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**Preferential binding of positive nanoparticles on cell membranes is due to electrostatic interactions: a too simplistic explanation that does not take into account the nanoparticle protein corona**

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## **Abstract**

The internalization of nanoparticles by cells (and more broadly the nanoparticle/cell interaction) is a crucial issue both for biomedical applications (for the design of nanocarriers with enhanced cellular uptake to reach their intracellular therapeutic targets) and in a nanosafety context (as the internalized dose is one of the key factors in cytotoxicity). Many parameters can influence the nanoparticle/cell interaction, among them, the nanoparticle physico-chemical features, and especially the surface charge. It is generally admitted that positive nanoparticles are more uptaken by cells than neutral or negative nanoparticles. It is supposedly due to favorable electrostatic interactions with negatively charged cell membrane. However, this theory seems too simplistic as it does not consider a fundamental element: the nanoparticle protein corona. Indeed, once introduced in a biological medium nanoparticles adsorb proteins at their surface, forming a new interface defining the nanoparticle “biological identity”. This adds a new level of complexity in the interactions with biological systems that cannot be any more limited to electrostatic binding. These interactions will then influence cell behavior. Based on a literature review and on an example of our own experience the parameters involved in the nanoparticle protein corona formation as well as in the nanoparticle/cell interactions are discussed.

## **Key-words**

Nanoparticle, Protein corona, Nanoparticle/cell interaction, Cellular uptake.

## **1. Introduction**

The knowledge of nanoparticle/cell interactions is of paramount importance in nanomedicine and nanotoxicology. Indeed, when nanoparticles are used for biomedical purposes, therapeutic and/or diagnostic agents must generally enter the cells to reach their targets. Furthermore, because of their size, high reactivity and large surface area nanoparticles can interact with cell components, potentially inducing side effects and toxicity. Therefore, the investigation of the underlying mechanism of cellular uptake is a crucial issue for the understanding of the biological fate of nanoparticles as well as potential adverse aspects [1].

Nanoparticle/cell interactions are largely influenced by the nanoparticle physico-chemical characteristics, for instance features such as size, shape, surface chemical functions, etc seem to have a major impact on nanoparticle binding to cell membrane and the subsequent cellular uptake [2,3]. In most cases, the nanoparticle surface provides the driving forces (electrostatic, hydrophobic and hydrophilic (polar) forces) for the cellular internalization and decides the uptake pathway [4]. This latter will have a deep effect on cell response.

In particular, it is commonly admitted that positively charged nanoparticles interact more with cell membranes than neutral or negatively charged nanoparticles. This preferential binding is supposedly due to favorable electrostatic interactions, as cell membranes are negatively charged. However, and as already expressed in a previous opinion paper [5], this explanation seems too simplistic as it only considers the nanoparticle charge whereas many other parameters are involved. For example, the nanoparticle protein corona is now acknowledged to play a critical role. The aim of this review is to draw attention on this issue and further demonstrate with details that the above-mentioned theory is too reductive and that other parameters should not be neglected.

## **2. General considerations on cellular uptake of nanoparticles**

Nanoparticles can be uptaken by cells through endocytosis. As reviewed by Wang et al. [6], endocytosis can be subdivided into phagocytosis (cell eating, mainly for large particles) and pinocytosis (cell drinking, rather for small particles, fluid and solutes). Phagocytosis primarily occurs in macrophages and polymorphonuclear neutrophils. In contrast, pinocytosis occurs in all types of cells through at least four distinct mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae independent endocytosis [6]. It is also worth noting that endocytosis can be either non-specific or receptor-mediated [7]. The uptake of nanoparticles by cells occurs through two steps: binding to the cell membrane and then internalization. The first one seems to be most affected by the physico-chemical characteristics of the particles and especially the surface charge [7,8].

As previously mentioned, it is commonly acknowledged that positively charged nanoparticles are more internalized by cells than neutral or negatively charged nanoparticles [1,2,4,7,9–15]. Some authors have even observed a strong correlation between the amount of positive charges and internalization into cells [2]. The accepted explanation lies in the fact that electrostatic interactions are favored with cell membrane that is negatively charged.

This observation seems to be a general tendency, observed with nanoparticles of various chemical bulk compositions (silica, gold, iron oxide, etc) and in various cell types. Indeed, several studies aiming at getting insight into the nanoparticle uptake efficiency by cells compare cell internalization of nanoparticles of similar size and shape, differing only in their surface charge owed by different surface chemical coatings. For example, Kralj et al. [9] used silica-coated maghemite nanoparticles functionalized either with amine groups for positive surface charge or with carboxyl groups for negative surface charge and clearly showed that the positively charged nanoparticles were internalized into the cells to a much higher extent than the negative ones in two different cell lines. Similarly, Lankoff et al. [16] modified the

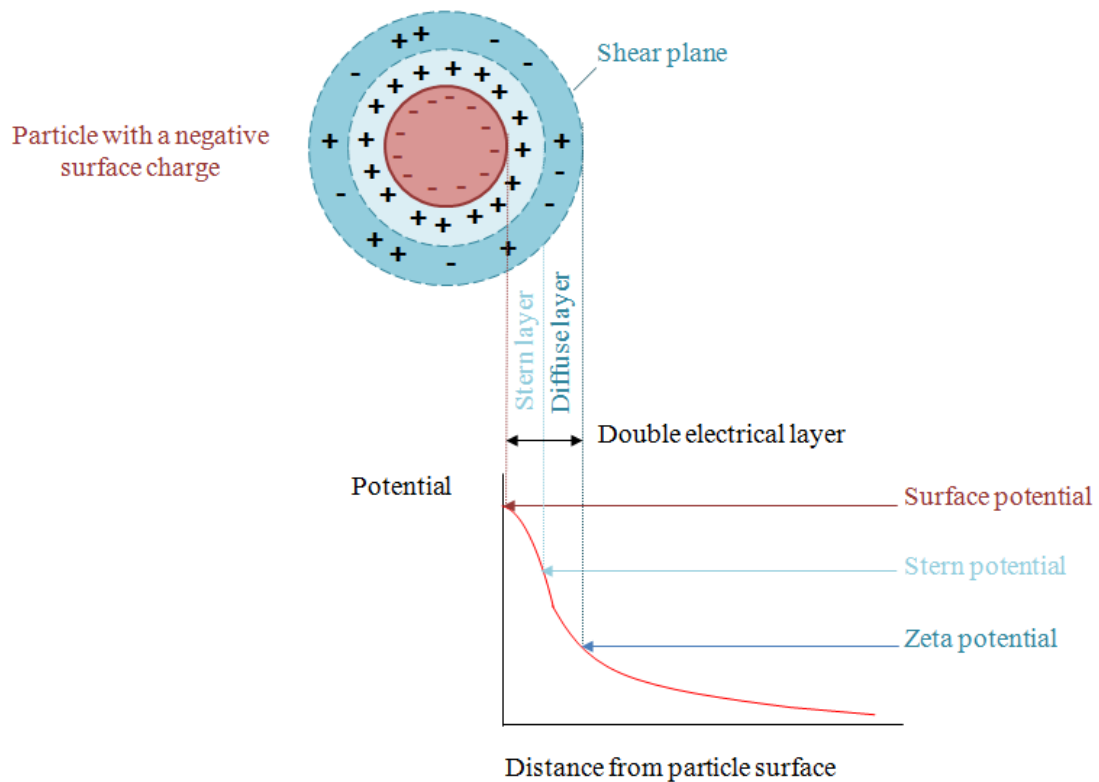
surface of silica nanoparticles with vinyl or aminopropyl/vinyl groups and observed that the uptake of these latter by lymphocytes was more efficient than that of the former. The same conclusion was reached by Ge et al. [17] using magnetic iron oxide nanoparticles coated with chitosan (positive charge) or with dimercaptosuccinic acid (DMSA, negative charge) in a model of oral squamous carcinoma cell.

All these examples illustrate why positively charged nanoparticles are usually chosen as carriers for drug or gene delivery [1,2,15]. Consequently, nanoparticle surface functionalization by modifying surface charge is an efficient and easy way to alter cellular uptake rate [4,9,11,18].

At this point, we should specify some definitions as behind the term of charge many concepts could be hidden depending on the level of observation (macro- or micro-scale) and they should involve different levels of subtlety and complexity.

### **3. Nanoparticle charge and cell membrane charge, what are we precisely talking about?**

Concerning nanoparticles, the term charge is confusing as it could refer to distinct and complex physical quantities. Indeed, when a nanoparticle is exposed to a fluid, a double electrical layer appears on its surface, consisting of two parallel layers of charges surrounding the particle, as illustrated in Figure 1.



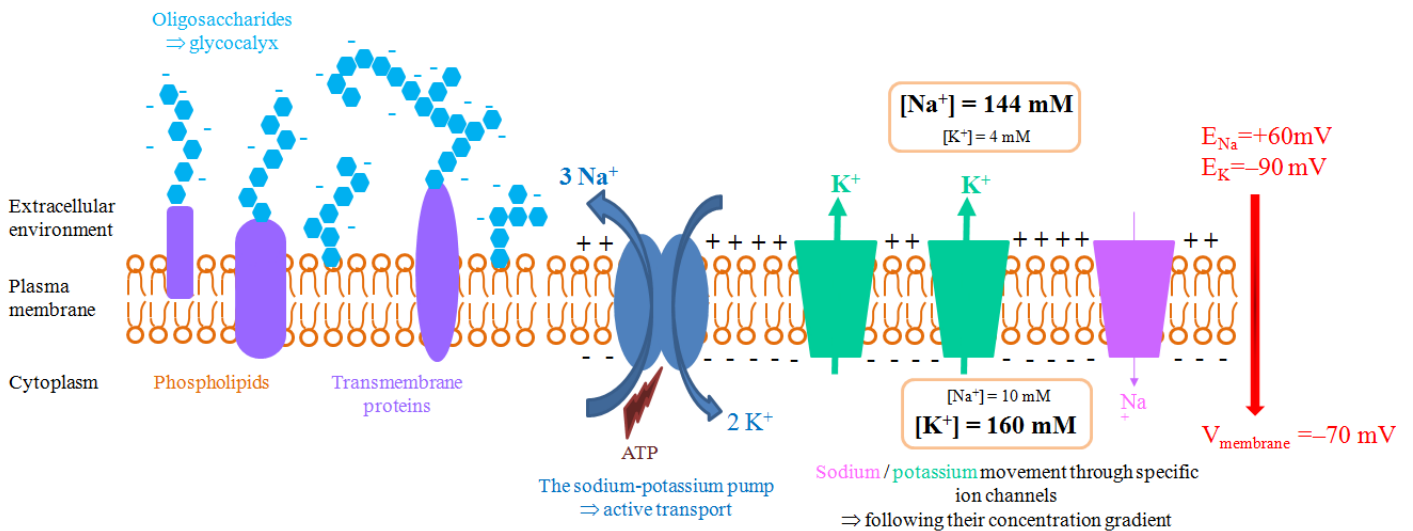
**Figure 1** – Double electrical layer formation at a particle surface leading to the definition of different charge potentials.

The first layer, called the Stern layer, corresponds to the primary electric surface potential (caused by protonation/deprotonation reactions on the surface) and ions from the bulk electrolyte strongly bound to its surface. Indeed, when immersed in an electrolyte, a nanoparticle develops a surface charge mainly associated with the hydroxylation of its surface and the specific ion adsorption due to chemical interactions. The second diffuse outer layer is composed of free ions attracted to the primary electric surface potential of the particle under the influence of electric attraction and thermal motion rather than being firmly anchored. The diffuse layer electrically screens the Stern layer. In other words, the surface charge of the nanoparticle interacting with dissolved ions in the bulk dispersant induces an electrical neutralization by accumulation of counterions. As the particle moves, a boundary exists between the ions in the diffuse layer moving with the nanoparticle and free ions that remain

with the bulk dispersant. From a theoretical viewpoint, the electric potential is defined as the local electrical potential at the slipping plane that separates the mobile phase and the stationary layer of fluid attached to the particle. Thus the zeta potential is widely used in the literature for quantification of the magnitude of the nanoparticle charge. However, the zeta potential is rigorously not equal to the electric surface potential nor to the Stern potential because these are defined at different locations in the electrical double layer [19].

Regarding cell membrane, surface charge and membrane potential are often confused and a sharper distinction should be done between the two as they refer to different concepts. Indeed, surface charge corresponds to the distribution of charges in a surface whereas membrane potential is due to ion distribution between both sides of the membrane following the Nernst principle [20]. To better understand let's briefly come back to the structure of plasma membrane. As shown in Figure 2 (left part), mammalian cell membranes consist of phospholipid bilayers where proteins are inserted. Lipids and proteins of the outer leaflet are generally glycosylated (with oligosaccharides oriented toward the extracellular environment). Consequently, the surface of the cell is covered by a carbohydrate coat, known as the glycocalyx, bearing negative charges [20].





**Figure 2** – Schematic representation of the cell membrane structure explaining surface charge distribution (left part) and membrane potential (right part).

On the other hand, membrane potential originates from the difference in ion concentrations between the cytoplasm and the extracellular compartment and the selective permeability of the plasma membrane for ions [20]. Many ions have a concentration gradient across the membrane but potassium (K<sup>+</sup>) plays a major role because cell membrane is particularly permeable to this ion. As illustrated in the right part of Figure 2, following its concentration gradient K<sup>+</sup> diffuses outside the cell through specific ion channels leaving behind uncompensated negative charges and thus creating a voltage difference between the two sides of the membrane. Membrane potential originates from this positive charges distribution at the external leaflet and negative charges at the internal leaflet. The membrane potential is thus physically located only in the immediate neighborhood of the membrane. Ion pumps also known as ion transporters or carrier proteins are also involved as they actively transport specific types of ions from one side to the other against their concentration gradient, this process requiring energy provided by the hydrolysis of adenosine triphosphate (ATP). In this regard, the sodium-potassium pump greatly contributes: on each cycle, it exchanges three Na<sup>+</sup> ions from the intracellular space for two K<sup>+</sup> ions from the extracellular space, giving a net

movement of one positive charge from the intracellular to the extracellular, thereby contributing to a positive voltage difference. Consequently, it gives the intracellular space a negative voltage with respect to the extracellular space [20]. This transmembrane potential, also referred as to resting potential, is held at a relatively stable value. Depending on the organism and on the cell type, typical values of membrane potential range from -20 mV to -200 mV [20].

Coming back to the nanoparticle/cell interactions, some contradictory results have been reported in the literature and there has been evidence of uptake of negatively charged particles despite the unfavorable interaction between the particles and the “negatively charged cell membrane” [2,11]. Patil et al. [7] have even reported a preferential uptake for the negatively charged cerium oxide nanoparticles by a model of lung adenocarcinoma cells. Explanations to such observations can be found in the fact that although cellular membrane is, in general, negatively charged, it is patchy and also exhibits some areas with cationic sites allowing the binding of the negatively charged nanoparticles resulting in a clustering of the particles in these domains followed by subsequent endocytosis [2,7,9,12]. Another hypothesis has also been proposed by Zhao et al. and is based on the occurrence of oxidative stress to the cell membrane that leads to a significant decrease in negative charges on the cell surface [1].

Anyway, the preferential cell uptake of positively charged nanoparticles over neutral or negatively charged nanoparticles can only be partly explained by favorable electrostatic interactions with cell membrane. This theory, although widespread, seems simplistic and reductive as it only considers the nanoparticle charge (and by this we mean the surface potential) whereas many other parameters are involved in the equation. For instance, it did not take into account a fundamental element which importance is increasingly documented: the protein corona.

#### **4. The key role of the protein corona**

Once introduced in a biological medium nanoparticles are rapidly covered by proteins which adsorb at the nanomaterial surface. This so-called protein corona by altering the original nanoparticle physico-chemical features (i.e. its “synthetic identity”) generates a new interface defining the “biological identity” of the nanoparticle. And it will have a direct impact on biological responses [3,10,12,21–25].

The formation of the protein corona is a dynamic process. It corresponds to the competitive binding of biomolecules at the nanoparticle surface. Adsorption of proteins is fostered by several forces such as hydrogen bonds, solvation forces, Van der Waals interactions, hydrophobic and electrostatics interactions [21,26]. The most abundant proteins in the medium first adsorb, but over time they are replaced by proteins of higher affinity due to a Vroman’s effect [3,22,23,26,27]. Depending on the dynamic exchange of proteins, induced by their different adsorption kinetics and affinities to the nanoparticle surface, the “hard” corona is distinguished from the “soft” corona. The molecules that have a high affinity for the nanoparticle and that can hardly be removed constitute the “hard” corona, whereas the “soft” corona is made of proteins with faster exchange rates [12,28,29]. Therefore, the composition of the nanoparticle corona is constantly evolving with time due to constant adsorption and desorption of proteins [28]. It suggests that the nanoparticle properties could be different at different times of biological experiment and can therefore lead to diverse intracellular responses and toxicological outcomes. This is an important point that should be taken on consideration for *in vitro* and *in vivo* studies in nanomedicine and nanosafety [22].

Similarly, the protein corona composition can vary depending on the environment of the nanoparticle. As nanoparticles can travel through various compartments (especially *in vivo*, but also *in vitro* at the cellular level), the nature of the protein corona evolves and it is now admitted that the final composition of the protein corona depends on the environments that

nanoparticles has moved through, rather than only on its current environment. In other words, the final corona contains a fingerprint of its history [3,21,22,27,28,30].

The structure and composition of the protein corona depends on the physico-chemical properties of the nanomaterial (size, shape, composition, surface functional groups, hydrophilicity/hydrophobicity, surface charge, etc), on the nature of the physiological environment (blood, interstitial fluid, cytoplasm, organelles, etc), and on the duration of exposure [22–24,27,28].

Concerning the nanoparticle physico-chemical characteristics, size was found to play a significant role in determining the nanoparticle coronas on different particles of identical materials [29]. Brun et al. [24] have demonstrated that the smaller gold nanoparticles, the higher protein corona form. This can be due to the fact that the overall size is influenced by the degree of the surface curvature and curved surfaces compared to planar surfaces provide extra flexibility and enhanced surface area to the adsorbed protein molecules. This suggests dependence of protein adsorbance on the nanoparticle size [27,28,31,32]. But, among the nanoparticle parameters which affect the protein corona, the surface properties such as hydrophobicity and surface charge were found to play a more significant role than other parameters. Especially, nanoparticle surface charge has been shown to be one of the main factors driving protein binding and corona formation [27,30]. It has been shown that increasing the surface charge of nanoparticles results in a protein adsorption increase. Unsurprisingly, it has been observed that negatively charged serum proteins preferentially adsorb on positively charged nanoparticles because of electrostatic interactions [7,24,27,33].

The nature of the biological milieu also plays a crucial role in the protein corona formation. For instance, when comparing the protein-gold nanoparticles interactions in two classical cell culture media it has been demonstrated that while DMEM elicits the formation of a large time-dependent protein corona, RPMI shows different dynamics with reduced protein coating

[34]. Also, physical factors such as pH and solution electrolyte concentration have a considerable impact on the strengths and types of electrostatic charges on the adsorbent and thus can lead to different protein and surface interactions under different conditions [7].

All these examples argue for a thorough nanoparticle characterization into relevant conditions (i.e. in cell culture medium in which subsequent experiments will be carried out) in addition to the initial physico-chemical characterization. This is a necessary step to understand the effects elicited by cell culture media on nanoparticle which is crucial as they can impact the interpretation of the results of subsequent toxicological assays [9,22–25,27,28,34–36]. Many authors agree that this issue is often underestimated and can represent a partial explanation of conflicting results reported in the literature for a same chemical nature or surface charge of nanoparticle.

Due to the complex and dynamic nature of a protein corona, it is quite challenging to determine its composition, because there is no universal protein corona and as mentioned before its formation depends on many parameters. However several proteomic studies were undertaken and allowed to collect some information. For instance it was clearly established that the composition of the corona does not reflect the relative abundance of the proteins in the surrounding medium [24,27,34]. Moreover the quantitative aspect should be distinguished from the qualitative one, as in terms of biological response, the more abundantly associated proteins do not necessarily have the most profound effect and as a corollary, a less abundant protein with high affinity may instead be a key player [37]. It cannot be excluded that some proteins present at a minor level and so not cited in the literature could be responsible for major biological consequences [24].

Although difficult to characterize precisely, the existence of this protein corona is beyond doubt and it is recognized that its formation induces changes in the hydrodynamic diameter and zeta potential of the nanoparticle [21,23,25,38]. Zeta potential is the potential at the

boundary of the hydrodynamic shear plane of a charged particle and is usually used to predict the surface charge and stability of a nanoscaled system in solution [33]. When nanoparticles are incubated with serum such a change can occur that initially positively charged nanoparticles can turn negative [29,33]. It has been well documented, with different types of nanoparticles such as polystyrene [26,30], gold [39], silica [23] or magnetic nanoparticles [40].

The presence of a protein corona can significantly alter the surface properties of a nanoparticle (and especially their electrical surface potential) sometimes masking the expected effects of purposely grafted molecules [24]. As it is what cells “see”, the new interface may have a deep impact and by modifying interactions between nanoparticles and cell surfaces, it can consequently deeply affect the biological responses, nanoparticle biodistribution and generally nanoparticle fate [1,8,12,21,22,27,28,32,35,38,41]. For instance, it has been demonstrated that the capacity of nanoparticle surfaces to adsorb protein is indicative of their tendency to associate with cells [26]. Indeed, as adsorption of proteins on the nanoparticle surface can take place almost instantly, it was suggested that interaction of the nanoparticle with cellular structures is indirect and occurs mostly via the protein corona and not the bare nanoparticle surface. The protein corona can thus influence the uptake of the nanoparticle by the cell [28,42].

Although a clear correlation was established between the nanoparticle corona and cellular uptake, discrepancies are reported in the literature some studies showing that protein adsorption on nanoparticle decreases their cellular internalization while other tend to demonstrate the opposite. Among the former Brun et al. reported that the smaller corona gold nanoparticle corona induced the higher uptake. And this observation has also been reported for carbon nanotubes, graphene oxide nanosheets or biopolymeric nanoparticles in several cell lines [24]. Smith et al. quantified this difference in cell internalization and showed that the

presence of serum reduced the cellular association of carboxylate-modified fluorescent polystyrene beads up to 20-fold, relative to cells incubated in serum-free media [41]. The proposed explanation was that the addition of serum generates a highly fluidic protein corona with a rapid exchange rate which is less likely to adsorb to the plasma membrane, possibly due to the resulting reduced zeta potential. This prevents binding to the plasma membrane and subsequent internalization [41]. Using silica nanoparticles, Docter et al. showed that corona formation reduced nanoparticle cellular uptake [25] and Lesniak et al. reported that nanoparticles exposed to cells in absence of serum have a stronger adhesion to the cell membrane and a higher internalization efficiency in different cell lines, in comparison to what is observed in medium containing serum, when a pre-formed corona is present on their surface [35]. Once again the explanation lied in the fact that bare nanoparticles exhibit a higher degree of adhesion on the cell membrane which could, at least in part, contribute to the higher uptake efficiency [35]. Similarly, the uptake of FePt nanoparticles by HeLa cells was suppressed in the presence of a protein corona [28].

On the contrary, studies have emphasized that protein binding on the nanoparticle surface facilitates its uptake. Thus, it was described that the particles that bound more proteins also associated with cells more [26]. This correlation was further highlighted by Qiu et al. using a model of gold nanorods, and more particularly, positively charged nanorods had the highest adsorption capability for proteins, which was later related to its high favorability to be internalized by cells [33]. Similarly, it was shown that after the protein corona formation, magnetic nanoparticles undergo an uptake process that is either quicker than or interacts to a greater extent with cellular membranes, which enhances their internalization process [40].

An explanation to these observations could be that nanoparticles with more proteins on surface may have a higher possibility to expose ligands which can recognize the membrane receptors and facilitate the transmembrane internalization [1,33]. Therefore the reported

conflicting results may be related to the endocytosis pathway as will be further discussed in a following section.

For all these reasons it seems improper to propose, as mentioned before the widespread theory of “the positively charged nanoparticles interact more with cells than negatively charged nanoparticles due to the negative charge of cell membrane”. Therefore, the picture is more complex than that claimed by Pyshnaya et al. or Hühn et al. who state that the initial charge of nanoparticles determines penetration into cells rather than the presence of a corona [39]. Even if it is a fact that the initial charge of the nanoparticle actually influences the nature of the proteins that adsorb and consequently has an indirect impact on nanoparticle/cell interactions.

## **5. A concrete example**

Our experience underscores the influence of the protein corona on nanoparticle/cell interactions. We worked with fluorescent silica nanoparticles (70 nm), functionalized either with amine or carboxylic groups at variable density defining variable surface charge from highly negative to highly positive through moderate negative and positive charge. A neutral form was also produced. Depending on their surface charge the nanoparticles were referred to as NP(- -), NP(-), NP(0), NP(+) and NP(++)[43]. They were characterized just after their synthesis (in water) and in the cell culture medium used for our subsequent nanotoxicological assays: DMEM supplemented with 10% fetal calf serum (referred to as DMEMc, c standing for complete). The zeta potential and the hydrodynamic diameter of the nanoparticles are reported in Table 1.



**Table 1** – Physico-chemical characterization of the nanoparticles

Nanoparticle type	Zeta potential in water (mV)	Zeta potential in DMEMc (mV)	Hydrodynamic diameter in water (nm)	Hydrodynamic diameter in DMEMc (nm)
NP(- -)	-30	-96	82±1	104±4
NP(-)	-25	-13	62±5	85±3
NP(0)	0	-11	76±7	88±5
NP(+)	5	-20	75±5	90±5
NP(++)	12	-94	89±2	111±10

The zeta potential of the nanoparticles exhibiting the highest charges (i.e. NP(- -) and NP(++)) experienced the highest change in DMEMc. Interestingly, the zeta potential of all types of nanoparticles became negative. They decreased up to -96 mV due to the buffered culture medium. These changes between water and culture medium, as well as the hydrodynamic diameter increase, are very likely due to the adsorption of proteins from the culture medium at the nanoparticle surface. Therefore, chemical groups initially grafted onto the nanoparticle surface are hidden by proteins and nanoparticle surface charge is rather related to the nature of the adsorbed proteins. Thus, initially positively charged and initially negatively charged nanoparticles may exhibit a similar global negative zeta potential in cell culture medium as previously discussed.

Then, the adsorption at the cell membrane and the uptake efficiency of the nanoparticles was evaluated after contact with macrophages from the RAW264.7 cell line. As illustrated by Table 2, we observed that initially positively charged nanoparticles were less uptaken than initially negatively charged nanoparticles.

**Table 2** – Semi-quantitative evaluation of nanoparticle/cell interactions and cellular uptake of the different nanoparticles. - means no interaction or uptake, + a weak and ++ a strong level of interaction or uptake.

	NP(- -)	NP(-)	NP(0)	NP(+)	NP(++)
Adsorption at the cell membrane	+	+	+	++	++
Cellular uptake	++	++	++	-	-

Interestingly, the most adsorbed nanoparticles at the cell surface were NP(++). But this observation cannot be directly related to electrostatic interactions with the negatively charged cell membrane as initially positively charged nanoparticles exhibited a negative zeta potential in cell culture medium. This suggests that the assumption of a high adhesion of positive nanoparticles to cell membrane through electrostatic interactions should be revisited in favor of an indirect effect due to the protein corona [43].

In addition, we evaluated the amount of proteins adsorbed at the surface of the nanoparticles and while positively charged nanoparticles were able to bind a large amount of proteins from the medium, negatively charged nanoparticles exhibited a lower capacity. Finally, no or very few proteins could adsorb at the surface of neutral nanoparticles (unpublished data). These data are consistent with findings from Qiu et al. and Oh et al. [8,33].

Taken together, these results suggest that the initial nanoparticle charge can indeed influence the interactions with cells but indirectly, through the chemical nature of the corona. These interactions need further investigations to be better understood and special care should also be paid to alternative parameters.

## **6. Other parameters to consider**

### *6.1. Cell type*

Many parameters can impact the nanoparticle/cell interactions.

First, the cell type can be a key factor [1]. Thus, Osaka et al. reported that magnetite nanoparticles with positive charge showed higher internalization into human breast cancer cells than the nanoparticles with negative charge, while the degree of internalization of the positively and negatively charged nanoparticles into human umbilical vein endothelial cells (HUVEC) was almost the same [44]. This suggests a cell line-dependent uptake, as also demonstrated by others [4,45].

### *6.2. Nanoparticle aggregation/agglomeration*

Second, nanoparticle aggregation/agglomeration should not be neglected as agglomeration rate and agglomerate size has a tremendous impact on particle transport, dramatically affecting diffusion and gravitational settling [23]. This has consequences on nanoparticle uptake and on the subsequent biological response [22]. Indeed it was clearly established that nanoparticle aggregation leads to a lower cell internalization [46]. Moreover, many authors report that aggregation is much diminished in a medium containing serum compared to a serum-free medium [22,31]. This is due to the fact that proteins immobilized onto the nanoparticle surface provide an effective protection against nanoparticle/nanoparticle interactions leading to aggregation [22]. In addition, zeta potential is an important parameter in the repulsive electrostatic force between the particles. The higher the zeta potential, the longer the range of repulsive forces. Therefore, suspension stability is enhanced by increased zeta potential [31]. One possible explanation for the lower cell internalization is that the adsorption of proteins affects the agglomerate size and surface charge of the nanoparticles, which alters the electrostatic binding affinity with cells [38]. Concerning the nanoparticle functionalization, positively charged nanoparticles tend to agglomerate faster and to a larger

extent compared to negatively charged or neutral nanoparticles [22,23]. And Guarnieri et al. demonstrated that although positive nanoparticles were more internalized than negative nanoparticles this was contrasted by the tendency of particles to form agglomerates leading to lower internalization efficiency. This is consistent with our observations where NP(++), exhibiting the highest agglomeration rate, were less internalized than NP(0) and NP(- -) [43]. Finally, Albanese et al. showed that nanoparticle aggregation decreased cellular uptake in a cell type-dependent way suggesting that different uptake mechanisms might be involved [47]. This leads to a third, fundamental parameter to consider: the endocytosis pathway.

### *6.3. The endocytosis pathway*

The mechanism by which nanoparticles enter the cell has important implications not only for their fate but also for their impact on biological systems [10]. As mentioned earlier, cells have numerous uptake mechanisms like phagocytosis, clathrin or caveolin-dependent endocytosis, pinocytosis... and cell internalization can occur through a combination of these pathways. Depending on the presence or absence of a protein corona the uptake mechanism solicited might be different and might explain, at least in part, conflicting results in the literature (i.e. studies reporting that protein corona favors uptake while others state that on the contrary it has an inhibitory effect). At this point non-specific uptake must be distinguished from specific uptake. Specific internalization is regulated by membrane receptors that are only activated by receptor-specific ligands to trigger internalization. Non-specific uptake is a random process without specific biomolecular control by the cell [24]. Therefore, the uptake in absence of serum proteins may be due to direct recognition of the particles at the cell surface whereas in the presence of proteins, the uptake probably proceeds by interaction of the adsorbed proteins, specifically with the protein receptors on the cell surface [3,24,38]. In support to this conclusion, Walkey et al. have demonstrated that variations in serum protein adsorption correlate with differences in the mechanism and efficiency of nanoparticle uptake by a

macrophage cell line [48]. Similarly, Lesniak et al. showed that a protein-rich corona was associated with a decreased particle cellular uptake [36], which is in agreement with our findings (NP(++)) exhibiting the larger protein corona and the lower cell internalization). In other words, serum protein binding can change the surface charge and therefore accelerate the cellular uptake of nanoparticles through receptor-mediated endocytosis [1,4,31,33]. Moreover, as the protein-rich corona may interact with multiple receptors multiple mechanisms may be involved simultaneously. In contrast, particles having one type of protein adsorbed at their surface may be restricted to a specific receptor [38]. This argues for the need of identification of the nanomaterial-protein corona complex to better understand the uptake mechanisms [40]. Relative to protein adsorption, the earliest studies identified opsonins, plasma proteins including immunoglobulins and complement proteins that adsorb to particle surfaces, creating a “molecular signature” which is recognized by immune cells and determines the route of particle internalization. Opsonization targets foreign matter for clearance by the reticulo-endothelial system and mononuclear phagocytic system, primarily via the liver and spleen [26,32]. To illustrate how the opsonisation of the nanoparticle surface by serum proteins remarkably influences its uptake one can quote the study from Saptarshi et al. where the NH<sub>2</sub>-polystyrene nanoparticle uptake by macrophages in a protein free medium was shown to change from clathrin-mediated endocytosis to phagocytosis when incubated in serum enriched media. Adsorption of complement protein C3 and opsonizing protein IgG on polystyrene nanoparticles was also shown to increase with time and this directly influenced their uptake by murine Kupffer cells [28].

#### *6.4. The nanoparticle charge*

The nanoparticle charge can also play a key role in the choice of the uptake mechanisms. It is generally admitted that nanoparticles with positively charged surfaces can be effectively internalized by cells through electrostatic interactions with the negatively charged cell plasma

membrane i.e. through non-specific endocytosis (possibly involving clathrin-mediated endocytosis), whereas negatively charged nanoparticles are more likely to take advantage of caveolae-mediated pathways i.e. involving specific receptors [4,6,11]. Such findings are supported by the fact that transferrin for example, which has been demonstrated to adsorb to the surface of negatively charged nanoparticles is well known to internalize via receptor-mediated endocytosis [11]. Another example is apolipoproteins, these molecules were found to be one of the major proteins of human serum immobilized on a number of nanoparticles surfaces. Apolipoproteins participate in lipids transportation in the bloodstream and, as such, are expected to affect the intracellular trafficking and transport of nanoparticles [22,28]. Indeed, there are multiple receptors for apolipoprotein complexes at cell surfaces that nanoparticles with surface-adsorbed apolipoproteins can potentially exploit to enter cells [21]. Besides the classical endocytosis pathways, alternative mechanisms can also be envisaged. For instance, Kim et al. proposed that positively charged gold nanoparticles could modulate cell membrane potential, ultimately disrupting the lipid bilayer. Such depolarization of cell membrane allowed enhancing the cellular uptake of cationic nanoparticles [18]. In the same vein, Smith et al. demonstrated that nanoparticles were able to permeabilize the plasma membrane to a similar extent as a detergent, suggesting that nanoparticles, at least a part, potentially have the capacity to enter the cell through permeabilization of the cell membrane [41].

#### *6.5. The nanoparticle functionalization*

Finally, the type of nanoparticle functionalization should be taken into account. Graf et al. discussed that one type of positively charged particle (AHAPS) was easily internalized by macrophages, while another type of positively charged particle (short alkyl chain aminosilanes) was uptaken by cells in a lower amount, this could be due to a difference of functionalization in group types and density. Similarly, nanoparticle can be coated with

polyethylene glycol group (PEG) to inhibit opsonization due to steric hindrance and hence display minimal cellular uptake [2,12,26]. And the uptake efficiency of PEGylated nanoparticles is closely related to PEG grafting density and molecular architecture of PEG [2,8].

Brun et al. suggested that as the formation of a protein corona seems unavoidable it should be exploited and further studies are needed to know how to take advantage of such corona [24]. A better knowledge of the relationship between the initial nanoparticle physico-chemical properties, the protein corona and cellular uptake efficiency could guide the design of nanoparticles. This could be of particular interest for the development of new nanocarriers with surface design for enhanced cellular uptake. This objective could be reached “simply” by modifying nanoparticle surface properties [1,7,27,33,35].

## **7. Conclusion and recommendations**

A better understanding of the nanoparticle/cell interactions is of paramount importance in the context of nanotoxicology. However, it is quite difficult to draw firm conclusions on this relationship as first, it depends on many parameters and second, studies from the literature often report conflicting results. To improve our knowledge on this topic some general recommendations can be made:

- All the parameters involved in a system should be carefully considered. In the example developed in this paper, we clearly underscored the key role of the protein corona (that is only a parameter among others). Typically, with proteins adsorbed at the nanoparticle surface, the situation is more complex and a preferential binding of positive nanoparticle to cell membrane cannot be only explained by favorable electrostatic interactions any more.

- Each parameter should also be defined precisely. For instance when one talks about the nanoparticle charge does he refer to the surface potential, the Stern potential or again the zeta potential? If the parameters are explicitly expressed comparisons between different studies can be made easier.
- The nanoparticle physico-chemical features are usually well characterized after their synthesis but they should also be systematically re-evaluated in the context of their use (for instance in the biological medium where subsequent assays will be carried out). Indeed, these features can dramatically vary from a medium to another, as previously illustrated by a positive zeta potential in water that can turn negative in a cell culture medium. This can potentially result in misleading conclusions.

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