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FORMATION OF THE PROTEIN CORONA: THE INTERFACE BETWEEN NANOPARTICLES AND THE IMMUNE SYSTEM.

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Abstract. The interaction of inorganic nanoparticles and many biological fluids often withstands the formation of a Protein Corona enveloping the nanoparticle. This Protein Corona provides the biological identity to the nanoparticle that the immune system will detect. The formation of this Protein Corona depends not only on the composition of the nanoparticle, its size, shape, surface state and exposure time, but also on the type of media, nanoparticle to protein ratio and the presence of ions and other molecular species that interfere in the interaction between proteins and nanoparticles. This has important implications on immune safety, biocompatibility and the use of nanoparticles in medicine.

Introduction. The physical and chemical properties of engineered nanoparticles (NPs) make them attractive for use in the industrial, manufacturing, agricultural and medical fields [1]. Due to their small size and high surface area, these materials have outstanding electrical, optical, magnetic, structural and chemical properties which have been exploited for many new industrial and consumer products [2-5]. The fact that inorganic NPs are similar in size to intra- and extracellular biological matter, allows them to specifically interact with molecular and cellular processes, and to manipulate biological states, structures and functions in a radical new way, which makes them extremely attractive for biomedical applications [6-10]. At the same time, and by the same reasons, as NPs can either intentionally or unintentionally enter the body and the environment, concerns have risen regarding their potential human and environmental hazards. Thus, the same properties that offer great promise to provide scientific and technological breakthroughs may also lead to unexpected biological effects not anticipated from materials of the same composition in the bulk form [11-13].

The potential biological impact of engineered NPs are not only determined by the physicochemical properties of the NPs *per se*, but also on the interactions of these NPs with the immediate surrounding biological environments. In this sense, to assess the impact of engineered NPs on the immune and defensive responses of organisms is especially important. Immunity is a major mechanism for the survival and fitness of practically all living organisms. The particulate nature of NPs dictates a preferential interaction with cells of the immune system deputed to recognition and elimination of foreign particulate matter [11]. It is therefore of key importance that, even for NPs that are non-toxic according to regulatory approved standard assays (*i.e.*, unable to kill cells or organisms), additional evaluations of their interaction with the immune system are performed [14]. It is the importance of defensive mechanisms (that ensure survival but also physical fitness and consequently reproductive capacity) where resides the need of assessing the effects of NPs on the immune response not only in humans but also environmental organisms. Thus, the immunosafety of NPs is a major issue for human health, because of the possibility that NPs, even if not directly toxic, may alter the functionality of immune cells, thereby posing significant health risks [15]. Importantly, defensive immune responses are present in practically all living organisms and some of

the basic mechanisms are remarkably conserved throughout evolution, in particular those of the so-called “innate” immune system [16-18].

The highly conserved system of innate immunity deserves special attention here since it has been observed to be the one responsible for managing exposure to nanoparticulate matter [14]. Phylogenetically ancient, innate immunity allows the host to differentiate self from pathogen. It provides a sophisticated first line of defence against infections and initiates a protective inflammatory response within minutes [19]. In the case that an intruding object is not eliminated, the innate immune response precedes and empowers the adaptive immune response. As innate immunity is the rapid and non-specific defence system that reacts to and eliminates foreign materials that enter the body (infectious microorganisms, dusts and particles), it is reasonable to think that it will also react accordingly with NPs [5, 20]. Whether NPs may induce an anomalous innate reaction or interfere with a protective reaction (*e.g.*, against an infectious agent) is an issue of high relevance for predicting a nanorisk.

The key feature of innate immune cells that enables them to detect and categorize infection seems to be their repertoire of Pattern-recognition receptors, such as the human Toll-like receptors, which are practically identical to invertebrate receptors and to the pathogen receptors found in plant cells. These receptors bind certain general types of molecules and particular molecular patterns absent in healthy self-cells that are expressed across broad classes of pathogens [19], enabling the innate immune system to induce, when needed, an appropriate response [21].

The interactions of nanoparticles and the innate immune system. It has been observed that NPs can induce/inhibit/alter the innate immune response [14]. The innate immune system naturally works at the nanoscale. Cells of the innate immune system interact with foreign matter, including NPs, which are within the size range of viruses, *professionally* recognised by immune cells. It is therefore expected that NPs would be recognized as foreign material by the innate immune system and processed by it. Direct interaction with immune receptors has been suggested for both natural NPs, like urea crystals [22], or engineered NPs, like silica NPs [23], although formal proof is still missing. Indeed, the data on this are conflicting, since many of them indicate the high relevance

of indirect effects [15, 24]. The interaction of NPs with immune cells can have opposing effects in the long term: NPs can act as immunosuppressants [25], either due to toxicity that impairs or kills immune cells [26] or to activation of down-regulatory mechanisms [27-29]; but they can also activate the immune system, *e.g.*, by inducing cytokine production and inflammation [30], which may eventually lead to the initiation of adaptive immunity (adjuvant effect) but also to anomalous responses such as in allergy and chronic inflammation [31]. Especially important are molecules absorbed on the NP surface that can either act directly as immunomodulators or, due to the repetitive structural pattern, induce unexpected recognition and response, *e.g.*, as in the case of PEGylated NPs that could induce an anti-PEG immune response and immunological memory, which did not occur in the absence of NPs [32, 33]. In this context, epitope concentration and repetition play a key role, with repeated and spatially organised structures being readily recognised as dangerous [34]. Needless to say, NPs may act as efficient and tunable epitope concentrators and organisers for vaccination [33, 35]. Note that the immune effects of NPs can be exploited for therapeutic purposes [24, 36]. Thus, selective and localised stimulation of innate/inflammatory immune reaction may, for example, be a highly welcome additional effect in tumour therapy using NPs. Similarly, immunosuppressive effects can be exploited for the use in autoimmunity [15].

Additionally, the study of immunological reactions has also a critical role when addressing the question of how sick people or organisms react to NPs. In the same line, immunotoxicology studies usually do not cover hypersensitivity. Contact sensitizers are an important issue in workplace safety, and the possible effect of co-exposure to allergens and NPs needs to be investigated. Allergens (*e.g.* from plant pollens, house dust mites or animal hair) are in most cases proteins and are likely to bind to NPs. Structural deformation of the allergen, clustering or patterning may occur upon binding, and influence recognition and immune reactions, which could result in enhanced or reduced allergenicity [37, 38].

The mechanisms of nanoparticle interaction with physiological media. Large surface area and low coordination at the NP surface determines the high energy potential of NPs and consequently their behavior and reactivity profiles. This applies to both the use

of the NPs as catalyst (such as small Pt for fuel cells) [39, 40] or as a reagent (such as zero-valent iron for environmental remediation) [41]. Due to their higher percentage of surface atoms and their colloidal nature, once being brought into contact with a physiological medium, NPs experience processes that transform them towards more stable thermodynamic states [42, 43], including aggregation, corrosion, dissolution and interaction with media proteins. Indeed, it is common to observe that at the same time, as NPs become unstable in the biological media they corrode while aggregate and are coated by proteins, what in turn stabilize them against aggregation and sedimentation **(Figure 1)** [44]. When salinity is increased (**Figure 1a**), the screening of electrostatic repulsion by adsorbed salt ions causes fast homo-aggregation between NPs. When proteins are present in the medium (**Figure 1b**), they provide a stabilizing electro-steric effect upon adsorption (hetero-aggregation) on NPs: this effect can prevent particles from precipitation only if a sufficient concentration of proteins is available. Later on, NPs may undergo chemicals transformations that lead to their dissolution (**Figure 1c**), the third mechanism able to further lower NP colloidal stability, where electrolyte ions (together with dissolved oxygen and/or helped by acidic environments) start oxidizing surface atoms. The kinetics of these three separate but often co-existing processes are strongly influenced by the respective concentrations of the causing chemical agents. Indeed, proteins in solution have to be at much higher concentration than NPs in order to avoid NP aggregation when dispersed in media of high ionic strength as physiological media, indicating the stronger tendency of NPs for homo-aggregation than hetero-aggregation [45]. These coupled processes are mediated by the different interactions between the NPs and components of the biological medium in which they are exposed, and ultimately determine the nature of the nano-bio interface [42, 46-51]. Remarkably, small modifications on the nature of the conjugate and the dispersing media have a strong influence on conjugate interactions and consequently different biological behavior and fate [52]. Since NPs can be produced with different functional groups on their surface, by modifications of NP's surface coating, charge and hydrophobicity, their reactivity can be modified altering its interactions with the biological surroundings [53].

From the NP point of view, the different interactions can cause phase transformations, particle aggregation, surface reconstruction and dissolution. All of these processes having a significant influence on their reactivity, bioavailability and pharmacokinetics [20], affecting their persistence and ultimately leading to (immuno)toxic effects [47, 54-56]. For example, extracellular agglomeration of NPs, or the agglomeration occurring prior or during exposure to *in vitro* or *in vivo* models has a significant impact on the observed biological effects and conclusions about their size-dependent (immuno)toxicity [57, 58]. In this regard, one interesting trend found was that in the majority of NPs tested, the addition of serum to the cell culture media (CCM) helped to mitigate the tendency and effects of NP agglomeration [59, 60]. The reason for that was that proteins present in serum did stabilize the NPs against aggregation [45, 60].

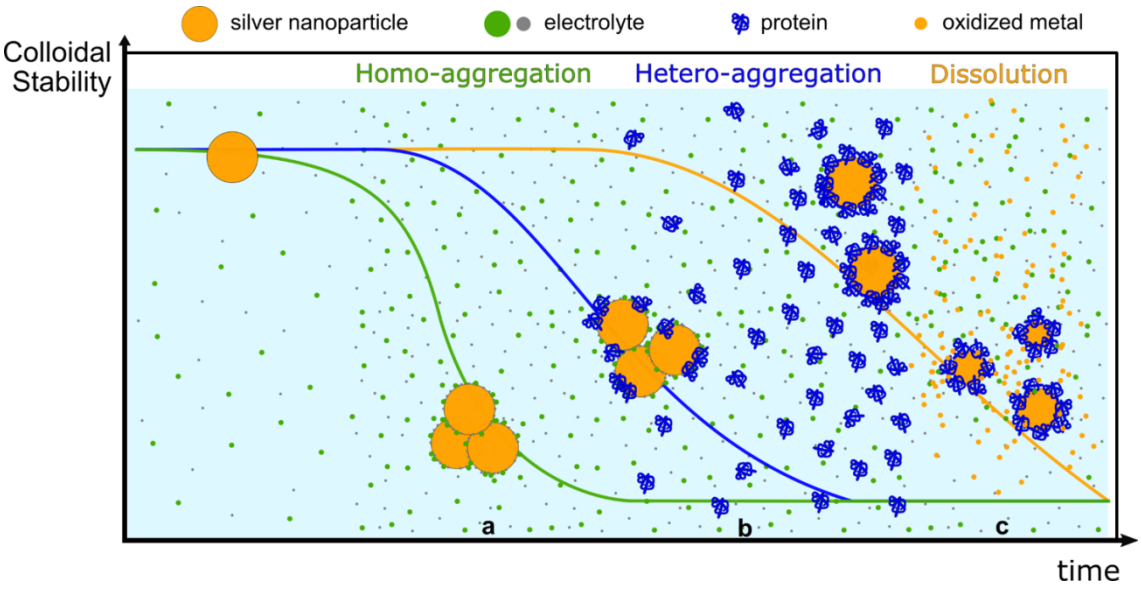

Figure 1. Colloidal and Chemical Stability of NPs. *a) Homo-aggregation induced by high ionic concentration; b) Hetero-aggregation between NPs and proteins at different concentration rates; c) chemical degradation, corrosion and dissolution of NPs incubated longer incubation times in physiological media.*

From biomolecules point of view, these interactions may lead to the opsonization, formation of Protein Coronas (PCs) of a different nature [45, 60-62] (the so-called *Soft* and *Hard* corona), denaturation of proteins [63], and the formation of NP-protein complexes [45], which inevitably provide them with a new biological identity [64, 65], eventually promoting the activation of signaling pathways [66-68] and ultimately

determining their physiological response and toxicity [69, 70]. The impact of the PC on cytotoxicity, and immunotoxicity has been widely studied [53, 69-73]. One interesting example is the immunogenic epitope generation, *i.e.*, a deformation of the protein tertiary structure (promoted by the interaction with the curved NP surface) that can induce protein aggregation or make self-proteins immunogenic, thereby inducing autoimmune reactions [74, 75] or that antigens can be absorbed on to NPs becoming more immunogenic [76]. In addition, opsonins and proteins of the complement system are able to recognize proteins adsorbed on the surface of the NP or to directly attach to the NP surface, triggering an immune response [77].

Among the innate effector molecules (antimicrobial peptides, degrading enzymes, complement agents) complement proteins have been identified as subject of research given the high affinity of NPs for proteins [69]. The complement system consists on a tightly regulated network of inactivated proteins that when absorbed into a surface get activated [78], inducing a sequential cascade of reactions which generate proteolytic enzymes in each step. Literature reports several studies focused on understanding the immunological response that leads to complement system activation when exposed to different types of NPs, including superparamagnetic iron oxide [79-81] and gold NPs [82].

In order to understand the interaction between inorganic NPs and biological fluids, it is worth to study the most important factors that define the relationships between biological fluids and inorganic solid surfaces. Hydrophobicity and surface charge have historically been the factors taken into account to describe the process of protein adsorption to surfaces. This was illustrated in the work of Prime and Whitesides [83] using self-assembled monolayers (SAMs) supported onto gold films. Those SAMs consisted on alkane chains with different terminal groups, that provide different hydrophobicity, and the more hydrophobic the conjugate the greater the degree of adsorption. Also the global charge of the protein can drive its adsorption. A higher adsorption of positively charged proteins onto negatively charged polystyrene surfaces and *vice versa* was observed by Norde and Lyklema [84]. Several examples of the role of surface charge and hydrophobicity on the protein adsorption process to surfaces and NPs can be found in the literature [85, 86].

Undoubtedly, the mechanism by which proteins stick to surfaces are principally hydrophobic and electrostatic interactions, and hydrogen bonds, all of them weak interactions. Over recent decades, many studies of interface phenomena involving proteins have identified their adsorption to surfaces as an irreversible process (hence, the failure in fitting protein adsorption data to the Langmuir equation) [87]. At first, that proteins are provided with multiple, although weak, anchor points was the strongest argument for this irreversibility. Different studies aimed to describe the hardening of the protein adsorption process through different mechanisms. Norde and Anusiem [88], for example, reported that Bovine Serum Albumin (BSA) that adsorbed to silica surface and later desorbed had more affinity for silica surfaces than BSA that had not undergone this process. In a further work [89], they suggested that the attachment BSA-silica surface caused an increase in the internal entropy of BSA, perhaps a slight modification of its structure, resulting in desorbed BSA being more stable on silica surface than "new" BSA. The work of Nygren and Aleadine [90] showed that, contrary to what might be suspected, proteins do not distribute on surfaces randomly. Instead, once the first proteins are attached, an initial cluster of proteins forms around, stabilizing them (*crowding* effect), and this mechanism is repeated until the entire surface is filled. These and other attempts to explain the irreversibility of this process seem to have in common that the initial attachment of a protein to a surface is followed by a series of movements and/or rearrangements to make more stable and ultimately irreversible this attachment. Therefore, not only affinities 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.

The fact that adsorption of proteins turns irreversible through these time-dependent mechanisms has important implications in the case of complex mixtures of proteins such as blood serum, plasma, etc. According to the Vroman Effect [91], initially fast exchange proteins with low affinity (i.e. proteins that when adsorbed are rapidly desorbed) fail in generating strong attachment to the surface, they are not able to stabilize on the surface despite occupying it earlier than slow exchange proteins with higher affinity. These

higher affinity proteins, despite delayed occupation of the surface, are able to stabilize onto it.

Thus, the “Soft” corona is formed initially, with more abundant and mobile proteins coating the NPs surface, but with weak and dynamic interactions, setting an equilibrium between bound and unbound proteins in solution. As time goes by, the adsorption equilibrium shifts towards the attachment of proteins with higher affinities for NPs surface, modifying the initial corona composition resulting into a “Hard” corona. At the same time, an ensemble of thermodynamic stabilizations mechanisms (i.e. conformational rearrangements, crowding effects) hardens the proteins layer bringing it to a steady, irreversible minimum energy state [45].

This process can be followed through a set of common techniques, namely UV-Visible Absorption Spectroscopy (UV-Vis) and measurements of hydrodynamic radii by Dynamic Light Scattering (DLS) and surface charge by ζ-potential. Initially **(Figure 2a)**, only a weakly bound layer of proteins (grey) adsorb on the particle surface, stabilizing the colloid in the saline medium but being in dynamic equilibrium with the unbound ones in solution. When the incubation is extended for longer times, the corona stabilizes progressively through different mechanisms, leading to the stronger attachment of proteins onto the particles surface (black). If purified through centrifugation and re-suspension in protein-free physiological media **(Figure 2b)**, protein-coated NPs display different colloidal stabilities depending on the duration of the incubation time. After short ones, loosely bound proteins readily detach from NP surfaces causing them to irreversibly aggregate in the saline medium. Colloidal stability increases gradually with longer expositions to proteins, which become more and more tightly adsorbed onto NP surfaces and do not get lost with purification. PC hardening kinetics strongly depend on each experimental parameter (i.e. NP material, size and concentration, protein type and concentration, pH and ionic strength of biological media). For a gas-like adsorption process, proteins will attach randomly on the surface showing no cooperative behavior or surface organization; if cooperativity is present (either positive or negative), proteins will reorganize on the particle surface through conformational rearrangements and crowding effects, leading to the formations of domains. Consequently, these effects

could influence strongly the accessibility of functional proteins such as antibodies and enzymes, hindering/enhancing their activity [92].

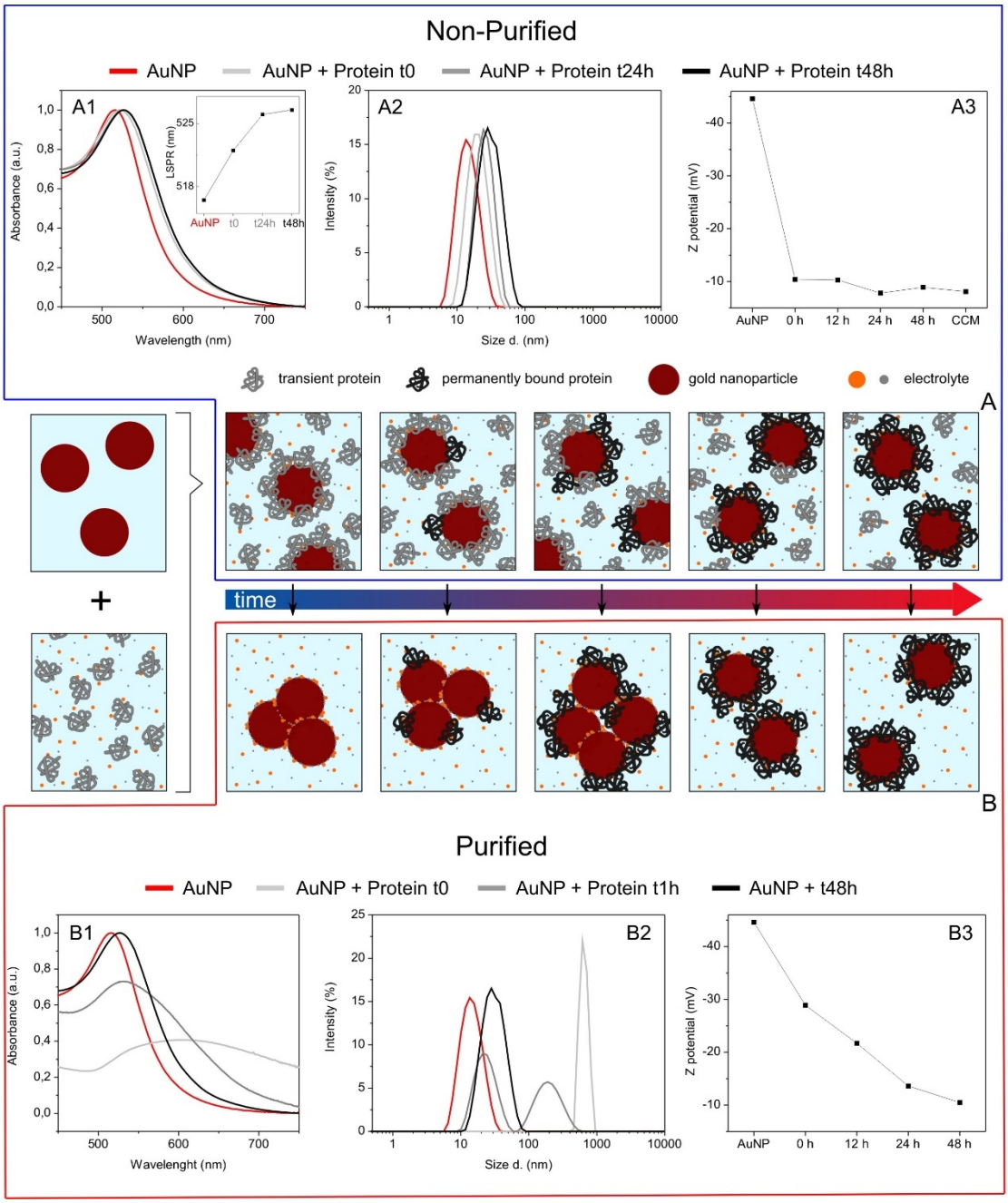

Figure 2. The hardening of the Nanoparticle-Protein Corona. (A1) *UV-Vis,* ***(A2)*** *Dynamic Light Scattering (distribution by Intensity),* ***(A3)*** *ζ-potential characterizations of 10 nm AuNP@SC before (Red) and after the exposition to complete culture media (CCM+FBS) at time 0 (Light Gray), 24h (Gray) and 48h (Black).* ***(B1)*** *UV-Vis,* ***(B2)*** *Dynamic Light Scattering (distribution by Intensity),* ***(B3)*** *ζ-potential characterization of the same 10 nm Au NPs solution after purification procedure at different time of exposition to the complete CCM. 10 nm AuNP@SC before (Red) and after the exposition to complete CCM at time 0 (Light Grey), 1h (Grey) and 48h (Black).*

Regarding biological and medical applications, it is important not only the adsorption of proteins themselves but also the implications that this adsorption entails for the protein, especially the maintenance of its tertiary structure, since biological function depends largely on it. Indeed, the crowding effect facilitates the maintenance of the native state of proteins. As noted in the works of Norde [93], the size of the adsorbed protein layer was closer to the size of the native protein. This, together with the success in methods of immunoassay [94], is an indication that at least a part of adsorbed proteins maintain their active structure. On the contrary, other studies observe an unfolding of the proteins when adsorbed on a surface but not always followed by aggregation [74]. It may happen that the closest model to reality is that surfaces are coated by proteins in a mixture of states. The first in adsorbing are more prone to lose their original conformation and denature while last ones have no room for denaturation and more easily maintain their native structure.

These modification may have important effects, as induction of exposure of hydrophobic residues and the consequent aggregation; or modifications in how proteins are recognized, employed and processed [95-97]. The various characteristic of the NP (material, dimension, surface charge), the different nature of the protein involved (primary/secondary/tertiary structure, molecular weight, hydrophilicity, melting temperature, number and exposition of disulfide bridge) and the several exposure conditions seem to highly influence the possible protein conformational changes. Goy et al. [74], after studying the interaction between sodium citrate-coated Au NP of different diameters and Human Serum Albumin (HSA) they suggested a decrease of the biomolecule mobility due to a small change in the secondary and tertiary structure of the HSA. Interestingly this effect was found to increase as the NP curvature decreases. In addition, studying the thermal protein unfolding profile, they observed an enhancement of the unfolding temperature when the HSA is adsorbed onto the Au NPs, revealing that the observed conformational changes brought a higher resistance to the complete thermal denaturation. Under fibrillation conditions they did not observe an increase in the HSA amyloid-like fibrils formation when NPs were present, seeming instead that Au NP smaller than 40 nm reduced the fibrils formation, and this effect was ascribed to the enhanced stability of the bound proteins. Conversely, Zhang et al. [75],

investigating the interaction of sodium citrate-coated 90 nm Au NP with lysozyme, showed that the protein forming the PC were partially unfolded with a partial dissociation of the disulfide bonds bringing to an Au-S coordinate covalent bonding, not detected in previous works. In here, in the presence of NPs and in a not fibrillation conditions, they observed a formation of extended, amorphous protein-NP assemblies and also large protein aggregates not containing NPs. This process was attributed to the NP colloidal destabilization and aggregation. The comparison of these two studies shows how several NP-protein exposure conditions with different proteins (Lysozyme, 15 kDa protein with only 4 S-S bonds, and the large HSA 67 kDa with over 17 disulfide bonds) can bring to distinct proteins conformational changes and consequently modify the NP properties and behaviors.

At this point, to know which is the composition of the Hard and Soft PCs depending on material and environment is critical. Corona compounds may influence signalling, when extracellular proteins arrive inside the cells, or when binding to NPs changes structure or association patterns of self-proteins. The effects of different corona compositions on intracellular alarm mechanisms has been explored, with the objective of linking the NP bio-shell composition to induction of cellular stress and inflammation [98, 99]. It is difficult to draw conclusions on similarities and differences between different materials and proteins since experimental conditions have been very different inter- and even intra-laboratories (proteins coming from different suppliers, with different stabilizers, adsorbed onto NPs with very different surfaces and morphologies, etc). Our group [60], dispersing same batch of Fetal Bovine Serum (FBS) proteins in different CCM -consisting on DMEM with different supplements- observed the time evolution of the PC in all cases but the PC formation rates differed depending on the final complete CCM (CCM+FBS) used. Further, in the same study [60], we compared the same DMEM composition with proteins coming from FBS and FCS (Fetal Calf Serum) with similar results as previously described: the same pattern of PC formation was observed but at different rates.

We also studied the surface modifications of metal (Au, Ag) and metal oxide (Fe_3O_4, CeO_2 and CoO) NPs, with sizes ranging from 7 to 20 nm, dispersed in the same CCM supplemented with serum [61]. Results showed that all the tested NPs adsorb proteins onto their surface through the hardening process (i.e. evolving towards an irreversible

coating). And, despite the fact that the studied nanomaterials have similar characteristics in terms of hydrophobicity and surface charge, different temporal patterns of the PC formation were found. In the case of metal NPs, two days were enough to stabilize the Hard corona, while up to one month was needed in the case of metal oxide NPs. This finding is of special relevance since different interactions between NPs and biological systems take place at different time scales (e.g: removal from the blood stream may be a question of minutes and interaction with cells of distant organs may be relevant hours to days after exposure [100]). Similarly, biodistribution and residence times in different biological environments will affect this NP–protein equilibrium what in turns will determine its biological interaction [72, 101]. Also, tightly associated proteins may stay adherent to the particle when the particle is endocytosed from the extracellular fluid to an intracellular location, whereas proteins with a fast exchange rate will be replaced by intracellular proteins during or after such transfer [102]. Thus, the same NPs can give different biological responses depending on portal of entry, history, pre–incubation in serum, etc., illustrating the importance of characterizing the NP–PC for each nanomaterial in a particular biological environment.

Cedervall et al. [66, 103] evidenced how association and dissociation rate of proteins were affected by the NP physicochemical properties as well as by the incubation and purification conditions. At the equilibrium, the serum apolipoproteins bond to the copolymer NPs with higher affinity than the more abundant albumin and fibrinogen, which probably dominate on the particle surface at short times. Monopoli et al. [71], reported that the concentration of many of the highly abundant proteins in the PC of different polystyrene NPs (for example apolipoprotein, fibrin and fibrinogen) are independent from the NP size and surface charge, and being they are always present at low concentrations together with a large amount of other possible different proteins on it (inter-alpha-trypsin inhibitors, serum albumin, clusterin, and vitronectin). Indeed, rather than NP morphology, different plasma concentrations can instead lead to a different Hard corona composition, which suggest a progressive selectivity adsorption by affinity [71]. Dobrovolskaia et al. [104], reported that the proteins that bind polymeric, iron oxide and Au NPs, and liposomes and carbon nanotubes, are mainly albumin, apolipoprotein, immunoglobulins, complement, and fibrinogen, which are the

most abundantly species in plasma. Tenzer et al. [99] quantitatively determined the time-resolved profiles of the human plasma corona formed on silica and polystyrene NPs of various sizes and surface functionalizations, observing the rapid formation of a complex and specific corona with more than 300 different proteins. The composition of the corona changed only quantitatively with the increasing of the incubation time, while the nature of the PC was preserved, contradicting Vroman observations [91, 105-108]. Casals et al. studied the time evolution of the NP-PC on citrate stabilized Au NPs in complete CCM (DMEM + FBS) revealing that albumin was the most abundant component in the Hard corona [60].

Despite initial studies, size has been shown to critically influence protein binding to NPs. For example, a denser protein layer has been observed on larger copolymer and Au particles compared to the smaller ones [60, 103]. This is in accordance with results from Lacerda et al. [109], who found that the strength of citrate-stabilized Au NPs interaction with common human blood proteins increased with NP size together with an enhanced protein packaging and to more efficient screening of the surface charge in the large particles compared to small ones. Other groups have also reported that larger SiO_2 and Au NPs with nearly flat surfaces tend to induce larger changes on the protein conformation rather than smaller particles, which possess a more curved surfaces [45, 97, 109]. However, aggregation triggered by complete protein denaturalization has been rarely observed [109], and the tendency is that proteins remain active after adsorption. Other studies found that for copolymer and SiO_2 particles with varying diameters, the amount of bound protein varied with size and surface curvature, but the total protein pattern remained identical [103, 110]. In contrast, other studies using similar polymer, metal and metal oxide NPs reported no only significant quantitative but also compositional size dependent changes in the obtained fingerprints [33, 97, 99, 104, 111, 112]. A more recent study conducted by our group [45] gave a throughout overview of size increase and charge reduction of the PC in citrate-stabilized Au NPs of sizes ranging from 3 to 100 nm. The obtained results indicate that different NP-biological interactions take places at different time-scales, and that PC from smaller particles matched with a faster kinetic evolution and thinner/incomplete protein layer. This emphasizes that is

very difficult to formulate size-dependent rules about protein interactions that apply to all types of NPs and conditions.

Indeed, the affinity of NPs surfaces for proteins has been exploited to biocompatibilize NPs. This is the case of CeO_2 NPs pre-albuminized prior their use to decrease liver inflammation in fibrotic model rats [29] or the case of superparamagnetic iron oxide NPs for biotechnology applications [113]. Otherwise, in the absence of proteins, when the inorganic NPs are dispersed in media of high ionic strength, they do irreversibly aggregate and get expulsed from the solution phase **(Figure 3)**.

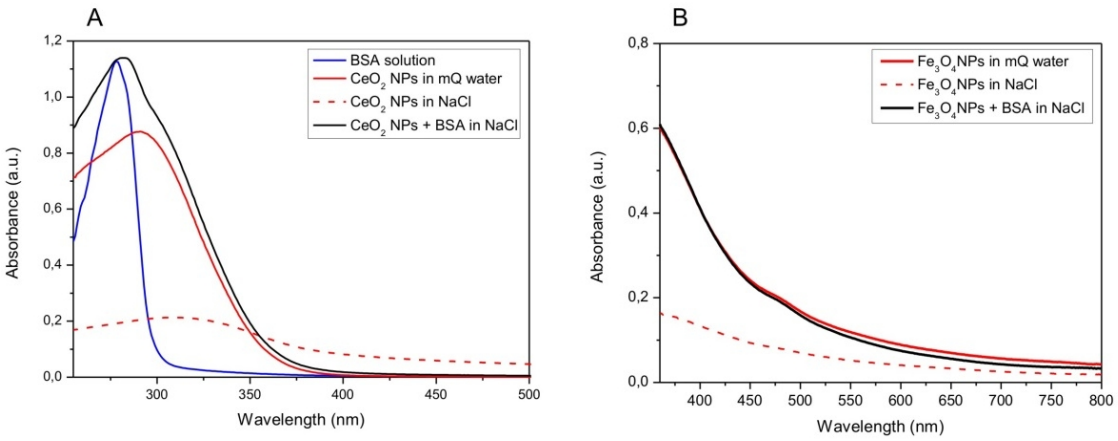

Figure 3. (A) *UV-vis spectra of 8 nm CeO_2 NPs before and after exposure to 150 mM NaCl solution, with or without presence of BSA. (Blue line) BSA solution; (Red line) CeO_2 NPs diluted in milliQ water, (Red Dash line) CeO_2 NPs diluted in NaCl; (Black line) CeO_2 NPs + BSA diluted in NaCl.* ***(B)*** *UV-vis spectra of 8 nm Fe_3O_4 NPs before and after exposure to media with certain salinity, with or without presence of BSA. (Red line) Fe_3O_4NPs diluted in milli-Q water, (Red Dash line) Fe_3O_4 NPs diluted in NaCl; (Black line) Fe_3O_4 NPs + BSA diluted NaCl. Experimental conditions: NPs as synthesized and diluted 1:50; NaCl 2g/L; BSA 20g/L; Spectra collected 2h after NPs addition.*

The majority of previous studies regarding protein adsorption have been performed on larger and polymeric NPs of few hundreds of nm, whereas less studies have addressed NPs in the small size regime (4-40 nm). PC formation studies in the case of inorganic NPs smaller than few tens of nm have been done, among others, on metallic NPs (Au [60, 61, 104, 109], Ag [61] and FePt [114]) metal oxide NPs (SiO_2 [52, 72], Fe_3O_4, CoO and CeO_2 [61], TiO_2 [101] and TiO_2, SiO_2 and ZnO [112]), and Quantum Dots (CdSe [115, 116] and CdSe/ZnS [114]). These NPs have diameters similar to the proteins found in serum and

it has been observed that at short incubation times they can easily escape from opsonisation and from the Mononuclear Phagocytic System [44, 117, 118].

Summarizing, inorganic NP surfaces have strong affinity for proteins. This strong affinity may compensate the destabilization forces that experience colloidal NPs in media of high ionic strength and help to stabilize NPs. This interaction is immediate and evolves with time, being the NP-Protein construct what the immune system will encounter in its surveillance work. The diversity of observed behaviors is broad but smaller than the NP/media variations, indicating that conditions for controlling these interactions exist and that this can be used to determine the safe and therapeutic use of NPs.

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