e.,* the inorganic core of the FeO_x NPs can be labeled either during the synthesis or by neutron activation of already synthesized material. Recently, a fast, gentle, and quasi on-demand method for post-synthetic labeling of monodisperse iron oxide cores with ^{59}Fe has been developed, which allows for studying the distribution and metabolism of these cores in detail [86]. After intravenous injection in mice, the fate of FeO_x NPs was studied, *cf.* Figure 3. After a lag phase of 3-7 days, ^{59}Fe from the administered FeO_x NPs appeared in the hemoglobin of newly formed erythrocytes, indicating the intracellular degradation of the FeO_x NPs. ^{59}Fe was released from the cores and channeled into the physiological transport ways for iron. However, a substantial part of the label from the NPs was obviously retained in organs and tissues, probably also indicating storage of intact or only partially degraded FeO_x NPs, presumably in cells other than macrophages.

It should be noted that the so-called ^{59}Fe-erythrocyte incorporation rate (^{59}Fe-EI) is a long-known and very unique parameter for following the *in vivo* processing of iron from any given iron supplement, including NPs. This has been monitored with ^{59}Fe-labeled dextran or carboxydextran coated FeO_x NPs used as MRI contrast agents (Endorem®, Resovist®), during studies involved in the registration procedure [164, 165]. The information obtained from ^{59}Fe-EI in living animals is whether iron is released from the core of a NP at all, to what extent, and at which time scale. In comparison to a test substance (*e.g.*, ionic Fe-salt), the degradation of a given FeO_x core and the incorporation of released iron in erythrocytes can be quantified. This technique, if available, is an optimal complement to EPR, which quantifies the persistent FeO_x core through its magnetic properties and to multiscale imaging techniques including TEM in *ex vivo* samples, which can give more insights into the mechanisms of uptake and intracellular processing. However, without a reliable quantification technique, these techniques always lack the information about how relevant the collected data is for the studied *in vivo* system. Highly fluorescent QDs with the same surface modification as FeO_x

NPs can be alternatively used, to follow the cellular distribution and uptake kinetics *in vivo* with intra-vital fluorescent microscopy. This technique shows for example the special role of vascular endothelial cells in the liver or outside the liver for the uptake of different NPs [166].

Together with the quantification at the organ level (*i.e.*, the recording of biodistributions), following inorganic cores at the nanoscale in biological environments, is essential for evaluating the degree of degradation of the nanostructures over time and possibly, demonstrating the recycling of degradation products in proteins. In that regard, the multi-functionalities of TEM are of great interest because on the one hand, analytical methods (energy dispersive X-ray (EDX) and electron energy loss spectroscopies) allow studying the local biodistribution of exogenous materials (from cellular up to the single NP or protein levels) and on the other hand, high-resolution imaging techniques provide the unique opportunity to probe the atomic-structure of NP cores in the organism. Although the morphological degradation of polydisperse 7-8-nm spherical FeO_x NPs is difficult to ascertain *in vivo*, FeO_x nanocubes and gold/iron oxide heterostructures show evident features of erosion in the liver and spleen seven days after administration, *cf.* Figure 6. This is particularly conclusive for heterostructures, because iron oxide crystals degrade around the gold cores, leaving the less reactive gold remnants as long-lived witnesses of iron oxide dissolution in lysosomes. Nanoscale observations also show that FeO_x NPs become increasingly surrounded by monodisperse (6-nm) iron-storage ferritin proteins, which are rich in iron, but present different atomic structures than the NPs and can thus be identified by high resolution TEM (Figure 6, day (D)14) [50, 110, 163]. The presence of ferritins proximally to FeO_x NPs suggests that iron released from degraded FeO_x NPs could be locally transferred to endogenous ferritins through a process regulated by iron homeostasis [167]. Consistently, an increase of non-magnetic iron in spleens was observed, confirming the local transformation of superparamagnetic FeO_x NPs into non-magnetic iron species. Such a scenario has a perfect plot: the cell firstly confines the NPs within the lysosomes, where the iron remains bound in crystals until the proteins are synthesized and recruited in the vicinity of NPs. Secondarily, in order to allow a close approach of endogenous proteins, which tightly and safely unload the iron cargo, while avoiding the cell deleterious Fenton's reaction, the cell progressively isolates the NPs within the lysosomes, as observed with TEM [163]. If confirmed, the mechanisms of metal transfer from NPs to endogenous proteins would exemplify a quintessential process in which biomolecules and homeostasis regulate the local degradation of NPs and recycle their by-products.

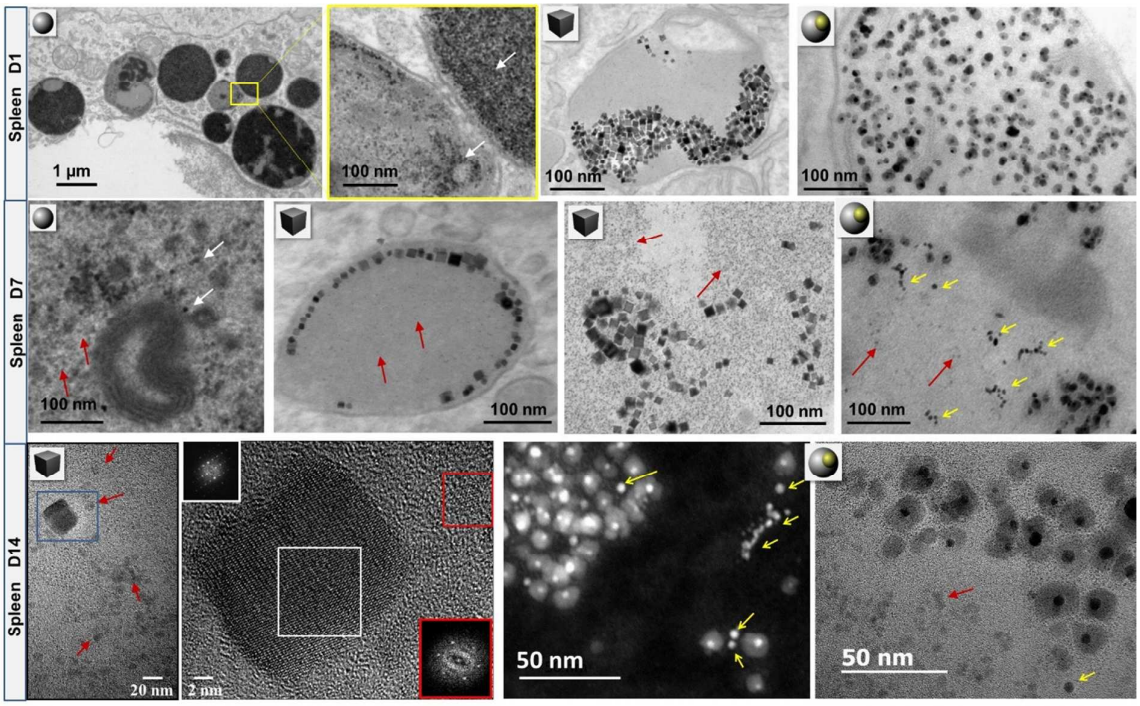


Figure 6: Intralysosomal degradation of FeO_x nanospheres, nanocubes and gold/FeO_x heterostructures in murine splenic macrophages. At day 1 (D1) post-injection, FeO_x are concentrated within lysosomes of macrophages. After one week (D7), a fair share of NPs arranges on the margins of lysosomes or appear more isolated. Red arrows point to NP-adjacent monodisperse iron-rich ferritin proteins. Some parts of gold/FeO_x heterostructures have been locally degraded leaving gold remnants (yellow arrows). Scanning transmissions electron microscopy (STEM) - high-angle annular dark field (HAADF) and elemental nanoanalysis by STEM-EDX confirm the partial or total disappearance of iron oxidesurrounding the resilient gold core. The Fourier transformation (FT) of the degraded and resilient nanocubes at day 14 (D14, white square on high resolution TEM micrograph) shows that nanocubes maintain their initial lattice structure (spinel inverse or vacancy-ordered γ-Fe_2O_3 structures). The FT of the ferritin protein core (red square) shows a hematite structure, suggesting local transfer of iron from degraded NPs to the storage protein. Image adapted from previously published work [50, 110, 163].

Advanced NP design could allow for a new generation of inorganic NPs with controlled degradability of the inorganic cores. For screening the factors that impact the lifecycle of NPs in the body, one could track the aging of single cores in a medium that mimics some features of lysosomal environment, *i.e*,. acidic pH (4.7), and the presence of iron chelators [109, 168], *cf.*

Figure 7. Consistent with *in vivo* observations, the surface coating raises a more or less efficient shield to the effect of the microenvironment and clearly governs the kinetics of FeO_x NP dissolution. At the single NP level, the areas of the FeO_x NP with the less dense polymer coverage (*e.g.*, the vertex of nanocubes) are the most prone to degradation [163], *cf.* Figure 8. In addition to the above mentioned argument of increased surface-to-volume ratio present at edges, the degradation may be sterically facilitated at these positions. As observed *in vivo* for gold/iron oxide dimers, hydrophilic PEG coating is less effective in retarding the FeO_x NP degradation than a double chain amphiphilic polymer [50]. In the case of Ag NPs this effect has not been observed in the same way [107].

Concerning NP geometry, the NP's architecture also appears as a major factor that impacts the NP's bio-persistence. Multicore flower-shaped FeO_x NPs, formed by the coalescence of magnetically oriented iron oxide seeds, rapidly disintegrate in lysosome-like medium, losing the outstanding properties that they had owing to their cooperative structure [169]. Importantly, the junctions in the multicore structures are the most vulnerable sites. Apart from organic coating, the association of different materials allows for modulating the biopersistence of NPs. For example, the disintegration of multicore FeO_x nanoflowers is more or less delayed when they are covered by a layer of gold depending on the thickness and porosity of the gold shell [169]. In a general manner, the degradation process of inorganic cores is a step-by-step corrosion governed by surface reaction mechanisms. Therefore, the efficiency of organic or inorganic engineered coatings relies on their ability to prevent the access of the cellular medium to the core surfaces. *In vivo*, the 5 nm gold seeds associated to iron oxide spheres persisted much longer than the iron oxide crystals in splenic and hepatic macrophages, but also showed reorganization (as chains and assemblies) as well as degradation into smaller structures one year after injection, once the iron oxide part had been dissolved, *cf.* Figure 6 [50]. Importantly, the size diminution of poorly reactive NPs could enable size-dependent elimination processes such as renal clearance, which could not occur in the case of originally injected NPs. In addition, the variation in the state of aggregation of the NPs in the lysosomes also plays an important role in the possibility of degradation and clearance. The way by which NPs could be excreted from macrophages or translocated and cleared in other organs is another issue. It has been shown recently that macrophages, endothelial cells or mesenchymal stem cells that have first internalized FeO_x NPs [170, 171] Au NPs, QDs [172], or CNTs [173], can expulse NPs in the extracellular medium within microvesicles when the cells are stressed by starvation. Microvesicles are constitutively released by virtually all cell types in body fluids

and are considered as potent vectors of intercellular communication *in vivo*. Such vesicles can spread NPs across the body and transfer these nanomaterials to distal cells. This propagation process, mediated by underestimated vectors of NP dissemination, additionally increases the importance of the *in vivo* fate of NPs. Despite the non-ambiguous local degradation processes of NPs, it must be noted that a few resilient intact NPs could be observed even one year after injection, regardless of the NP's nature, even in the case of highly biodegradable iron oxide. As observed *in vitro* during single NP tracking, evidence shows that the degradation process is a non-linear and uneven process, which integrally dissolves some NPs, while other particles remain unmodified. The exact mechanisms of degradation, enzymatic attack and involvement of cell metabolism in such process still remain an open question. Ideally the design of complex nanostructures should help modulating both the time frame of NP activity and the duration of degradation/excretion processes. Interestingly, the recent advances of TEM in liquid environment could also help contemplating nanomaterials at the nanoscale within biological environments, with the possibility of observing the interaction between NPs and wet-cell cultures *in situ* with unprecedented resolution [174, 175]. In addition, TEM in liquid media opens many avenues for studying oxidative transformations of NMs, by directly observing the effects of reactive-oxygen-species (ROS) created by the electron beam by radiolysis processes, on the atomic structure of NMs. As oxidative stress play a critical role in the cellular processing of NMs [176], such dynamic nanoscale investigations allow recapitulating the ROS-induced aging of NMs in cellular media. For example, the *in situ* monitoring of CNT degradation induced by hydroxyl radicals provided a mechanistic understanding of the stigmata of degradation observed on nanotubes after aging into macrophages [177].

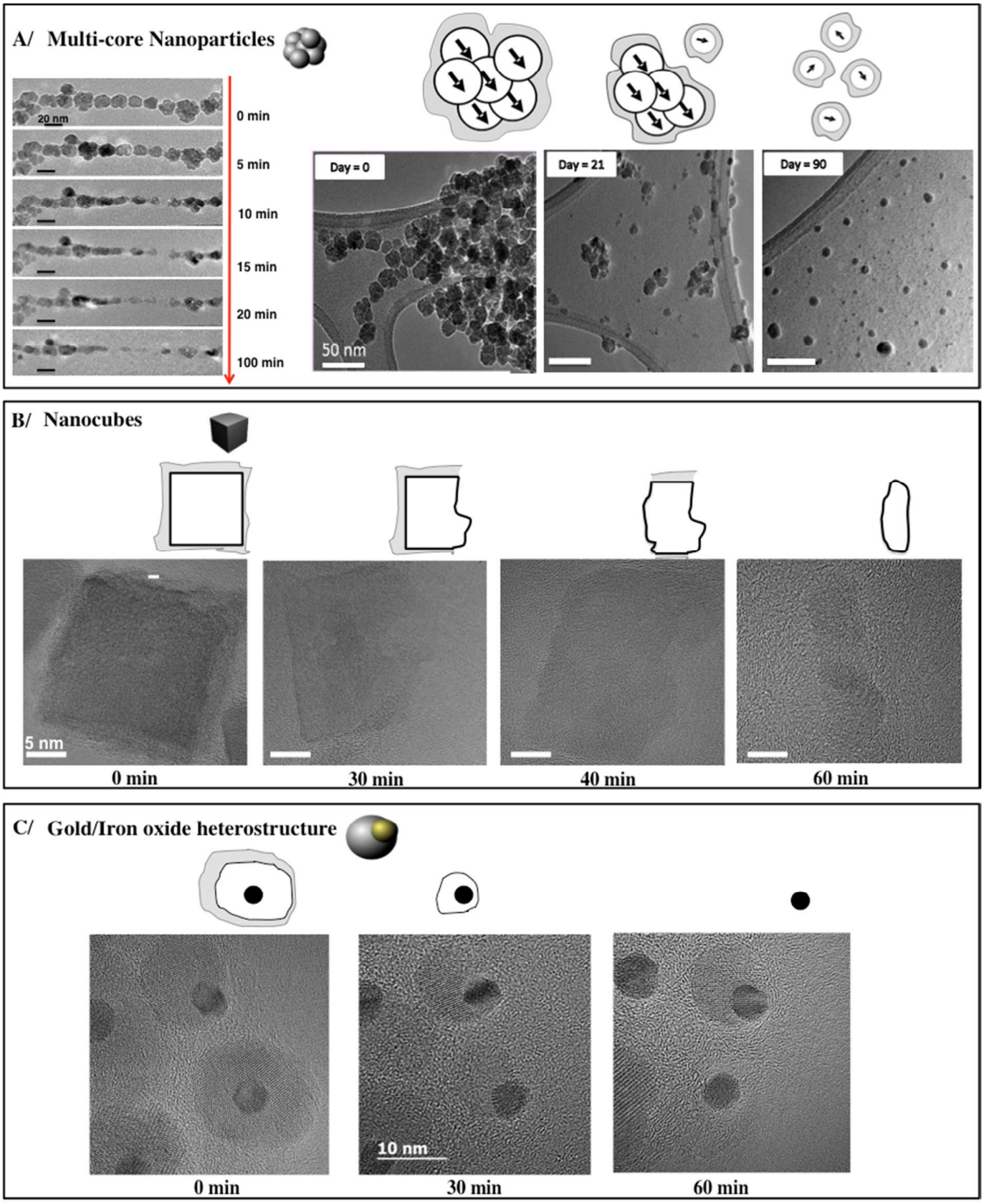


Figure 7: Progressive erosion of different FeO_x NPs in lysosome-like medium. Image adopted from previously published work [50, 163, 169].

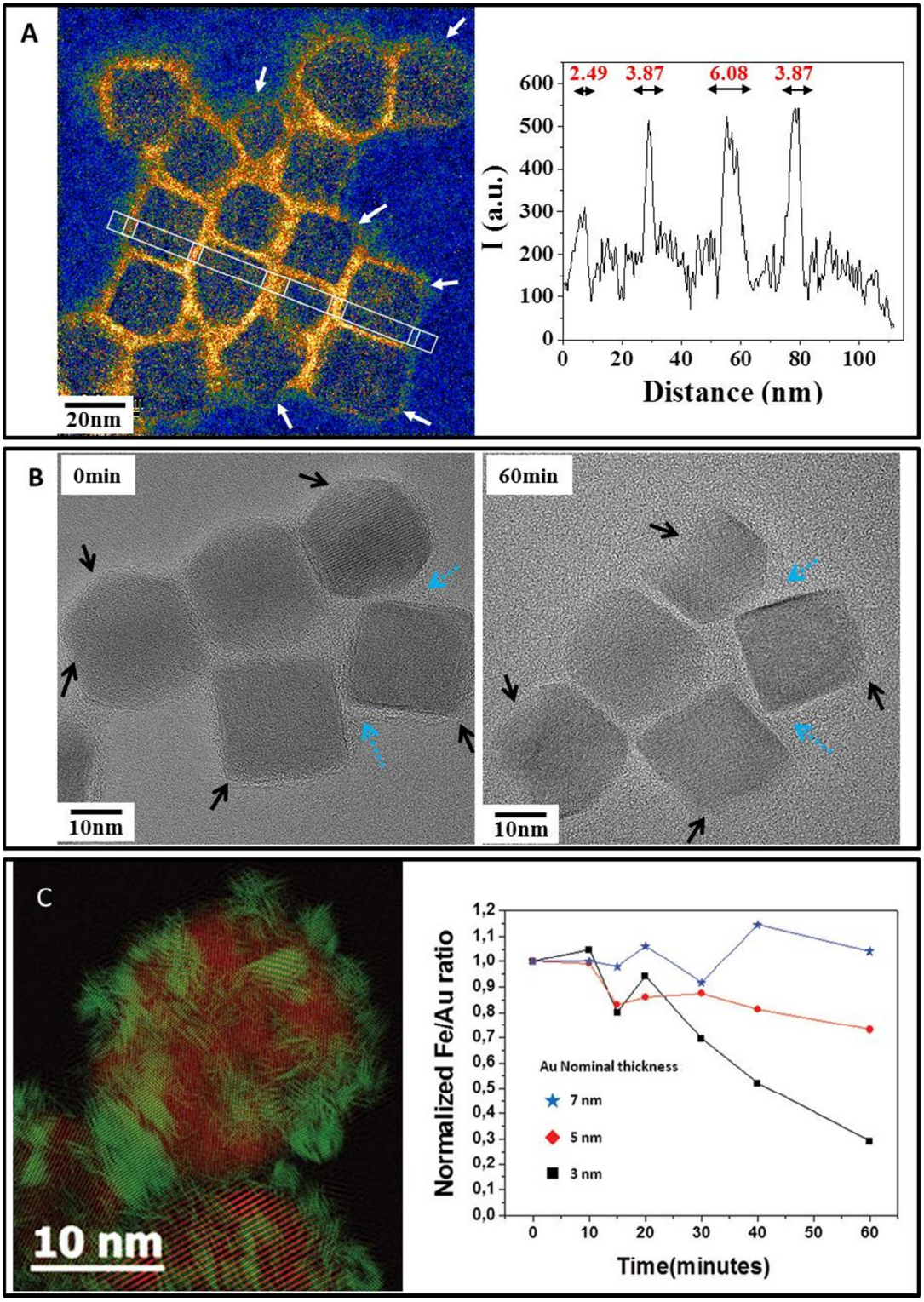


Figure 8: Protective role of coating on NP degradation. A) Carbon mapping obtained by Energy Filtered TEM (EFTEM) evidences the uneven coverage of the amphiphilic polymer layer surrounding the cubes. White arrows point to polymer-poor regions where the degradation begins. Image adapted from Lartigue *et al* [163]. B) Step-by-step degradation of

nanocubes showing faster degradation in zones with poor polymer coating (black arrows) and protective effect of dense coating areas (blue arrows). C) The coverage of multicore FeO_x NPs with a 3 nm gold layer (iron featured in red and gold in green on Bragg filtered high-resolution micrograph on the left) protects the NPs from degradation. The EDX quantitative measurement of the Fe/Au ratio shows that the thickest gold layer efficiently delays NP degradation and iron release in lysosome-like medium.

The examples listed in this review clearly demonstrate that inorganic NP cores can be degraded *in vivo*. While this conclusion is commonly reached in literature for different materials [105], such as Ag, FeO_x, ZnO, the discussion about degradation of other materials, such as Au, has just begun. While some cores do degrade, the exact mechanisms of degradation have not been fully understood yet. Can NPs be completely dissolved and these products excreted from the body? For NPs of "inert" materials such as Au, what are the detailed mechanisms of intracellular gold dissolution? What is the role of the PC in core degradation, following the observation that the PC changes over time, in particular after NP internalization in lysosomes [118], and what is the role of intra-lysosomal proteins? In case NP concentrations in one organ are reduced over time, how to distinguish between translocation of intact NPs (with or without coating) from organs where local degradation occurs? If degradation happens, are the byproducts less toxic than the original non-reactive persistent NPs? Does degradation depend on NP concentration, *i.e*,. will lysosome overload with NPs lead to impairment or acceleration of the degradative capacity of cells and autophagy? Importantly, corroborating nanoscale information (*e.g.*, by TEM) with tissue level investigations (*e.g.*, by ICP-MS, magnetic or optical techniques) and biological studies (genetic or proteomic techniques) is a cornerstone for addressing the many open questions on the degradation of NP cores.

Conclusions

As interactions of NPs with their environments are dominated by their surface and in this way by their engineered surface coating together with adsorbed biomolecules, the biodistribution and fate of NPs need to be correlated to their physicochemical properties as well as their biocoating. While the physicochemical properties and biocoating of NPs can be measured in different biological fluids, there are only few appropriate techniques to (kinetically) determine NPs *in vivo* and evaluate their evolution over time through their route in the body. The most drastic change in NPs' properties may involve *in vivo* degradation, and in this way the fate of

all NP components - the inorganic core, the engineered surface coating, and the adsorbed biological molecules - need to be analyzed. Assessing biodistribution and clearance would involve multiple labeling strategies, in which all different components can be traced and analyzed separately. There are increasing experimental evidences that all of these compounds may degrade *in vivo*. Thus, the hybrid nature of NPs eventually transforms when they lose their integrity during their voyage through the human body. While detailed extracorporeal NPs characterization provides information on the products we put "in" and take "out" of the body, the intracorporeal processes are still shrouded in mystery.

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