Nanomedicine

The impact of nanoparticle protein corona on cytotoxicity, immunotoxicity and target drug delivery

In a perfect sequence of events, nanoparticles (NPs) are injected into the bloodstream where they circulate until they reach the target tissue. The ligand on the NP surface recognizes its specific receptor expressed on the target tissue and the drug is released in a controlled manner. However, once injected in a physiological environment, NPs interact with biological components and are surrounded by a protein corona (PC). This can trigger an immune response and affect NP toxicity and targeting capabilities. In this review, we provide a survey of recent findings on the NP–PC interactions and discuss how the PC can be used to modulate both cytotoxicity and the immune response as well as to improve the efficacy of targeted delivery of nanocarriers.

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Nanomedicine is the field of science devoted to the medical application of nanoparticles (NPs). Recent years have seen a surge in the development of nanomaterials as therapeutic and diagnostic agents, paralleled by major advances in the development of nano-sized delivery systems for targeted drug administration. In this scenario, it is important to understand the interactions occurring at the interface between NPs and biological components in order to predict the fate of injected NPs. Once NPs interact with biological fluids and come into contact with tissues, they are exposed to active biomolecules that form a 'crown' (*corona* in Latin) around them, thus transforming the bare NP into an NP that has a biological component: the so-called protein corona (PC). The PC is primarily composed of proteins that are the basis of most of the investigations conducted so far in this field [1–3]. The presence of other biomolecules such as sugars, nucleic acids and lipids is expected but so far little studied [4,5]. The first studies on the interactions between

NPs and plasma proteins were conducted between 1996 and 2000 [6–8], but it was the group of Dawson that first introduced the NP–PC complex concept [3]. The synthetic identity of the NP was replaced by a new biological identity, namely, the NP–PC complex (Figure 1) and as a consequence, researchers are now starting to consider NP–PC complexes as the new NPs. During their journey in the body NPs are subjected to significant changes in their physicochemical properties and in their biological identity. Therefore, the characterization of the properties of NP–PC complexes has become a mandatory step in an experimental plan involving the use of NPs. On the other hand, the physicochemical characterization of bare NPs before exposure to body fluids remains crucial to reveal the correlations between the properties of NPs and PC composition (Figure 1). During the recent years, a large number of studies have been devoted to the characterization of NP–PC complexes [9–23]. Furthermore, the scientific community is now moving from the
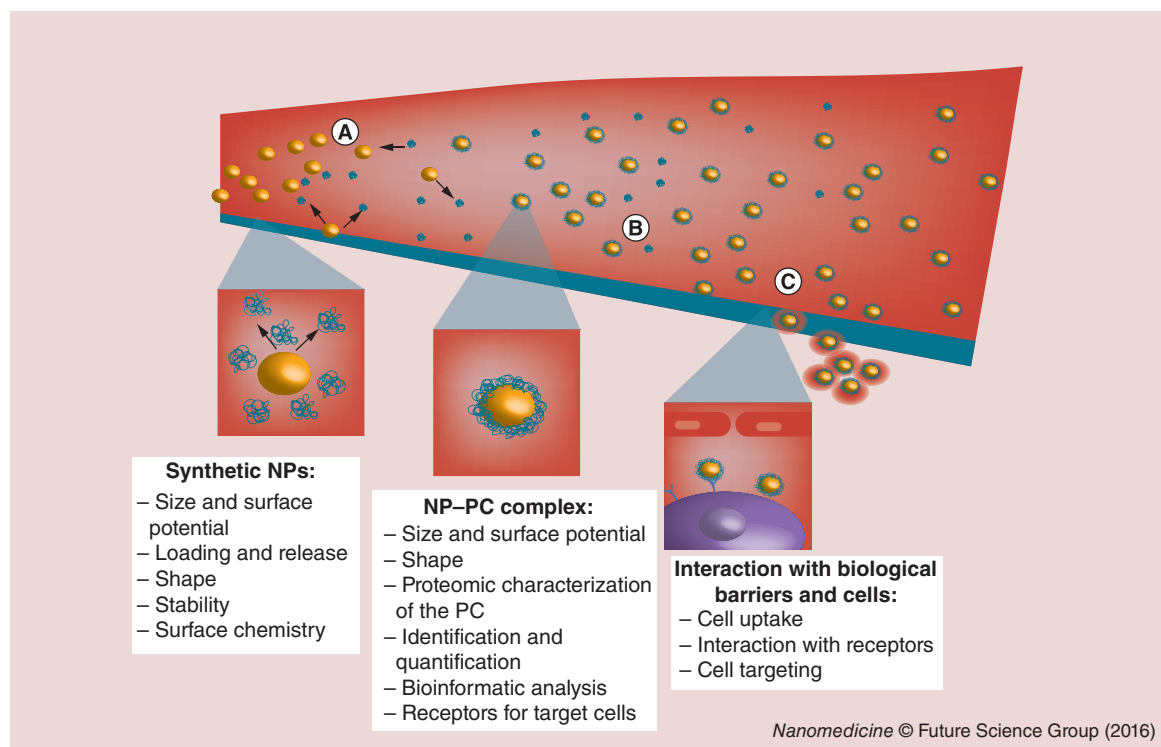
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Figure 1. The nanoparticle journey in the body: from synthetic identity to physiological response through biological identity. (A) The bare NP (synthetic identity) that has a specific shape, size and charge is injected into the body. **(B)** Once exposed to biological fluids, synthetic NPs come into contact with active biomolecules that surround them thus giving rise to the NP–PC complex (biological identity). **(C)** These NP–PC complexes are responsible for the interaction with biological barriers and cells. The NP can reach the target site using a specific receptor endowed upon them by the PC as part of their new biological identity (physiological response). The experimental steps associated to the study of bare NPs, NP–PC complexes and their physiological behaviors are also reported.
NP: Nanoparticle; PC: Protein corona.

mere evaluation of the impact of the PC on the physical and chemical properties of NPs to the impact on their behavior in physiological systems.

In addition to a discussion on the dynamics of PC formation, its composition and the experimental approaches used, in this review we discuss the most recent advances regarding the impact of the PC on NPs targeting ability, toxicity and immunological recognition.

Dynamics of PC formation

Time evolution studies on the composition of the PC have demonstrated that the process of NP–PC complex formation is in continuous evolution and that the adsorption of proteins on the surface is mainly governed by affinity interactions of proteins towards the NP surface and by affinity-based protein-to-protein interactions [24]. For example, although blood plasma is constituted by thousands of proteins (≈4000) and some of which are more abundant than others [25]), their abundance in the plasma does not correspond to their abundance in the PC [26–30]. Moreover, the affin-

ity-based competition between proteins for adsorption on the NP surface is responsible for the changes in the PC composition over time [31]. Based on the exchange time of its composition, the PC is classified into hard and soft (Figure 2). The hard corona is considered the first tightly bound layer of proteins that has a long exchange time (many hours), while the soft corona is represented by the second layer of proteins (not directly bound to the NP) that undergoes fast exchanges over time (seconds or minutes) [11]. The proteins adsorbed on NPs are considered to be in a continuous flux of desorption/adsorption mainly controlled by the so-called 'Vroman effect' [32] as the group of Vroman observed this phenomenon at the plasma–solid interface in 1962 [33]. According to this 'effect', an initially attached protein can, at any time, desorb from a NP and be replaced by a different one with major affinity. This substantially changes the PC composition while the amount of adsorbed proteins remains relatively constant. In theory, proteins that are more abundant in the biological fluids are the first to be adsorbed during the initial phase, after which

they are replaced by proteins of lower abundance but higher affinity that remain around the NP for a longer time [3,34,35]. This model has been widely discussed and implies the existence of a highly dynamic PC formed over time. More recently, Tenzer *et al.* described the early evolution and impact of the NP–PC complex. They demonstrated that an interaction between NP surface and plasma proteins is already established at 0.5 min in all the formulations under investigation (silica and polystyrene NPs of various sizes and surface functionalization) [36]. They also found that the composition of the generated PC did not change qualitatively but only quantitatively over time [36], thus showing results in contrast with previous reports discussed above. These findings led to the introduction of a novel model of PC formation, which implies new binding kinetics independent from the Vroman effect [37] (Figure 3).

Composition of the PC

The PC can be considered unique for each given nanomaterial and even in the case of NPs constituted by the same material, it greatly depends on the physicochemical properties of NPs. Size [38], shape [39] and surface charge [38,40–42], together with the surface modifica-

tion [22] are key factors in determining the composition of the PC.

The characteristics of the biological environment also play a determinant role in the formation of a PC: the type of plasma (e.g., human or murine), incubation time, temperature, pH and the physiological state of the plasma (alterations due to disease/medical conditions) [36,42–44] may also affect the protein adsorption on the NP surface.

Despite this great variability, a similar subset of about 125 proteins has been shown to be adsorbed on various nanomaterials in different amounts when incubated in plasma [2]. These proteins are involved in the same cellular processes, namely, complement activation, pathogen recognition and blood coagulation. Despite this similarity in function, they have a different physiological impact on NPs depending on their material [2,45]. Among these, immunoglobulin G, serum albumin, fibrinogen, clusterin and apolipoproteins are generally present in the PC of most analyzed NPs after exposure to plasma [2,46].

Since the majority of NPs are intended for intravenous administration [47], most of the studies in the current literature are devoted to the characterization of the PC adsorbed on NPs after incubation in
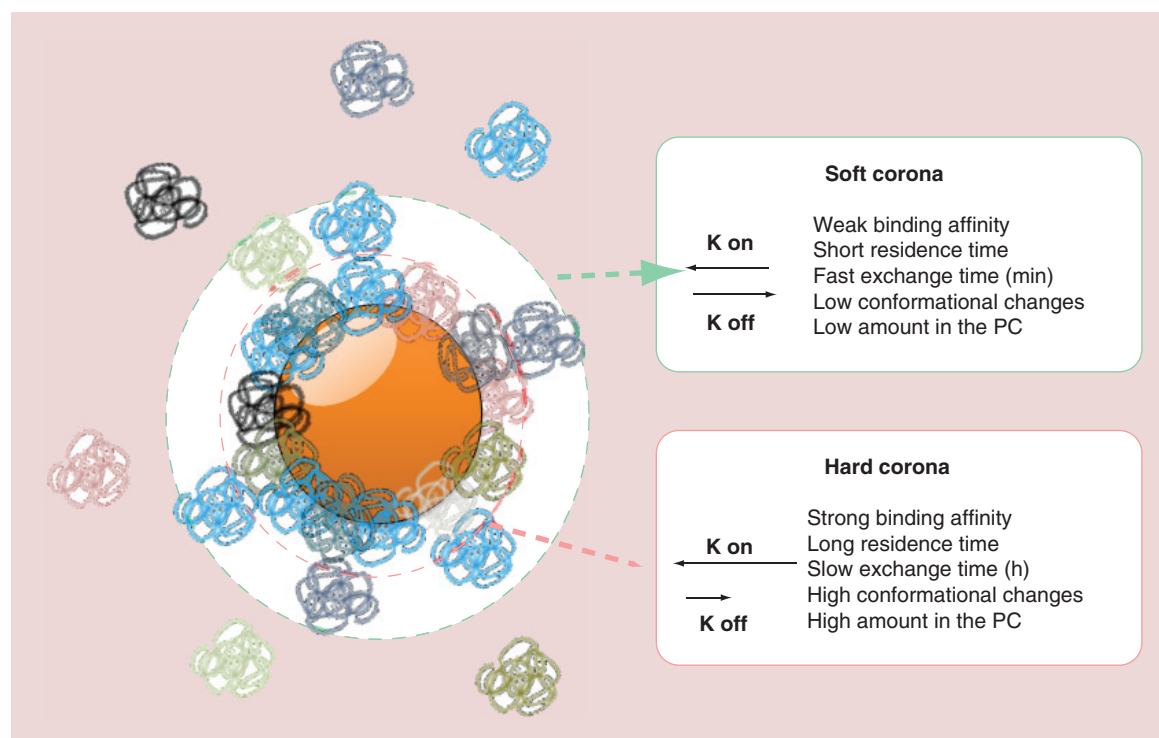

Figure 2. Nanoparticle–protein corona complexes: characteristics of hard and soft coronas. The protein corona represents the biological entity of a nanoparticle. Hard corona proteins are directly adsorbed on the nanoparticle surface due to their strong binding affinity. These proteins also have a slow exchange time. Soft corona proteins associate with the hard corona via weak protein–protein interactions, thus showing a short residence time around the nanoparticle and a fast exchange time. The objects are not drawn in scale.
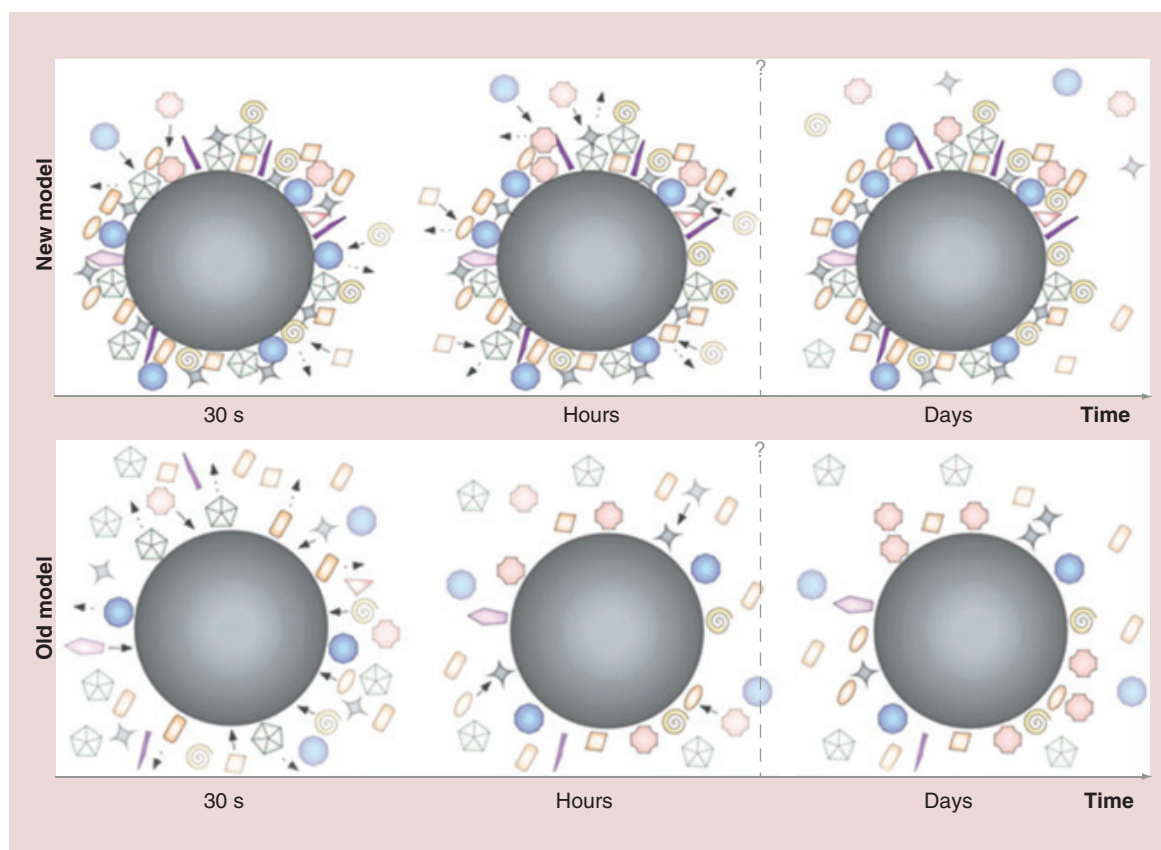PC: Protein corona.

Figure 3. Formation of the protein corona: the old versus the new model. During the very early phase: a highly complex corona is established (new). A corona of low complexity evolves slowly (old). During the late phase: the protein corona composition remains stable and shows quantitative rather than qualitative changes and binding kinetics dependent and independent of the Vroman effect (new). A highly dynamic protein corona changes significantly over time, controlled by the Vroman effect (old). The objects are not drawn to scale. Reproduced with permission from [37].

plasma [19,48–50]. Similarly, information about the nano-plasma interface and the effects it may have on the biocompatibility and efficacy of nanotherapeutics is now becoming available [51,52].

However, when other administrative routes are used, NPs are subjected to interactions with biomolecules of other biological fluids before reaching the plasma. For example, inhaled NPs are first exposed to lung fluid [53], which has a very different composition than blood plasma [54]. A first PC, rich in surfactant proteins and other biomolecules, is generated around the NPs in the lungs [55–57]. This is then displaced by a new corona as soon as the NPs enter the bloodstream [9]. Instead, NPs administrated via oral ingestion are subjected to contact with saliva and then with fluids of the gastrointestinal tract: an environment characterized by a low pH and the presence of enzymes that may hinder the bioavailability of NPs. Little information is available about the interactions of NPs with the biological milieu of the GI tract [58]. It is becoming clear that a deeper understanding of the role of PC generated in biological fluids other than blood plasma is needed.

Experimental approaches used to characterize the PC

Many studies have revealed the identity [21,35,38,39,42,59–61] and, more recently, also the quantity [28,36,62–64] of the proteins in the PC using proteomics-based approaches coupled with MS. In a typical experimental plan, NPs are incubated in a biological fluid that ideally should replicate the features of the *in vivo* biological milieu. After incubation, the NPs are recovered by centrifugation, ultrafiltration [60,65] or, more recently, by a combination of size exclusion chromatography and ultrafiltration [66], and extensively washed to avoid contamination of nonbound proteins. Finally, the adsorbed proteins are eluted from the NP surface and analyzed by two different methods: gel-based or gel-free proteomics [67]. The first implies the separation of proteins through mono or bidimensional sodium dodecyl sulfate electrophoresis followed by *in situ* digestion and identification by MS [9,36,66]. In the gel-free proteomic approaches, instead, complex peptide mixtures are separated using last generation mass spectrometers during the chromatography step that precedes the identifica-

tion by MS. Among the latters, label-free MS [68] is becoming the gold standard procedure with which to obtain simultaneously a quantitative and qualitative analysis of PC components [64,69–71] thanks to its benefits that include a low number of steps in the protocol and high reproducibility.

Do *in vitro* models of the PC reproduce the physiological complexity?

Despite the progresses made in the field of NP–PC, the majority of the *in vitro* studies seem to be only partially relevant from a biological point of view because the incubation of NPs inadequately replicates the conditions of an intact biological system. The most widely used *in vitro* models of the PC are based on a single protein [72,73], usually bovine serum albumin, or plasma in different variations and/or concentrations [4,71].

These types of models oversimplify the studies and cannot serve as a reliable indicator of the much more dynamic and complex *in vivo* scenario.

Once in the body, NPs traverse various compartments that have different protein concentrations/compositions, pH values, ionic concentrations and temperatures [74]. Moreover, injected NPs are subjected to blood flow, the velocity of which is another parameter that dynamically changes depending on the environment (e.g., aorta 60 cm s^{-1} vs tumors 0.1 mm s^{-1}) [52]. Thus, despite many good attempts to simulate the biological systems, *in vitro* experiments lack several dynamic parameters that exist *in vivo* and are hard to reproduce. The lack of similarities between these two models could explain why NPs that seem promising *in vitro* are less promising in preclinical trials.

On the other hand, *in vivo* experiments are complicated by the fact that NPs cannot be readily recovered once they are injected in animals for protein adsorption analysis. Moreover, the biological identities adsorbed on the NPs in mice and humans could be different from one another [75]. However, several examples of *in vivo* experiments have been reported.

Recently, Hadjidemetriou *et al.* [66] investigated *in vivo* PC formation on commercially available liposomes that are used in the clinical setting (i.e., the anticancer agent Doxil). In particular, they compared *in vivo* and *in vitro* PCs in terms of morphology, function and composition. The results revealed that the two PCs differ in morphology: electron microscopy imaging showed that liposomes incubated in plasma *in vitro* create fibrillar structures, whereas liposomes retrieved after *in vivo* systemic administration did not. Moreover, the composition of the *in vivo* PC was more complex, in terms of variety of molecular species adsorbed, than the *in vitro* PC. Finally, both *in vitro* and *in vivo* PCs affect the targeting capabilities of NPs,

thereby decreasing cellular internalization. However, the impact of the *in vivo* PC was weaker [66].

Sakulkhu and collaborators [62] compared the *in vivo* and *in vitro* PCs of superparamagnetic iron oxide NPs (SPIONs), by exploiting the magnetic properties of these NPs to extract them from rat sera after *in vivo* injection. Even in this case, the results indicated strong differences between the *in vivo* and *in vitro* experiments in terms of PC composition and relative protein amounts. Taken together, the results of this subsection confirm the need to carefully consider the data acquired so far from *in vitro* studies, and to test their reproducibility *in vivo*, when possible.

Impact of the PC on the targeting capability of NPs

Over the past decade, various nanomaterials have been designed for delivery of drugs [76], DNA [77] and imaging agents [78]. These nanocarriers were designed in an attempt to: increase drug bioavailability and avoid drug inactivation, which is particularly important in the case of poorly soluble compounds (i.e., paclitaxel) [79], and in the case of macromolecules sensitive to degradation by blood proteases/peptidases or nucleases, (i.e., proteins/peptides or DNA/RNA-based therapeutics) [80]; minimize side-effects due to a nonspecific body distribution of the drug and to the high amount of drug commonly used [81]; and selectively deliver the payload to the affected area, thereby increasing its tissue accumulation [82]. While the first-generation nanocarriers, such as micelles, polyethylene glycol (PEG)-modified liposomes and polymeric NPs fully addressed the first two points, thereby increasing the pharmacokinetics and tolerability of nanoformulate drugs [83,84], the second-generation nanocarriers exploit active targeting strategies to ensure tissue-specific delivery [85]. These approaches are based on the functionalization of the carrier surface with active targeting molecules, (e.g., ligands for cell membrane receptors) [86], monoclonal antibodies [87] and aptamers [88]. Several receptor systems, are overexpressed on the cell surface in such disease conditions as inflammation and cancer [89]. The outcome of the aforementioned strategies has had a high impact on both pharmaceutical research and in the clinical setting. In addition, numerous nanotechnology-based platforms that have the potential to result in more effective and safe therapies have been developed or are currently in development [90,91]. Various carrier functionalizations aimed at improving cellular/tissue targeting and overcoming biological barriers have already been exhaustively reviewed [92–98], and will not be further discussed in this article. Herein, we discuss whether and how the PC affects the interaction between a NP (functional-

ized or not) and its cellular target, as well as the general mechanisms of that interaction. In the recent years, much attention has focused on the biological identity of NPs [9]. As reported previously, the PC strongly affects the fate and biological impact of NPs, as well as their therapeutic and pathophysiological effects [99,100]. It is now evident that the *in vivo* localization of a nanoplatform ultimately depends on the identity of the PC around its surface, rather than on its physicochemical properties [101], which instead are strong determinants of the composition of the PC [102]. The role of the PC in particle-targeting is still controversial. For example, Salvati *et al.* [103] recently showed that the targeting abilities of transferrin (Tf)-functionalized SiO$_2$NPs disappear after their incubation in biological fluids, due to the formation of a PC that prevents Tf from binding its receptor. Consequently, the Tf-related specificity is lost (Figure 4). In addition, Davis *et al.* [104] demonstrated that Tf-targeted NPs accumulated in tumor tissue independently of the presence of the targeting ligand (Tf), also if the Tf ligand itself provided greater tumor cell uptake. In another study, Varnamkhasti *et al.* [105] demonstrated how, after incubation in 100% fetal bovine serum (FBS), aptamer-targeted core-shell chitosan NPs carrying an active metabolite of camptotechin (i.e. SN-38) and nontargeted NPs showed similar cytoxicity activity toward human colon cancer cells HT29. Flow cytometry experiments revealed that the PC induced a reduction in the uptake of the targeted NPs. By contrast, Dai *et al.* [106] examined the effect of the PC on the targeting ability of a core-shell NP assembled by the layer-by-layer method, conjugated with a humanized A33 monoclonal antibody. They showed that the PC did not significantly affect the targeting ability of antibody-functionalized NPs toward colon cancer cells. Using different concentrations of human serum (0, 10, 50 and 100%), the authors demonstrated that, although 10 and 100% human serum induce a different composition of PCs (e.g., 100% human serum-derived PCs contained most of the proteins, while 10% human serum-derived PCs had a higher concentration of specific proteins as complement C1q protein subunits), none of these PCs significantly alter the targeting ability of functionalized NPs [106].

These findings prompt two considerations. First, they illustrate the importance of studying the physicochemical properties of NPs and the experimental conditions in which they will be applied. In fact, even if a simple dispersion, such as phosphate buffer solution, can reveal a specific function, a more complex system that mimics relevant physiological conditions could instead nullify it. In a very elegant study, Mahmoudi *et al.* [102] investigated the link between physical–chemical properties (size and surface charge) of SPIONs and PC composition. Specifically, they found that PC composition, in terms of relative amount and molecular weight of proteins, strongly depended on NPs size and surface charge. These factors are directly proportional, in other words, larger NPs preferentially adsorb proteins with a higher molecular weight, whereas proteins with a lower molecular weight accumulate on the surface of smaller NPs [102]. In addition, although surface charge plays an important role in the distribution and abundance of proteins that constitute the PC, it is insignificant compared with the size of NPs. Hadjidemetriou *et al.* [66] confirmed the clear difference between the *in vitro* and *in vivo* PCs, and highlighted the need of studies designed to advance our understanding of the correlation between the physical–chemical characteristics of NPs and the nature of the incubation medium (*in vitro* vs *in vivo*, human or murine models).

The second consideration is that the new NP identity provided by the PC may result in the loss of targeting properties, thereby affecting the pharmacokinetics of a nanocarrier and its payload. This is the case, for example, of 3.5 nm sized, negatively charged SPIONs [102]. When incubated *in vitro* with 90% FBS, they adsorbed higher amounts of the small proteins ApoA-I and ApoA-II (≈30 and ≈11 kDa, respectively) compared with positively charged and plain 3.5 nm SPIONs. As these apolipoproteins can cross the blood–brain barrier through a saturable transport mechanism, once SPIONs were injected *in vivo*, ApoA-I and II were adsorbed on their surface and drove the NPs to brain vessels. This was demonstrated by magnetic resonance imaging only 5 min after injection. If not considered in advance, this behavior may result in neurotoxic side-effects after injection of 3.5 nm SPIONs [102].

An understanding of the nano–bio interactions can be used to overcome the above reported limitations, for instance by modifying the NPs in a controlled manner to induce the binding of specific proteins. This concept is at the basis of the clever strategy recently developed by Prapainop *et al.* [101]. The judicious functionalization of the surface of quantum dots with 5,6-secosterol atheronal-B induced the binding to and misfolding of apolipoprotein B-100 (Apo-B100) in low-density lipoprotein (LDL) in the PC. The conformational change induced by the misfolding of Apo-B100 causes the exposure of epitopes that bind cell-surface receptors on macrophage cells, which ultimately favors the selective uptake of atheronal-modified LDL particles by cells and foam cell formation [101]. Experimentally, the incubation of atheronal-functionalized particles with macrophages cultured in medium supplemented with fetal calf serum (which contains lipoproteins), lipoprotein-deficient serum (LPDS) or delipidated LPDS, revealed
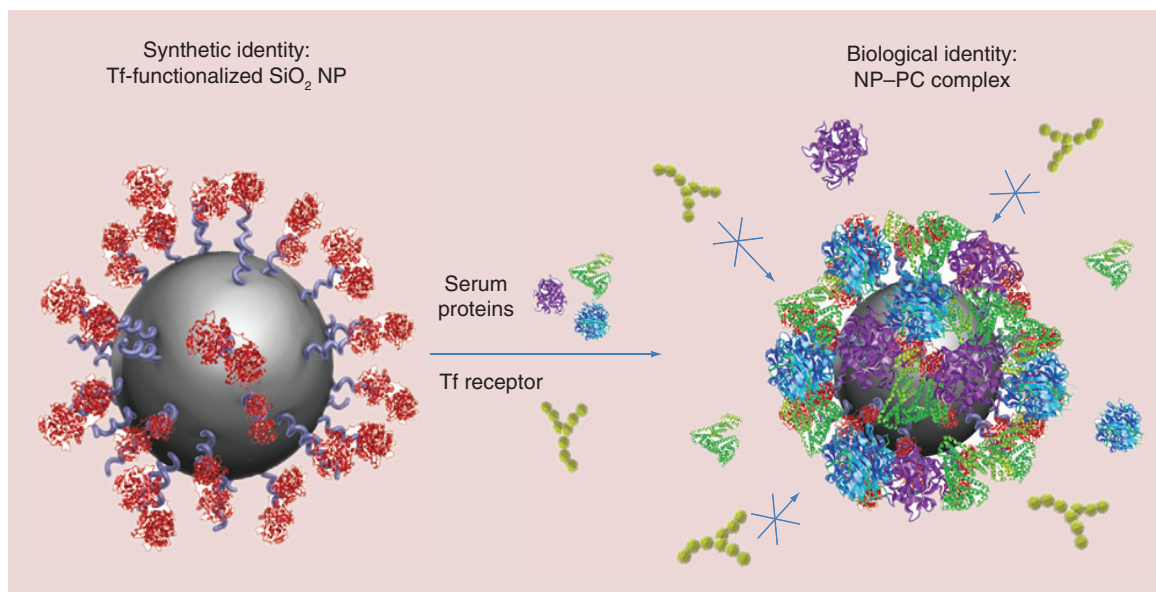
Figure 4. Negative impact of the protein corona on the nanoparticle-targeting ability. The PC hides the functionalization molecule (in this case, transferrin) on the NP surface, thereby inhibiting the interaction with its specific receptor on target cells.
NP: Nanoparticle; PC: Protein corona; Tf: Transferrin.
Adapted with permission from [103].

the presence of lipoproteins for cell uptake [101]. Other studies of the mechanisms of internalization revealed that CD36 is the key receptor on the macrophage surface that is responsible for interaction with atheronal-modified LDL particles [101]. This strategy could be used for the targeting of all CD36+ cells (e.g., adipocytes, skeletal muscle and retinal pigmental epithelial cells). In another study, PC formation was exploited to target cancer cells [107]. Briefly, the high affinity of vitronectin for the cationic-charged surface of lipoplexes was used to efficiently target the human breast cancer cells MDA-MB-435S, which overexpress two major vitronectin receptors. Internalization of naked lipoplexes (i.e., without a PC, and thus without vitronectin on their surface) was lower [107]. Figure 5 shows a schematic example in which the PC contributes to improve the NP targeting of a tumor cell.

Toxicological implication of the PC

The formation of a PC impacts not only on the targeting and delivery properties of NPs but it can also influence their toxicity and pathophysiology [36].

As for the NPs targeting, the chemical and physical characteristics of NPs and, eventually, the cellular phenotypes used in the study, play a relevant role in the potential toxic or nontoxic effects exerted by the PC. Moreover, even in the case of NPs with the same chemical and physical properties, it is difficult to compare the toxicological profile of the PC formed in different experimental conditions, because the relative quanti-

ties of the adsorbed proteins can change remarkably. Lastly, the use of very different toxicity tests has jeopardized attempts to extrapolate general rules to define the biological impact of the PC on human cells, and it is still a significant obstacle to determine NPs toxicology [108–110]. In this scenario, there is a race to develop quantitative models to predict the biological identity that occurs after PC formation and eventually their cytotoxic properties. Our literature search indicates that, in terms of particle biocompatibility, the formation of a PC can be advantageous or disadvantageous in function of several factors [19]. In the next subsections we report information about both these cases in various NPs.

When does the PC decrease nanoparticle toxicity?

Many toxicity properties of NPs derive from the reactivity of their surface in interfacing cellular membrane. For example, positive charged NPs can perturb the continuity of plasma membrane, due to their interaction with the negatively charged surface of the cells [80]. This can lead to barrier disruption and, eventually, cell death [111]. The presence of a coating can mask these properties and can be considered a factor that increases the safety of NPs. This is true for both inorganic and biological coating. For example, the coating of zinc oxide (ZnO) NPs with silica [112] or with a PC [113] was shown to decrease their toxicity toward human skin dermal fibroblasts and human hepatocarcinoma,
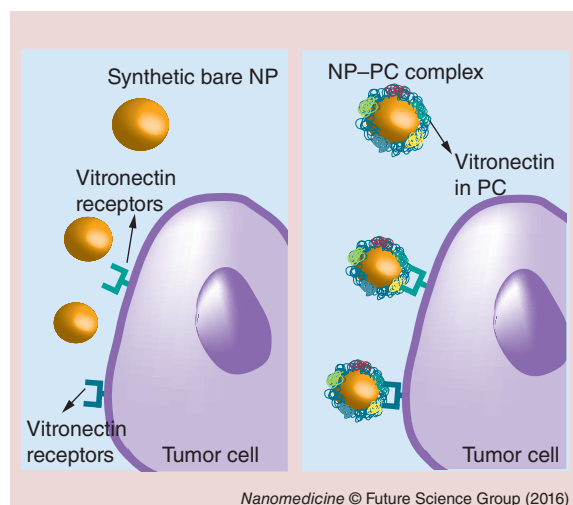
Figure 5. Positive impact of the protein corona on the nanoparticle-targeting ability. The PC contains proteins (in this case, vitronectin) that act as ligands for receptors on specific cells (tumor cells). This phenomenon, in theory, paves the way for induction of the PC formation to target selected cells. Elements not drawn to scale.
NP: Nanoparticle; PC: Protein corona.

respectively, by hampering direct surface contact between NPs and cells. Given their unique properties, carbon nanotubes (CNTs) have generated interest in a number of areas of medicine, including cancer therapy and diagnostics [114,115]. The toxicological implications related to their use have been widely discussed and are mainly due to the nonbiodegradable nature of these NPs [116,117]. However, even in this case, the binding of blood proteins to CNTs was shown to reduce their cytotoxic effect toward human acute monocytic leukemia (THP-1) and umbilical vein endothelial cells [29]. This is likely due to the effect of PC in protecting the cells from the direct contact with the bare surface of CNTs. Recently, the interaction between CNT–PC complexes and human blood platelets was also investigated [118]. De Paoli *et al.* demonstrated that the type of interaction between CNTs and platelets strongly depends on the identity of the proteins forming the PC [118]. Particularly, PCs made of different proteins may have opposite impacts: while albumin PCs decreased the platelets aggregation, histone H1 and gamma globulins PCs caused platelet aggregation and fragmentation, respectively [118].

The interaction between NPs and PC can also reduce systemic toxicity: Tenzer *et al.* [36] reported that freshly isolated human thrombocytes are activated by incubation with uncoated silica NPs. However, the same experiment performed with NP–PC complexes (obtained by incubating NPs in human plasma for 30 s) did not trigger this cell phenotype. Similarly, while uncoated silica NPs had a significant hemolytic effect

on red blood cells, the formation of a PC on the particle surface protected the cells from this damage [36]. In this scenario, the innate surface charge of NPs is a crucial factor in determining NP toxicity in function of the PC. As mentioned above, the beneficial effect of the PC formation can be better appreciated when NPs are positively charged, as this physical feature possesses toxic properties [119]. Recently, the role of the PC in protecting cells from damage caused by positively charged NPs was elegantly demonstrated by the group of Dawson [119]. They showed that positively charged polystyrene NPs bearing a PC were trafficked with their coating to the lysosomes. In this cellular compartment, the PC was digested, thereby exposing the positive groups of the NPs that eventually exerted toxic effects on the cells by damaging lysosomal vesicles [119]. This is a general model that can be used to avoid deviations due to the chemical and physical properties of NPs in order to study the effective contribution of PC to NP toxicity.

In addition to protecting the cell membrane against reactive surfaces of NPs, the PC increases the safety of the carriers in other ways. For example, the protein coating that occurs in biological fluids can increase the stability of particles, which is important from the standpoint of cellular toxicity if the secondary degradation products of the carriers pose a potential threat.

This phenomenon was shown in ZnO [113] and silver (Ag) [120] NPs in which the formation of a PC inhibited NPs dissolution and consequent release of Zn and Ag ions that eventually became toxic for cells. Lastly, the formation of a PC can increase the safety of NPs by inhibiting the generation of radical oxygen species (ROS), by which several compounds exert their cytotoxic activity [121]. Nanoparticle toxicity can be related to the formation of ROS when the particles are composed of materials with semiconductor features [122,123]. In this context, ZnO NPs are again an example of this phenomenon because their pristine surface is a natural trigger of ROS, but the formation of a PC on their surface inhibits this phenomenon [113].

When does the PC increase nanoparticle toxicity?

The adsorption of proteins on the NP surface can induce protein denaturation by decreasing their thermal stability and favoring conformational changes of the adsorbed proteins. For example, titanium dioxide (TiO$_2$) NPs were shown to induce conformational changes of tubulin, thus inhibiting its polymerization properties [124], while gold NPs instead can induce changes in the conformation of serum albumin [125]. It was hypothesized that the cytotoxic mechanism

underlying this phenomenon is the potential increased immunogenicity that the particles can exert by exposing protein epitopes on their surface in an aberrant conformation [34]. For example, it was demonstrated that the presence of poly(acrylic) acid on the surface of gold NPs induced unfolding of adsorbed fibrinogen that, in turn, interacted with the leukocyte receptor MAC-1, thereby triggering an inflammatory response [100]. The surface interaction of proteins at the nanoscale also affects protein fibrillation, which is at the base of neurodegenerative diseases such as Parkinson's and Alzheimer's [126]. This is an important observation, given to the role that was recognized to NPs in inhibiting the process of fibrillation and the consequent progress of the disease [127]. Recently, Mirsadeghi *et al.* found that the PC of gold NPs impacts the fibrillation process in a way that strongly depends on the protein source and concentration [128]. This is another demonstration of the need to review the data obtained without considering the presence of a PC, to better predict *in vivo* results.

Opinions differ as to whether or not the PC can affect cell internalization. However, the formation of a PC can increase internalization specificity, particularly in professional and non professional phagocytic cells such as macrophages and endothelial cells, causing an increase in the risk of cytoplasmic and systemic toxicity. Ag NPs were shown to be specifically internalized through scavenger receptors in endothelial cells, thereby activating intracellular pathways that increased the mRNA expression of the inflammatory cytokine IL-6 [129]. From this perspective, the elements of the innate immune system can exert a completely different action toward the presence of a PC and can lead to activation of the immune response.

Macrophages, considered the main players in the clearance of NPs from tissues, use phagocytosis to internalize NP–PC complexes, while naked NPs can be taken up through other mechanisms (i.e., clathrin- and dynamin-dependent endocytosis) [130]. In evaluating the inflammatory effects of NPs and their PC in monocyte cell lines *in vitro*, it is important to induce monocyte differentiation to a macrophage phenotype. Macrophages displayed a completely different profile of secreted inflammatory cytokines compared with undifferentiated monocytes when treated with PC-coated NPs [130]. Such leukocytes as macrophages, dendritic cells, eosinophils, neutrophils and mast cells express on their surface the Fc receptor, which binds with high-affinity opsonized material through the crystallizable fragment region of the antibodies. In some diseases in which the serum level of antibodies significantly increased, the use of NPs as therapeutic or diagnostic tools can be accompanied by the formation of a PC enriched in these molecules. As a result, the phagocytic process increases as do all the side-effects associated to NPs and their PCs related to the onset of inflammatory response [51]. This process is an excellent example of the complexity of the topic, and indicates that the formation of a PC can induce different toxicological responses in function of the specific cellular phenotype used in the study. A scheme of the pros and cons in terms of cytotoxicity is shown in Figure 6.

Impact of the PC on the immune response

The PC plays a role in cell uptake [131], accumulation, biodegradation and clearance of NPs [132]. It is becom-
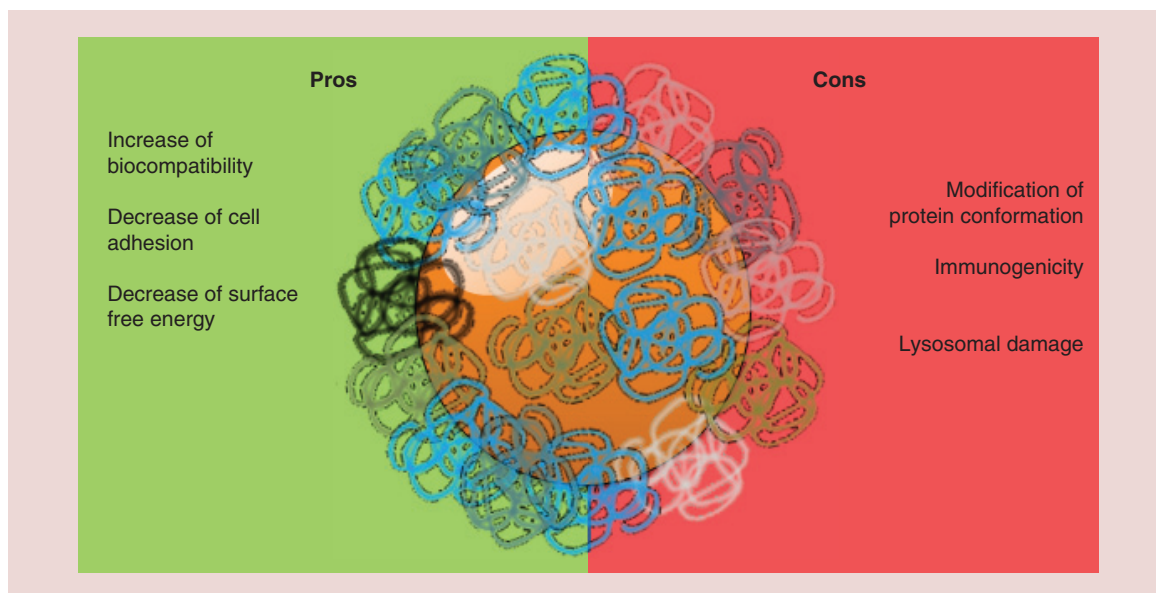

Pros

Increase of biocompatibility

Decrease of cell adhesion

Decrease of surface free energy

Cons

Modification of protein conformation

Immunogenicity

Lysosomal damage

Figure 6. Impact of the protein corona on nanoparticle cytotoxicity. Schematic representation of the pros and cons of the protein corona in terms of cytotoxicity.

ing clear that the PC also mediates and has the ability to alter the immune response. However, investigators are cautious and tend to refute the notion of differential immune stimuli dependence on the NP–PC. The immune system is expected to react to NPs and thus a better understanding of nano-immune interactions is critical for the safe application of engineered NPs in medicine [133]. In this section we discuss recent evidence that supports this meaningful mechanism. Specifically, we focus on the effect of the PC on the immune cell recognition of NPs, complement activation in response to NPs, the adaptive immune response and NP evasion of the reticulo-endothelial system.

The PC induces differential recognition of NPs by immune cells

The human body is equipped with mechanisms that protect the host against foreign entities. From a biological and physical point of view, injected NPs could be considered one of the potential body 'invaders' just like viruses or bacteria. Standing at the interface, the PC has a major impact on the interaction between immune cells and injected NPs. An example of this was provided by Yan *et al.* [134] who demonstrated the selective cell recognition for PC formation on poly-(methacrylic acid) nanoporous polymer particles. The levels of internalization of NPs in monocytes and macrophage-like cells differed significantly depending on the presence/absence of a PC (Figure 7). Compared to the uptake of bare NPs, NP uptake by monocytes (THP-1) was strongly inhibited by the presence of serum albumin. This phenomenon is most likely due to the conformational changes

of the albumin once adsorbed on the NPs. On the contrary, in the case of macrophage-like cells (differentiated THP-1), the presence of albumin on the NP surface induced an increase in the internalization rate accompanied by secretion of inflammatory cytokines as proof of the activated phagocytosis [134,135].

Complement activation by NP–PC complexes

The complement system identifies and induces the elimination process of nonself entities [136]. As expected, complement proteins were shown to be involved in the opsonization process and uptake of NPs [137,138]. While the complement can be activated through three pathways: classical, lectin, or alternative; NP–PC complexes seem to initiate the classical pathway [139].

Moreover, complement proteins that bind to NPs may lead to formation of a PC which implies activation of complement processes that eventually cause inflammation [140]. NPs may be designed to avoid such immune toxicity, and so improve their safety. *In vitro* experiments revealed that the physicochemical surface properties of NPs could mediate PC formation, and thus control the activation of complement pathways [141–143]. For example, it was shown that changing the surface chemical modification (e.g., methoxyl, carboxyl and amine groups) on polymeric NPs resulted in modulation of complement activation [141]. Another study demonstrated differential complement activation of gold NPs of different surface hydrophobicity by evaluating the complement protein (C1) bound to IgG adsorbed on the nanosurface. The levels of complement activation were dramatically lower
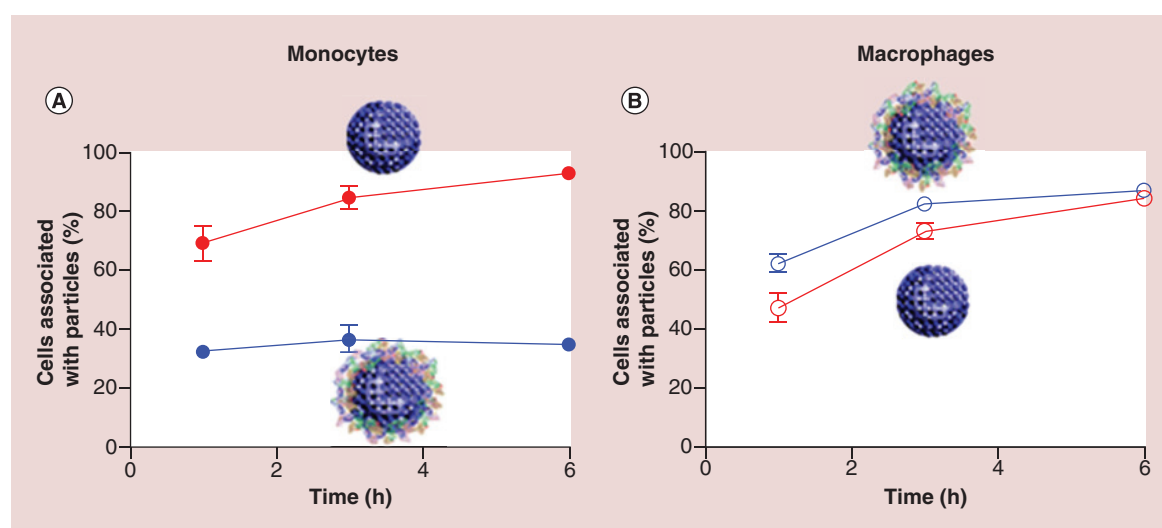

Figure 7. Mechanisms involved in nanoparticle-induced immunomodulation. The protein corona significantly affects nanoparticle–cell interactions (e.g., cell internalization and pathway activation). The different role of protein corona in nanoparticle uptake by monocytes and macrophages is represented.
Reproduced with permission from [134].

when the NP surface was hydrophilic than when it was hydrophobic [142]. Yu *et al.* instead used different conformational states of glycopolymer chains to induce the formation of a PC, which in turn, works as a 'molecular switch' of complement activation [143]. Taken together, these studies show that, because complement activation depends on surface modifications of the foreign body, it is possible to switch the complement cascade on/off through the modulation of the surface properties of NPs, among which, the PC plays a crucial role.

Adaptive immune response induction by NP–PC

The adaptive immune system recognizes NPs because they are clearly 'nonself' and occasionally share biochemical properties with viruses (same biological properties and size range). NPs smaller than 10 nm may elude the immune system, although most remain dispersed at low concentrations. Elevated concentrations often result in particle aggregation and the consequent formation of macrostructures that can no longer be ignored by the immune system.

Moreover, NPs can be designed as adjuvants in order to manipulate the cytokine secretion profile, which will influence T-cell reactions [144]. An adaptive immune response usually occurs in a site that is already compromised, in which an innate immune reaction is ongoing. Such a response can enhance the existing inflammatory process occurring in the site. Adaptive immunity may be directed against the NP itself, even after formation of a PC, particularly if only a 'soft corona' has formed. Moreover, adaptive immunity may recognize nonself proteins, if these proteins have been introduced from outside the body (which is possible if the NPs are not sterile). However, cells of the immune system may never 'see' the naked NP surface, and the question is: do NP–PC complexes present NP-associated molecular patterns to the immune system and induce its further activation? Rationally, the self-PC, when interacting with NPs, can stimulate the adaptive immune process if the complex created represents a 'danger' signal. Adsorbed proteins may undergo secondary and tertiary structure conformational changes, which ultimately lead to adaptive immune responses. The tertiary structure change from the NP–PC formation results in protein aggregation and induces the immunogenic potential of these self-proteins [145,146]. Candace *et al.* [72] characterized the changes in protein secondary structure that result from adsorption of serum albumin on NP surfaces. Such changes could initiate immune processes. For example, the PC has been reported to affect cell signaling involving cytokine production by activation of receptors, that in turn activate a signaling pathway (e.g., NF-kB), thus resulting in the secretion of inflammatory cytokines [100,147].

Evading the immune system

In order to prolong NPs circulation time and avoid their early recognition from the immune system and the organs of the mononuclear phagocyte system (MPS), 'stealth' NPs were developed by coating them with the hydrophilic polymer PEG. This procedure creates a hydrodynamic radius around the surface of NPs, which is efficient in reducing the overall amount of serum adsorbed proteins, thus reducing macrophage-driven clearance, but does not affect their qualitative composition [148,149]. Furthermore, PEG plays a critical role in triggering inflammatory and immunogenic phenomena [51].

As for the active targeting approaches, the biomimicry concept was applied also for MPS escape. Various cellular membrane markers have been identified to confer immune surveillance properties to the cells. Among them, CD47, also known as integrin-associated protein, is widely expressed in human cells and serves as marker-of-self on red blood [150]. Rodriguez *et al.*, for example, inhibited macrophage uptake of their NPs by conjugating a 'stealth' self-peptide derived from human CD47 to the NP surface [151]. Using a different approach, Ashley *et al.* designed the 'protocells' by coating mesoporous silica NPs with a lipid bilayer that mimics the plasma membrane. This coating enabled the incorporation in the bilayer of molecules important for the cell-like activity of these NPs (e.g., fusogenic peptides for the endosomal escape) [152]. Rather than using synthetic membranes, several groups recently began exploring strategies to obtain biological membranes from cells and using them to coat NP surface. We refer to these types of NPs (functionalized or made by cell membranes that provide them with cell-like functions) as 'bio-inspired NPs' [153,154] (Figure 8).

For example, red blood cell membranes have been used to coat NPs as well as to generate nanovesicles with liposome-like features in terms of drug loading and release capabilities [154,158]. Hu *et al.* demonstrated that red blood cell molecules were correctly transferred and oriented on the NPs surfaces [159]. Other bio-inspired NPs whose surface is functionalized through the integration of cell membranes, have been developed [153,156–157,160]. Among these, we used membrane proteins purified from leukocytes [161] to obtain the Leuko-like Vector (i.e., a nanopourous silica NP coated with leukocyte membranes). These vectors replicate white blood cell features showing a prolonged circulation time in the bloodstream and a natural tropism for the inflamed endothelium [156].

Using another biomimetic approach, Aimanianda *et al.* conjugated hydrophobin to the surface of porous silicon NPs. Hydrophobin is a surface protein that renders the spores of the human fungal pathogen *Aspergillus fumigatus* invisible to immune cells [162].
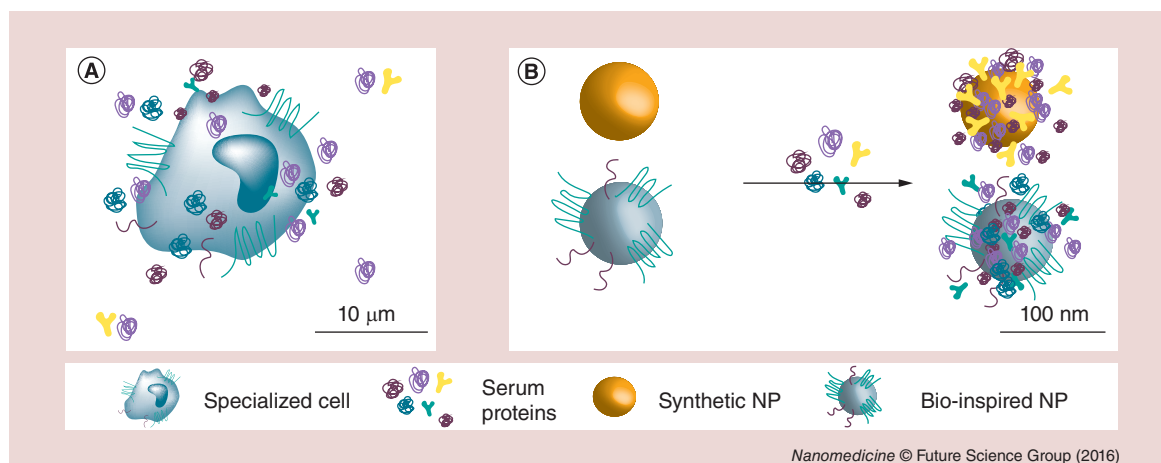
Figure 8. Protein corona of bio-inspired nanoparticles. The transfer of cellular membrane properties to synthetic NPs led to the development of the so-called biomimetic strategies. Specialized cell types **(A)**, such as red blood cells [155], leukocytes [156] or platelets [157], inspired the development of particles that share a similar cellular membrane composition. The resulting vectors, defined as 'bio-inspired NPs', are recognized as self by the immune system. This could depend on the protein corona (PC) adsorbed on their surface. To date, no studies have been performed in order to describe the PC of bio-inspired NPs. **(B)** We hypothesize that while a synthetic NP is mainly recognized as nonself and adsorbs many serum proteins; bio-inspired NPs (reported to possess cell-like capabilities [155–157]) absorb a specific PC similar to the PC of the cells used as membrane source for the NP synthesis. This confers self-like properties to the bio-inspired NPs and mediates their biological activity. NP: Nanoparticle.

The modified NPs displayed a significant change in the degree and composition of plasma protein adsorption *in vitro*. Moreover, when the bare NPs were injected intravenously, their accumulation was higher in spleen than in the liver (ratio of 2:1). However, hydrophobin coating changed the ratio of NP uptake by the MPS organs and resulted in an opposite trend of accumulation [163].

Again, the aforementioned examples underline how the chemical modifications of NPs surface affect their biological fate. As the PC represents the new bio-interface between NPs and biological fluids, then it is of great importance the understanding of the key players that regulate particle recognition and their mechanisms. While there are several studies about the PC-mediated clearance of conventional NPs, to the best of our knowledge, there are no studies related to the PC of such type of bio-inspired NPs.

If the biomimetic coating improved the biological performances of such NPs, one could speculate that the PC actively mediates the recognition of surface markers and, eventually, 'communicates' their presence as 'self' to the cells responsible for 'nonself' clearance.

In Figure 8, we illustrate a potential mechanism that governs the PC formation for bio-inspired NPs. Synthetic NPs react with serum proteins according to the mechanisms described in this article. Bio-inspired NPs attract serum proteins according to their physicochemical properties (as synthetic NPs). On the other hand, they further discriminate among the serum proteins depending on

the affinity of the latter to the biomimetic coating. The result is that bio-inspired NPs may absorb a PC similar to the one that the original cells present. This confers self-like properties to bio-inspired NPs, thus mediating their biological activity. Therefore, it is evident how further studies on the interaction between bio-inspired NPs surface and serum proteins are necessary in order to better understand the potentiality of the biomimicry strategies associated with the nanotechnology field. In addition, it will provide insights to the molecular mechanisms of PC formation and its biological relevance.

Conclusion

To reach its target, synthetic NPs enter into contact with a biological environment that confers them a bio-identity (i.e., the PC). During recent years there has been a remarkable increase in the number of studies about the PC (Figure 9). New concepts have emerged in nanotechnology and, as a consequence, the complexity of NP features needs to be addressed in an experimental set up characterized by new steps (Figure 1). Since the PC is the 'effective' particle surface, it interacts with cells and tissues thus affecting the function of NPs, namely targeting, toxicity and escape from the immune response. However, its specific role is still under debate, and discordant results have been reported and discussed in this article. How can these controversial results be explained and is there any way to predict the fate of a NP *in vivo* based on its surface chemistry and, *in vitro* properties?

These questions should be addressed based on three considerations. First, we should determine how the physicochemical properties of NPs (i.e., surface charge and, in particular, their size) differ from each other and affect the type of proteins that will be adsorbed on carrier surface once NPs are immersed into biological fluids. Tailoring these two parameters, for example, can help to include/exclude a class of proteins with respect to another one. Second, in the case of targeted NPs, we should consider that the targeting agents differ from each other in terms of molecular weight, size and charge. They also differ in affinity with their ligand on the target cell surface and in their density on the carrier surface [106]. In comparison to a small molecule (e.g., transferrin), an antibody may be not easily shielded by the PC, thus preserving the targeting ability of the functionalized NPs. Lastly, diverse biological factors, for example, serum concentration, *in vitro* or *in vivo* conditions, as well as murine or human serum, affect the PC composition, thereby altering the biological identity that the particles acquire and, consequently, their biological fate.

Future perspective

We can affirm that the PC concept is almost completely accepted, even if it is too early to claim that we have a clear understanding of its role in nanomedicine. Therefore, the scientific community is starting to reconsider the previously obtained data on NPs features and effects, in the light of the existence of a PC. The following items should be considered for the future studies: i) Continue characterizing PCs, taking into account that the PC is characterized by strong and weak interactions and we still need to understand the role of the latter. In fact, the experimental needs have given a greater space to the studies on the hard corona, but we should start to consider the idea of focusing more attention also on the other side of the coin: the soft corona. ii) Understand how to modulate the formation of the PC. This can be done either by trying to inhibit the formation of a PC through NP surface modification or by taking advantage of the formation of a PC to obtain specific NP properties. The ability to tune the formation of a PC represents an additional value in the design of novel drug delivery platforms. It may open the way to new possibilities in nanomedicine, such as decreasing toxicity and/or improving of cellular uptake and the potential driving of the targeting. More investigations are needed to better understand how the 'real' corona directs the NPs in the body. The intensification of studies that better reproduce the *in vivo* conditions is now crucial. Knowledge of the NP–PC interaction with blood components and the target site would

allow bio-engineers to better design the next generation of drug delivery systems. Furthermore, it has been recently demonstrated that the PC composition strongly changes, if plasma deriving from the blood of patients with different diseases is employed for the incubation of NPs [44,50]. Thus, the new concept of 'personalized protein corona' has been introduced. We believe that, in the next not too distant future, we may expect to see the engineering of patient-specific NPs in a disease type-specific manner for safer clinical applications. Lastly, as discussed above, the lack of information about the PC of bio-inspired NPs is an important challenge that has to still be resolved. The knowledge of the PC of these NPs, synthesized using membranes (lipids and proteins) extracted from live cells (e.g., leukocytes and red blood cells), is fascinating as it could help to better understand the potentiality of the biomimicry strategies in nanomedicine.

Summary

When immersed in biological fluids, the high surface free energy of NPs promotes the absorption of biomolecules thereby forming the PC. The formation of a PC causes changes in the charge, size and surface chemistry of a given NP, thereby impacting on its uptake and cellular fate. An understanding of the interaction between NPs and biological fluids is a prerequisite for the control of this phenomenon and for engineered development of NPs that have a favorable biodistribution and biocompatibility. The composition of the surface protein layer consists mainly of negatively charged proteins, with a molecular weight typically higher than 60 kDa. A subset of about 125 proteins involved in complement activation, pathogen recog-
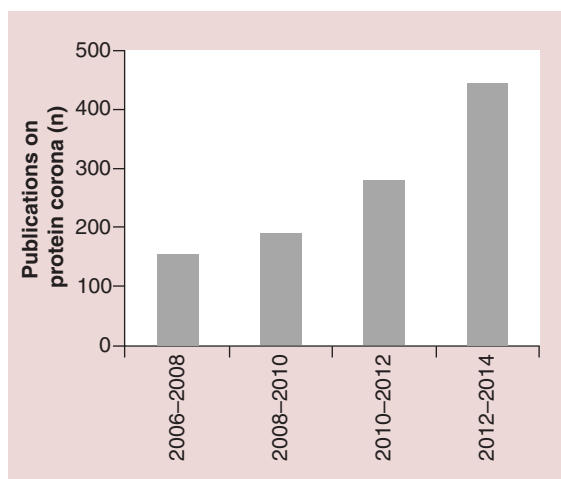

Figure 9. Number of articles devoted to the protein corona published from 2006 to 2014. The number of articles has tripled during the 3 years between 2012–2014 versus 2006–2008.

nition and blood coagulation is commonly adsorbed on nanomaterials. This review is devoted to recent acquisitions regarding the role of the PC in NP functions and in the development of new strategies with which to modulate the PC to more efficiently target specific cells. The impact of the PC on NPs properties is discussed.

Executive summary

Background
- Once injected in a physiological environment, nanoparticles (NPs) interact with biological components and are surrounded by a protein corona (PC).
- The PC can trigger an immune response and affect NP toxicity and targeting capabilities.

Dynamics of PC formation
- Time evolution studies on the composition of the PC have demonstrated for long time that the process of NP–PC complex formation follows the Vroman effect.
- According to the Vroman effect, an initially attached protein can, at any time, desorb from a NP and be replaced by a different one with major affinity. The PC is a highly dynamic over time. Its composition changes, while the amount of adsorbed proteins remains relatively constant.
- Instead, recent findings show that the PC is already established 0.5 min after injection. Its composition remains constant over time, while the amount of adsorbed proteins changes.

PC composition
- The PC is not universal, but is unique for each type of NP.
- The proteins generally identified in the PC are involved in complement activation, blood coagulation and foreign body recognition processes.
- Albumin, apolipoproteins, IgG and fibrinogen, are present in the PC of almost all NPs investigated.
- Proteomic-based approaches are widely used for the characterization of the PC. Among these, label-free MS has several benefits that include a low number of steps in the protocol and high reproducibility.
- The majority of the *in vitro* studies seems to be only partially relevant from a biological point of view because the incubation of NPs inadequately replicates the conditions of an intact biological system.
- Few attempts of *in vivo* experiments are now emerging.

Impact of the PC on the targeting capability of NPs
- The impact of the PC on the targeting capabilities of NPs is still under debate.
- It has been shown that the PC can have a dual role: in some cases, it hides the functionalization molecule on the NP surface, thereby inhibiting the interaction with its specific receptor on the target cells; in other cases, the PC contains proteins that act as ligands for receptors on specific cells.
- NP synthesis can be manipulated to favor the binding of specific proteins in the PC in order to target selected cells.

Toxicological implication of the PC
- Results about the toxicological implication of the PC are discordant.
- It has been demonstrated that the PC is cytotoxic (because it induces conformational changes of the proteins, or causes the NPs to aggregate).
- Unfolded or misfolded proteins lose their functional activity and are associated to various diseases.
- On the other hand the PC may increase the biocompatibility of NPs, especially in the interaction with cell membranes.

How does the NP–PC complex affect the immune response?
- The immune system does not see a 'naked' NP, thanks also to the PC.
- What role does the PC play in the immune system-NP interaction? What impact does the PC have on the immune response when it recognizes an NP? We address these questions by examining the different compartments of the immune system.
- The PC induces differential recognition of NP by immune cells. In addition, the response developed by either innate or adaptive immunity is PC dependent. Finally we discussed how the PC helps NPs evade the immune system.

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