Nanomedicine delivery: does protein corona route to the target or off road?

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The biological identity of the nanoparticles

Nanoparticles (NPs) in the context of biological environments always possess a hybrid nature [1]. Even if an NP itself is, in the simplest case, a completely homogeneous sphere of one material (e.g., metal, metal oxide and polymer, among others), interaction with molecules present in biological liquids such as ions [2] and proteins [3], will lead to modification of the NP surface and thus to the formation of a complex object, which has to be seen as one dynamic entity. From the viewpoint of an NP, adsorbed biomolecules, mainly proteins, on the NP surface form the so-called protein corona (PC). As the outermost part of the NP, the PC represents the NP's interface with the environment and thus significantly determines the biological identity of the NP [4]. Adsorption of proteins (as present in serum) to the surface of colloids, for example, changes the uptake of these NPs by cells [5]. Differences in the original physicochemical properties of the NPs, such as their charge, can be 'smeared-out', as their interface is effectively governed by the adsorbed proteins [6]. While this effect has been known for a long time [7], only recently the importance of the PC around NPs has been fully appreciated and detailed systematic studies have been performed [8]. From the viewpoint of a protein, adsorption to NPs leads to the formation of PCs [9–12], bringing together many proteins on the surface of one carrier NP. Such adsorption and agglomeration effects can significantly alter the function of proteins [13].

In a comprehensive picture one could predict which proteins are present on the surface of a NP after its exposure to a mixture of proteins. While some dependence of the physicochemical properties of the NPs on protein adsorption has been understood, a complete understanding of its formation and evolution has still not been achieved. One problem in this direction is that the adsorption kinetics of the PC is highly complex, due to the composition heterogeneity of the biological fluids. This adsorption is characterized, particularly during the initial stages, by a continuous dynamic exchange of proteins from solution to the NP surface and *vice versa*. This is known for planar surfaces as Vroman effect. While first most abundant proteins

reach the NP surface and are adsorbed, those are later replaced by less motile proteins with higher binding affinity. Human serum albumin originally adsorbed to the NP surface is, for example, replaced by fibrinogen in a dynamic competition reaction [14,15]. One important parameter that determines which proteins will be bound to the NP surface under equilibrium conditions is given by the apparent binding constant K_d (Figure 1A) [16]. Values for K_d have been experimentally determined for a variety of different NP coatings and proteins [17–19]. While the PC of inorganic nanomaterials have been extensively studied, the PC of self-assembled soft materials such as micelles, liposomes and polymers, among others, have not been deeply studied yet and only few examples are available in the literature [20,21].

One problem toward a better fundamental understanding of the PC lies in finding adequate measurement techniques. One has to distinguish between techniques that can be performed *in situ*, and others that require extraction of the NPs from the biological environment before measurements. The most straightforward approach used in the literature is based on quantitative mass spectroscopy and gel electrophoresis analysis, which allow identification of the proteins attached to NPs. However, in this case, excess proteins free in solution have to be removed before measurements, as the techniques do not distinguish between free and adsorbed proteins. Separation of free proteins can be done using filtration, precipitation or magnetic separation, for example. However, due to the dynamic nature of the PC, upon each washing step some originally adsorbed proteins will start to desorb thus modifying the original PC [16]. Therefore, such measurements requiring extraction and purification steps ultimately do not determine the original PC before extraction/purification. On the other hand, mass spectroscopy and gel electrophoresis allow for distinguishing between all the different adsorbed proteins and thus for the detailed analysis of the composition of the PC [8,23].

In situ techniques allow direct measurement of the PC in the original environment. However, in con-

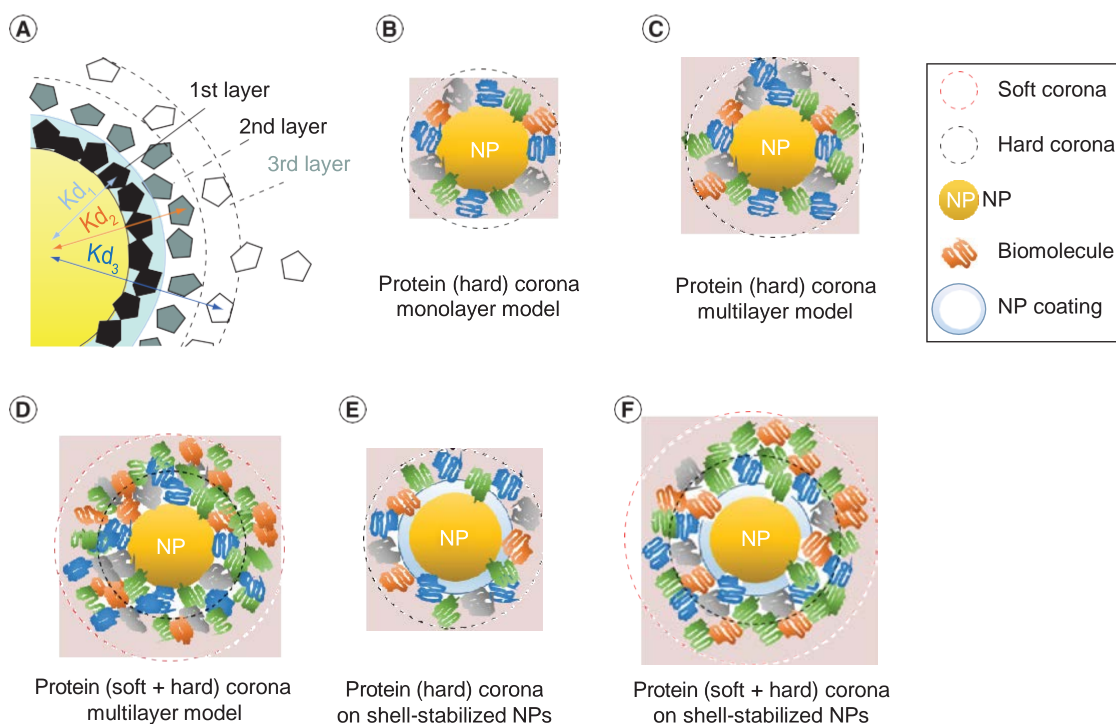

Figure 1. Examples for models of formation of the protein corona. **(A)** Scheme reporting a multilayered organization of the protein corona (PC). The binding of the different proteins to the NP surface is characterized by specific dissociation constants (K_d). Note that the possibility of the formation of multiple layers is still under discussion in the literature. **(B–D)** Illustration of some of the different models accounting for the formation of hard and soft PCs on NPs. **(E–F)** Model explaining the formation of respectively hard and soft PCs on coated NPs. Notably protein adsorption can be accompanied by polymer desorption or degradation due to presence of proteolytic enzymes in the biological fluids.
NP: Nanoparticle.
(A) Adapted with permission from [22].

trast to mass spectroscopy and gel electrophoresis as described before, current *in situ* techniques do not generally consent for a composition analysis of the PC. One type of *in situ* measurements is based on measuring changes in hydrodynamic diameter of the NPs. The more proteins that are adsorbed, the bigger the PC–NP complex becomes, which can be conveniently measured by fluorescence correlation spectroscopy, for example [17,22]. This analysis based on size measurements, however, does not permit one to tell which proteins have been adsorbed, and thus it is limited for model studies in which only selected types of proteins are present. Alternatively, one can directly detect adsorbed proteins when they change a readout signal as compared with proteins free in solution. One technique in this direction is surface-enhanced Raman scattering, in which only proteins adsorbed to the surface of plasmonic NPs provide sufficient signal to be detected [24,25]. Other spectroscopy tech- niques such as circular dichroism are less suited, as they allow for distinguishing between adsorbed and free proteins only in the case that proteins undergo conformational changes upon adsorption to the surface of NPs [26].

Due to such experimental limits our fundamental understanding of the PC is still limited. One example in this direction is, besides the question of the PC composition, also the question of the PC structure. While in some studies formation of a protein monolayer around NPs is claimed, mostly for single protein coatings, (Figure 1B) [17], other studies report a multiple layer model (Figure 1C) composed of a 'hard' PC directly attached to the NP surrounded by a diffuse outer layer of a 'soft' PC (Figure 1C) [22,27]. Unfortunately, experimentally addressing and unraveling this question is not straightforward, which is the reason why no uniform 'picture' yet exists in the literature. The problem starts with describing the NPs themselves. Completely uncovered NPs – particularly if solely made of hard materials such as metals, metal oxides or metal alloys – have been rarely employed for biomedical applications, in particular due to issues of colloidal stability. In fact, in order to apply these NPs they are usually stabilized by polymers and surfactants or conjugated to biomolecules. As such an organic shell around the NPs is soft and partly flexible, there is no longer a well-defined NP surface, which could be described by the smooth surface of a sphere. In this more complicated scenario it is difficult to ascertain if the PC is bound to the NP surface as a monolayer or as a multilayer, simply due to the fact that it is not straightforward to define where the NP surface ends. Moreover, protein binding is often accompanied by a partial degradation (Figure 1D & E) or desorption of the stabilizing shell [28], or, as in the case of NPs stabilized by PEG, proteins can interpose between different PEG chains [29]. The overall picture is also complicated by the discussed presence of a 'soft' PC that can play a role in the *in vivo* NP fate. 'Soft' adsorbed proteins are characterized by a high exchange rate with their free counterparts in solution [30], but they can still cover important functions in biological processes [31]. However, due to the high exchange rate and the low binding affinity it is quite complicated to experimentally detect this 'soft' corona. Most detection techniques are insensitive to loosely attached proteins, in particular in cases when purification steps are required. Even if less studied, due to the lack of suited analytical techniques for their characterization, soft PCs probably play an important function due to their more dynamic nature during the *in vivo* PC evolution. For example, a recent study shows the importance of protein glycosylation/deglycosylation in determining PC biological activity. Here the authors demonstrated how the transient binding of deglycosylating enzymes (soft interaction) to the 'hard' PC increases NP association to macrophage by removing sugar motifs on the hard PC [32]. Although this is a specific case and formation of soft PCs on preformed hard PCs should be evaluated case-by-case, it is clear that the evolution of *in vivo* soft coronas could have biological unscreened implications.

The development of a general PC model is also hampered by the fact that the dispersion of NPs in the biological environment generally may cause the formation of different populations of NP–PC complexes such as monomers and multimers (see Figure 2). These different NP–PC complexes are diverse in terms of structure and composition potentially leading to alternative biological outcomes. Thus, resolving PC structure and composition of these co-existing NP–PC complexes can help to better understand and predict the biological responses to NP *in vivo* administration [33]. Recently, ultracentrifugation in sucrose gradient has been shown to permit high-resolution separation and recovery of these PC-NP complexes from different biological fluids allowing the investigation of their biological response in a systematic way [34]. Overall, the generally accepted model of PC derives from studies in human plasma in 'test tubes' that represent a much simpler scenario than that *in vivo*. In this regard, a very recent paper reported that the *in vivo* biomolecule corona of blood circulating targeted PEGylated liposomes resulted in different morphology and composition with respect to the cognate one *in vitro*. Reassuringly, despite these differences, both coronas gave the same biological responses, in other words, restricted cellular internalization and compromised targeting [35].

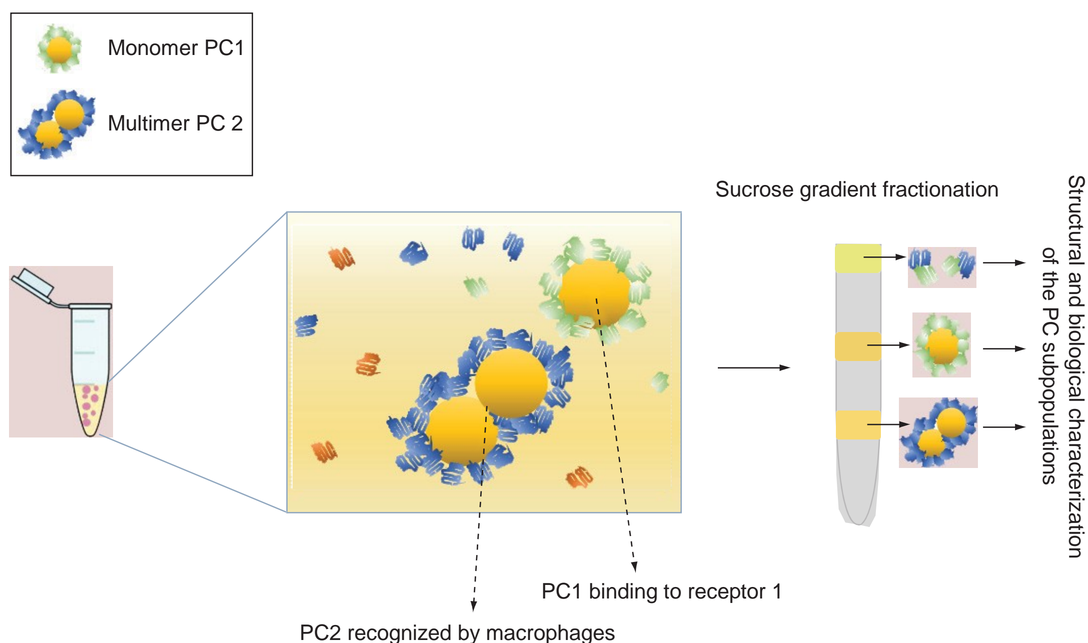

Figure 2. Separation of protein corona complexes. Nanoparticles when dispersed in a biological fluid can form mixed populations of monomers and multimers of PCs. The different subpopulations can lead to different biological outcomes. A potential approach to separate and analyse each of the subpopulation, based on sucrose gradient fractionation is presented in the main text.
PC: Protein corona.

Overall, to keep the discussion more fluid, we will refer to the complex biomolecule corona (see Figure 1F) around the NPs as PC without distinguishing between hard and soft.

Evolution of PCs within the body

Having pointed out the PC as biological identity of the NPs in a biological environment, it is clear that understanding its evolution and exchange processes *in vivo* becomes crucial to produce effective and non-toxic nanotherapeutics [36]. *In vitro* experiments have shown that the formation of the PC influences cellular uptake of NPs, which results also to be dependent on the nature of the adsorbed proteins [37].

To elucidate the fate of the PC during the delivery of the NPs *in vivo* is a key determinant for developing efficient nanotherapeutics. The complexity of the biological environment can strongly alter the composition of the PCs during their systemic journey into the body with possible biological implications (Figure 3) [38]. It is difficult to keep the discussion general as the specific fate of the NPs mostly depends on the chosen administration route (i.e., inhalation, topical and systemic, among others). For example, an NP formulation intravenously (iv.) injected will incur the following biological processes: vascular transport (blood), extravasation, interstitial passage (extra-cellular matrix), cellular uptake and clearance. Thus, engineered NPs and more realistically, cognate PCs, will need to overcome different biological barriers before reaching their final destination. As we explained in section 1, the formation kinetics of the PC is dependent on NP size, shape, surface functionalization and concentration.

However, it is reasonable to predict, keeping invariant the biological environment, a general trend in which PCs form almost immediately after contact with the biological milieu. So-formed PCs will then mature in approximately 10 min with small rearrangements in the following 1–2 h [11,40]. It is also envisaged that PCs will likely be subject to modifications in their composition during the journey into the body when they encounter different biological environments, as recently highlighted by Chan and co-workers [39] for NPs conceived to target tumors (see Figure 3A & B). Also post-translational modifications of the biomolecule PC such as glycosylation/deglycosylation, phosphorylation, alkylation, ubiquitination, sumoylation, can lead to different biological responses driven by a specific PC, as recently demonstrated in the case of PC deglycosylation [32]. Interestingly, change in protein glycosylation by enzymes produced by tumor cells can modulate immune response and tumor invasive behavior [41]. Thus, specific mechanisms triggered by the formation of the PC in specific biological microenvironments can induce *in vivo* PC modifications affecting the expected biological function of the injected NPs.

A preliminary study on silica NPs also demonstrated that PCs formed in serum and then transferred into the cytosol experienced composition changes retaining a sort of fingerprint of their history [42]. Bertoli *et al.* investigated the intracellular fate of silica-coated magnetite NPs by recovering NP-containing cellular organelles by magnetic separation. Again these studies showed that PCs associated to NPs extracted by different cellular compartments, although enriched with additional proteins, still retained a plasmatic fingerprint [43]. Thus, as mentioned in section 1, for experimental determination of the PC *in vitro/in vivo* extraction steps are possible, which by themselves may change the PC. Results need to be interpreted in this context. Nevertheless, the endurance of specific protein features in the PC from the different biological compartments encountered by the NPs could allow for a sort of reconstruction of the NP history in the body. Obviously, the extent of these rearrangements will be related to the NP surface functionalization as well as to the nature of the encountered biological environments. For instance, engineered NPs designed with antifouling features might be characterized by weaker bound PCs and in this way be subjected to more extensive exchanges during their *in vivo* journey.

Considering the *in vivo* journey of an injected NP formulation mentioned above and recalling that the exposed general concepts can be extended to the different routes just considering the specific biological environments encountered by the NPs [44], the so-formed PCs in the blood can be recognized and quickly removed by the mononuclear phagocyte system (MPS, constituted of monocytes, macrophages and dendritic cells) with further accumulation of NPs in the liver and spleen. Alarmingly, this accumulation can often be several magnitudes higher than NP accumulation in the desired region of interest (ROI) [45,46]. In fact, the recognition and removal by the MPS depends on the nature of the adsorbed proteins on the surface of the NPs (opsonization). To avoid this process and prolong the circulation time of the NPs in the bloodstream, many different chemical strategies have been developed in the last decades [47], which will be explained in the following section. If opsonization is reduced, theoretically NPs have sufficient time to cross the endothelial barrier to enter the extravascular space within the ROI. This can be done by passive targeting, in which the process is promoted by setting the correct size, shape and chemistry of the NPs depending on the chosen ROI. For instance, NPs designed to reach tumors

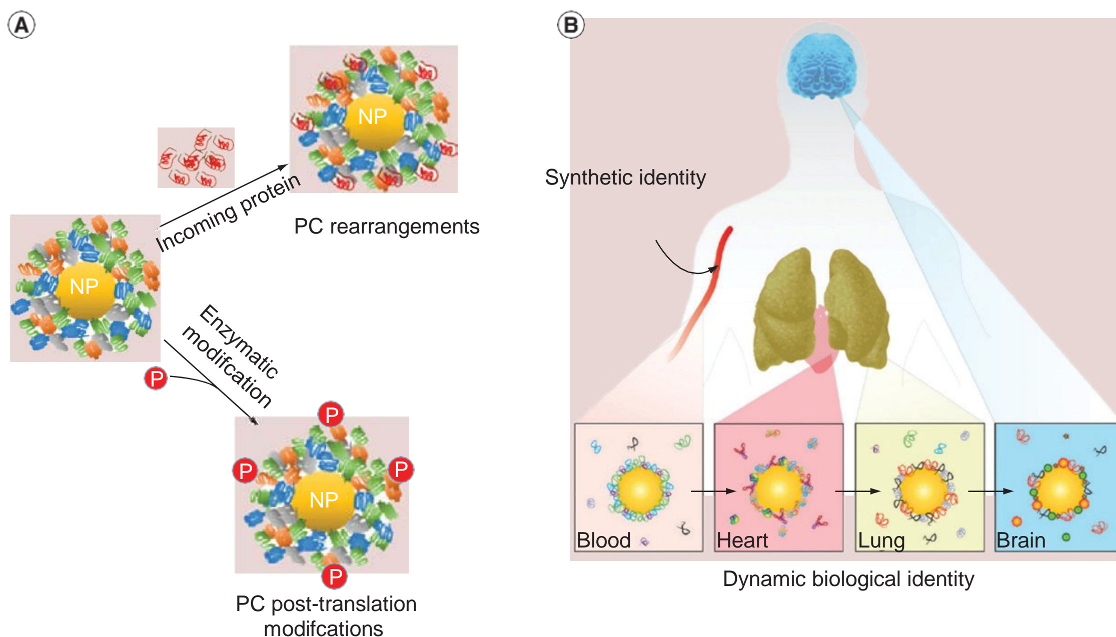

Figure 3. Evolution of the protein corona nanoparticles in the body. **(A)** PC can evolve in response to a new chemical/biological environment following different pathways: secondary binding of protein or PC replacement, or through enzymatic modification (depicted with a red P) of the primary PC binders. **(B)** Schematic drawing showing the same nanoparticles (NPs) in different *in vivo* environments. PC evolution can lead to a different NP fate. The PC associated to the NPs will be subject to a dynamic environment that will depend on the site of administration.
PC: Protein corona.

can exploit the Enhanced Permeability and Retention effect [48], which takes advantage of the abnormal vascular organization of tumor tissues (lesions bigger than 100 mm^3) with consequent accumulation of nano-sized objects of 20–100 nm size compared with healthy tissues [49]. Extravascular transport at the ROI can be enhanced by active targeting through the addition of ligands on the NP surface that can specifically bind the endothelia of the desired biological substrate [50]. Active targeting to solid tumors, for example, might have a more dramatic effect after extravasation, thus targeting ligands specific to cancer cells might be preferred with respect to tumor vasculature [51].

During extravasation the PC might be retained, but once the NPs reach the extra-cellular matrix, (i.e., the skeleton guaranteeing the high-order organization of cells within a tissue, formed by laminins, fibronectins, proteases, proteoglycans and collagens, among others, to cite a few), it is envisaged that major exchanges and rearrangements in the PC take place. Recently, Albanese *et al.* showed a 'conditioning effect' on the PC exerted by the secreted biomolecules in the extracellular environment in cell culture media. A sort of PC fingerprint deriving from serum was kept, but changes in terms of protein composition and PC structure were observed according to NP size, surface chemistry and cell phenotype upon incubation with the cells [52]. These changes were generally related to an enhancement of cellular uptake and retention of NPs, indicating that dynamic extracellular milieus can alter the biological identity of the NP and so NP-cell interactions.

Finally, a key-step in the successful delivery of the NPs, or better cognate PCs, to the ROI is their ability to preferentially be taken up by the desired cell type. The most exploited strategy has been to functionalize the NP surface with ligands for markers over-expressed on the surface of targeted cells. The formation of a PC can also alter the ability of the targeted NPs by shielding the ligand from the interaction with the marker, thus *in vitro* prescreenings of this interaction could strongly help for finding the optimal chemistry to obtain best targeting conditions in the biological environment [53]. Clearly, this will not automatically guarantee that the targeting will work *in vivo*, as the dynamic nature of the PC makes the scenario more complex, as already mentioned [53]. In addition enzymes may digest part of the active targeting moieties [28]. Targeting aspects related to the presence of a PC will be extensively described in the following sections.

Overall, the preliminary results performed on the evolution of PCs showed that dynamic biological environments encountered by the NPs on their *in vivo* journey likely alter their biological identity with respect to both their pristine surfaces and infancy PCs (i.e., for intravenously injection the blood-borne PC) in a so far not completely understood way. Interestingly, humans with different diseases may be injected with nanotherapeutics with different plasma PCs, with a possible impact on their biodistribution as well as on the efficacy of the administered treatment [54]. Thus, formation and study of personalized PCs should be carefully assessed case-by-case to optimize the selected biomedical treatments. The actuation of this would help to adapt a therapy for a specific disease also for a particular patient.

Thus, we envisage that systematic studies on the PC of engineered NPs should become routine tests in the standard protocol for developing nanoformulations for clinical use (as suggested in the scheme reported in Figure 4). Still, the clinical relevance needs to be tested.

Drawbacks from the PC formation

Reflecting about the PC formation from the point of view of the NP synthetic identity, one could probably consider it as an undesired effect bringing more problems than solutions. However, in the last couple of years, it is turning out that the PC has both *bad* and *good* sides (see Figure 3 & Table 1). As already said, the PC will be specific for different type of NPs and often the formation of PCs induces the self-assembly of agglomerates of different composition and structure with a drastic change of the NP surface properties and an increase of the averaged size of the nano-objects interacting with the cellular machinery.

NP size is considered critical in predicting NP biodistribution [65], in particular NPs with size >100 nm are easily recognized by the MPS as well as phagocytized by splenic macrophages and hepatic Kupffer cells [86], while NPs with size <10 nm are cleared by kidney glomerular filtration [71]. Thus, intermediated sizes between 10 and 100 nm seem favorable for long half-life circulating time of spherical NPs in the bloodstream, even if the final fate is heavily dictated by the composition of the associated PC. In contrast with this observation, it has been described that polymeric stealth filomicelles with length in the micro range are able to avoid phagocyte recognition and can circulate longer (up to 6 days) in the bloodstream of the injected mice [87]. This observation motivates the need to characterize more PC as well as the pharmacokinetics of soft nanomaterials. Mostly, a better understanding of the PC of anisotropic NPs lacks, in particular in bloodstream mimicking conditions. In fact, to date most of the published works focus on the escape of spherical NPs from the adsorption of specific proteins, called opsonins, which once adsorbed on the NP surface favor NP clearance by the RES. Chemical strategies aiming to increase plasma half-life have been proposed as possibilities to functionalize the

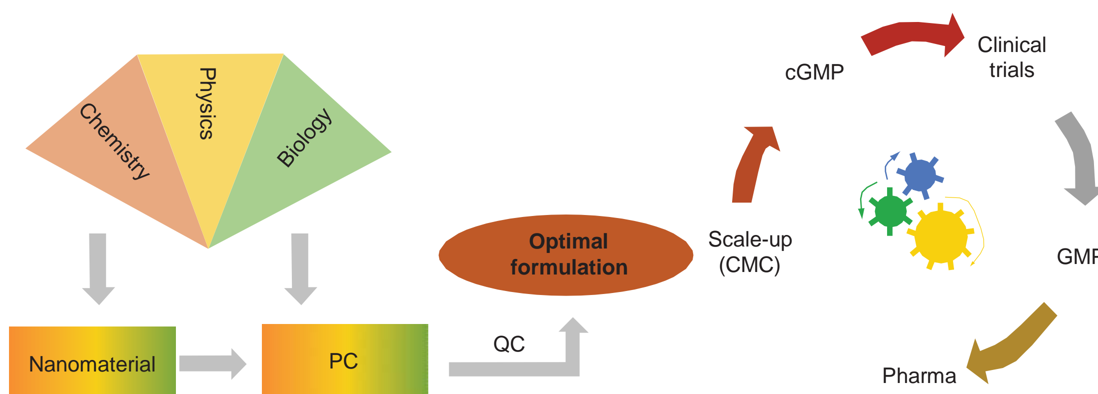

Figure 4. From the bench to the bedside path for nanotherapeutics. Schematic drawing indicating the optimal protocol for developing successful nanomedicines. The scheme highlights the need to make quality control procedures (QC) not only on the pristine nanoparticles but also on the cognate PC (protein corona). Once nanomedicines have been optimized, the resulting formulations can enter in the path to reach the market through the following steps: scaling-up (CMC, chemistry, manufacture and controls), cGMP (clinical trial-Good Manufacturing Practice), clinical trials, GMP (Good Manufacturing Practice for industrial production) to finally reach the Pharma market.

surface of NPs with a variety of different molecules. As examples, hydrophilic oligomeric or polymeric ethylene glycol units (PEGylation) [88], zwitterionic low molecular weight [89] and polymeric coatings [90] have been extensively used as stealth materials [91]. More recently, Welsher *et al.* proposed a hard silica shell, further modified with charged silane-reactive species, as NP coating resistant to nonspecific protein adsorption [92,93]. Ideally, iv. administered NPs should not aggregate in the blood, resist MPS detection, have prolonged circulation times and should selectively target the desired tissue. However, the real scenario is far from being like this depiction. The picture that is emerging is that the current strategies aimed to physically limit PC formation, when challenged by a real biological environment, fail in their role. As a consequence the PC in most cases hijacks the NPs from their ultimate target in the body sometimes leading to their sequestration by professional phagocytes.

For the same reason, obtaining an efficient NP targeting via (bio)molecular engineering [53] through conjugation of the NP with 'biochemical zip codes' [94] is not trivial. Indeed, grafted targeting elements on the NP surface very often lead to unpredictable outcomes [95], increasing the manufacturing costs of the potential nanoformulations, thus reducing their impact on the market. Said that, there are already some examples of targeted NPs in clinical Phase II indicating that for the treatment of untreatable diseases the companies might be prepared to make big investments when the technology seems to work [96,97].

At the same time the paradigm of controlling targeting by tuning the physicochemical properties of the NPs and the corresponding uptake is under examination [98]. In fact, controversial results in literature showed that, unless high-throughput screening is carried out, it is hard to extrapolate general mechanisms able to link the surface features of the NPs to their trafficking from organ to subcellular levels [98,99]. To counteract this drawback different strategies can be envisioned.

The most used strategy is again PEG backfilling to mitigate the negative impact of the PC on NP cell targeting. This strategy is not universally accepted and has to be evaluated case by case. It has been demonstrated that sometimes the thermodynamic shield imposed by PEG itself reduces the active binding of the ligands tethered on the NP surface to the targeted receptor [100]. In addition, recently there is indication about antibody formation against PEG and thus interaction of the immune system also with PEGylated NPs [101]. Besides this classic approach of engineering the NP bio-interface, perhaps a really attractive strategy might be using the organ-on-a- technology [102] to understand the unbalanced effect driven by the PC formation on targeting NPs at a more mechanistic level. Basically, these devices mimic all the characteristics of normal and pathologic tissues at a smaller scale, thus allowing for a rapid validation of the NP-receptor binding. Moreover, organ-on-a-chip systems also allow for growing cells in the presence of conditioned media and extracellular cues with the opportunity to test libraries of different targeted NPs in real systemic condition. This will in turn permit to decrease time and increase efficiency of the screening procedures and understand how evolution of the PC with possible hiding targeting moieties can bring NP off road.

Another important concern about the formation of a PC *in vivo* is the eventual activation of cell pathways

due to unpredictable unfolding of the adsorbed proteins with exposition of novel hidden epitopes in their native state. An elegant study performed by Deng *et al.* showed that fibrinogen unfolds upon adsorption on 5 and 20 nm negatively charged gold NPs which in some cases induces activation of the Mac-1 pathway depending on its conformation, while native fibrinogen does not promote it [67]. The same authors have also reported that different surface functionalized gold NPs, bearing similar PC composition in serum, stimulate a different biological response, indicating that the PC composition is not sufficient to predict the effects of the NPs on the cell machinery as it depends on its molecular and conformational structures [103]. In fact, not always a direct relation between the composition of the PC and biological functions is found. For example, it has been demonstrated that the presence of proteins involved in complement activation and coagulation in the PC does not predict the hematocompatibility of the cognate NPs, but relevant biocompatibility tests need to be run [104]. On the other side, *in vitro* studies on human blood cells have demonstrated that the formation of a PC *per se* reduced toxic effects working as a 'physiological coating' increasing biocompatibility of the engineered NPs [11]. These myriads of data on the different biological response of PCs indicate that, in an era in which the proposal of going toward personalized medicine is increasingly taking place as future perspective, general statements on PC behavior and effects cannot be done but specific experiments on novel nanoformulations have to be performed in relation to their clinical use.

Possible exploitation of the PC

PC is something intimately connected to the existence of the NP itself in a biological environment and results often in hiding the NP's pristine chemical identity.

For these reasons the idea of engineering NPs focusing merely on their synthetic identity has been replaced by the one that looks at the NPs as an ensemble and takes also their biological identity, in other words, the PC, in consideration [4]. Indeed, it is becoming clear that NP-bio interactions in complex fluids are a challenge and need to be tackled with an appropriate holistic approach.

At this purpose, the high affinity and reproducibility of the so-formed PCs [22,23,36] have been revalued and what was perceived as an unspecific and unwanted effect has nowadays sometimes been translated in an advantage and seen as the opportunity to add to the intrinsic features of an NP the extrinsic functions of the PC [105]. Moving in this direction PC exploitation has opened in the last years the path to a myriad of technological applications.

Pioneering studies from Hamad-Schifferli's group demonstrated the possibility to exploit the PC associated with gold nanorods and carbon nanotubes to transport a cargo whose release could be triggered

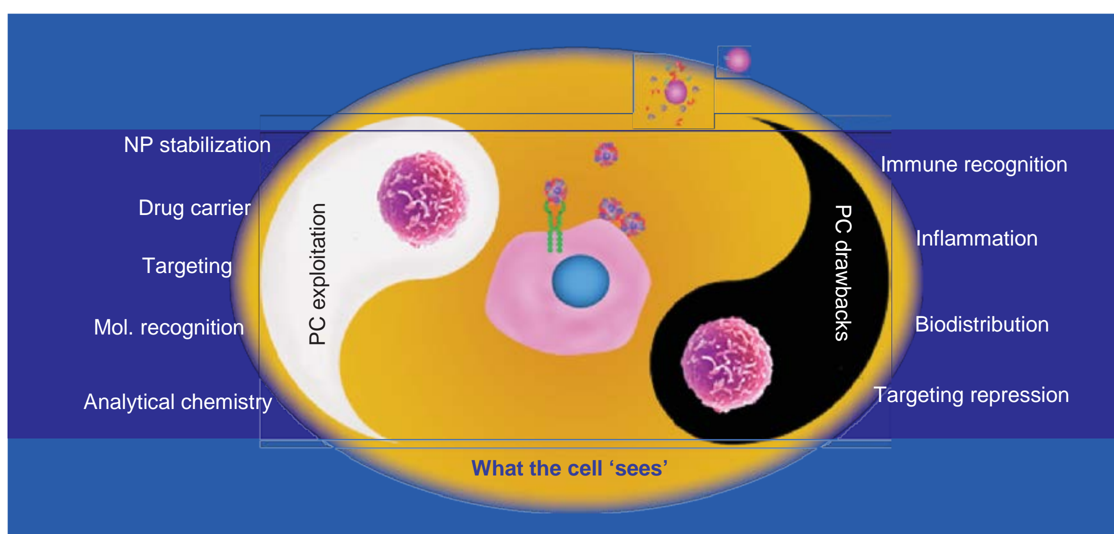

Figure 5. A comprehensive perspective of the possible effects of the protein corona on the nanoparticle interaction with the cell machinery. This image indicates the possible effects that the formation of the PC on the surface of nanoparticles can activate *in vivo*. On the right side, a list of the possible undesired biological outcomes induced by the PC is reported, while on the left side examples of positive uses of the PC in biomedical applications are outlined.
PC: Protein corona.

Table 1. Examples of the opportunities and drawbacks offered by protein corona.

Effects of the protein corona	Ref.
The 'good' side: exploitation of the protein corona	
Identification of new biomarkers	[55]
Cellular component isolation and quantification	[43,56]
Drug carrier and drug release	[57,58]
Molecular recognition and targeting	[59–62]
Biomolecule adsorption	[56,63,64]
The 'bad' side: protein corona drawbacks	
Off target biodistribution	[53,65,66]
Surface-induced protein unfolding	[61,67,68]
Complement activation	[69,70]
Nanoparticle clearance	[71–74]
Inflammation	[67,75]
Avoid protein corona formation: biological and synthetic strategies	
Biological shuttles	[76–78]
PEGylation	[79,80]
Zwitterionic molecules/polymers	[5,81–83]
Biomimicry	[84,85]

using both active and passive mechanisms [57,106,107]. Thus, they showed that it is possible to increase the payload capacity of a NP exploiting the 'sponge effect' of its associated PC. This interesting approach revealed some of the underestimated potentialities of the PC presenting it as a reservoir of a diverse set of biomolecules with different biological functions.

In this perspective another exploitation of the PC can be related to molecular recognition functions as a switch for triggering the release of a PC cargo. Proteins can in fact be seen as molecular switches that can change their structure in response to a molecular recognition event [108] or to changes of the surrounding environment (e.g., pH or ionic strength [109]). These recognition events if appropriately engineered can trigger release or adsorption of selected molecules as sketched in Figure 6, working as a drug release control and/or artificial antibody. Unfortunately, to date our limited understanding of the PC molecular structure *in situ* does not allow a selective control of the secondary adsorption of molecules from the surrounding environment. This is currently limiting the possibilities to have *switchable* PCs able to adsorb/release molecules in a controlled way. A deeper knowledge of the PC molecular structure in terms of protein conformation, position with respect to the NP surface as well as availability to exert their native biological functions when adsorbed in the PC is necessary to transform the approach proposed by Hamad-Schifferli *et al.* in a real exploitation of functional PCs.

On the other side, recent examples of exploitation of the PC can be seen in few bioanalytical applications: going from the capture of protein biomarkers [55], and cellular organelles [43] to determining protein concentration [63], or gauging extracellular vesicles [56]. In these examples, the high NP surface energy is exploited in terms of increased adsorption capability and could be easily applied to evaluate the purity and concentration of other biological entities like membrane coated viruses, or organelles isolated from cell materials.

Another interesting aspect of the PC so far not explored is the possibility, particularly *in vivo*, to bind to nucleic acids secreted by cells. Like exosomes or extracellular vesicles, in other words, vesicles secreted by cells carrying proteins and nucleic acids inherited by the secreting cells, NPs might harvest and concentrate DNAs and RNAs in their corona. The PC was used to entrap DNA *in vitro* [110], but its ability to harvest nucleic acids released by cells in the bloodstream or inside exosomes has to be demonstrated yet. Studies in this direction can be really important considering that nucleic acids are of particular clinical importance for the diagnosis of many pathologies and the evaluation of therapeutic treatments [111]. It is clear that, for example, cancer cells can release free nucleic acids or nucleic acids encased in exosomes that are implicated in the spreading and the growth of tumor cells [112]. The analysis of these molecules and their fractionation is actually difficult and time consuming. Design-

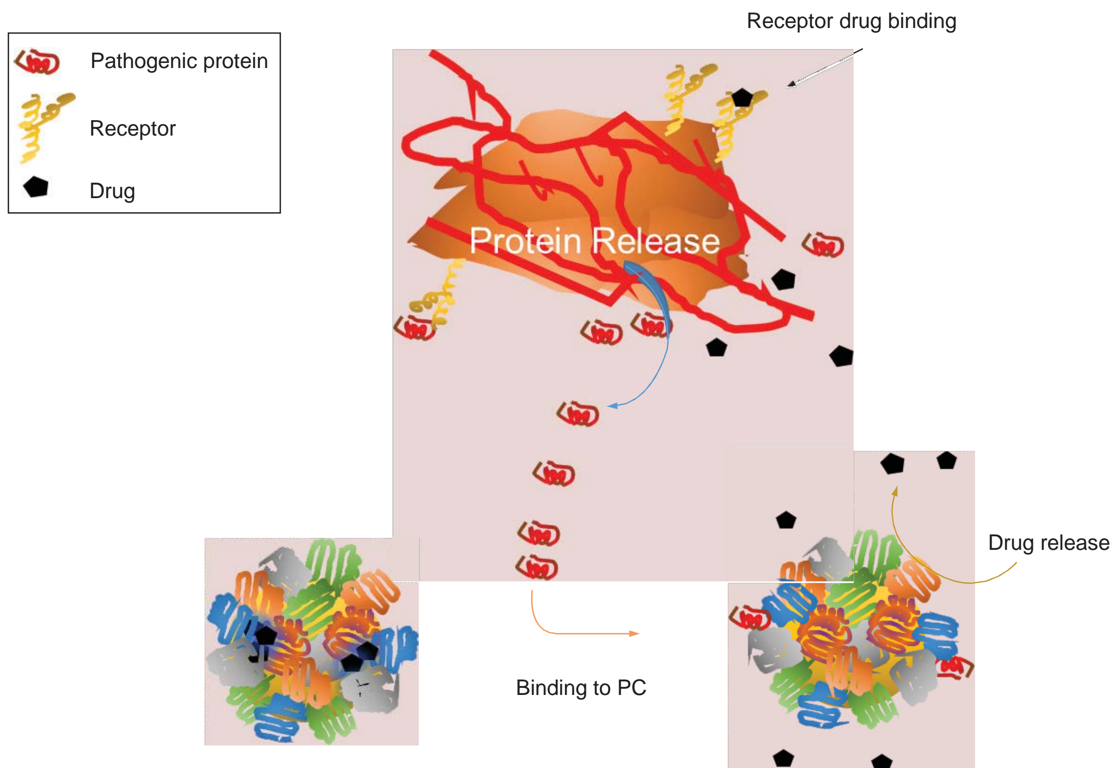

Figure 6. Exploiting the protein corona for triggering drug release. The PC molecular recognition functions can be used to sequester proteins implicated in the onset of pathologies and trigger drug release. On the top of the picture some cell secreting pathogenic proteins are schematically drawn in red. These proteins can be adsorbed or specifically bound by the PC of administrated nanoparticles. A specific interaction between those proteins and the PC can trigger the release of drugs previously encapsulated in the PCs.
PC: Protein corona.

ing a PC having molecules able to interact with such nucleic acids could represent a step forward to their purification (Figure 7).

Another chapter of the PC exploitation story is related to its use for targeting purposes: PCs are naturally endowed with (bio)molecular recognition ele- ments [95]. In this context, pioneering works have been carried out by Hoshino *et al.* who showed the use of polymer imprinted NPs as synthetic scavenger recep- tors for venom bee toxins [59,113]. Targeting was shown not only in *in vitro* but also in *in vivo* conditions [59,113].

The use of the PC for targeting purposes has several advantages with respect to using antibodies or peptides. Natural proteins are less immunogenic than exogenous antibodies or peptides. Moreover, natural proteins have usually a higher association constant than peptides for their cognate receptors [114]. Last but not least, the natural enrichment of the selected PC binders would decrease the production cost of nanotherapeutics. However, the PC intended for targeting delivery has to necessary fulfill some of the biochemical requirements (for instance, high receptor binding, ability to trigger endocytosis, etc.) necessary for a successful targeting delivery that are often neglected in the design of new nanotherapeutics [98].

To date few attempts to design a NP forming a smart PC *in situ* have been shown and most of them are focused on the adsorption of small molecules rather than proteins [62,115,116]. This is due to the difficulties encountered in the control of the adsorption of proteins at the NP interface that actually may be accompanied by unfolding, loss of function and stochastic surface confinement of the PC forming proteins [95]. The few studies demonstrating the potential use of the proteins forming a PC as targeting elements often show that the PC's molecular recognition functions derived from the protein misfolding due the adsorption at the NP surface [61,67]. As a result, PCs are recognized by scavenger receptors or integrins, expressed by cells of the monocytes macrophage system. Other studies instead show that it is possible to control PCs formation to obtain lymph-nodes and cancer cell targeting [117].

The successful delivery of NPs via a targeting corona is strictly related to our knowledge of drug delivery. One can try to develop new targeting PCs taking advantage of the lessons learned by drug anti-

body conjugates [118], expanding this knowledge to nanomedicine.

Engineering the NP surface and consequently controlling the PC formation *in situ* is perhaps the most important and difficult task for obtaining a targeting PC [119]. In the following the requirements to do this will be analyzed in a PC-centric perspective.

First, a PC intended for targeting delivery should be designed to be recognized by a receptor highly overexpressed by a specific cell population and highly recycled at the cell membrane [62,98]. This will in turn decrease off-target effects on nonmalignant cells increasing the therapeutic index of the drugs loaded into the NPs. Attention should be given to the presence, for example, of temporal barriers due to the time of expression of the interested PC binders and the receptors or also to anatomical barriers [98]. In this regard, it is really important to take in consideration different factors, such as the delivery route, the localization of the target cells and the localization of the receptors on the cell surface [84]. In polarized cells, for example, receptors are not homogenously distributed on the whole cell surface (an example is represented by the receptors expressed on the apical surface of an epithelial cell not in contact with capillaries, and connected between them by tight junctions) [120].

It is also essential to consider the ability of the PC to counteract the activity of the proteins in the cellular milieu under physiological and pathological conditions. It is important that selected PC proteins reach a local concentration higher than the one occurring in the cell microenvironment in order to compete successfully with the naturally expressed proteins for receptor binding. Finally, the proteins should be exposed, with epitopes responsible of receptor binding, on the outer layer of the PC in the native state. The interactions between a PC and a receptor are not sufficient to induce an efficient uptake, but the PC needs to trigger the clustering of the receptors at the cell membrane surface, an event responsible for the increased cell uptake [108]. At this purpose a PC with a certain degree of movement could be beneficial and this property is often reached by functionalizing NP surfaces with lipids or other smart stimuli sensitive surface binders [78]. If all these requirements can be satisfied, the interaction and clustering of NP-PC-receptor complexes should be followed by cell uptake and trafficking inside the endosomes. At this point or later in lysosomes it is necessary to have the release of the PC from the receptors in order to avoid the recycling of the NP–PC–receptor complexes and the following exocytosis of the NP from the cell. This in turn, will help to decrease off-target effects improving the therapeutic efficiency and decreasing side-effects of the NP cargo. On the other side, the lack of unbinding could hamper the release of the NPs inside the target cells increasing toxicity due to NP excretion and release of the cargo in the healthy tissues [121].

Finally, the use of biological relevant NPs is also emerging as an important way to deliver cargo to specific regions of the body [76]. Indeed exosomes, ectosomes, microvesicles, high density lipoproteins, lipoproteins and erythrocytes are increasingly becoming more important, not just because they allow for encapsulation of NPs inside their lumen, but also because they are endogenous NPs transporting DNA or siRNA cargos that can be delivered inside specific cells as they are naturally equipped with a set of targeting molecules.

As we have discussed, from the point of view of targeting many results are emerging. We envision that the next generation of PC engineered NPs will bring not just targeting elements but also functional ones. Therefore, a PC bringing targeting elements and immune escape elements would increase the bioavailability of nanotherapeutics. This, accompanied by the explosion of high-throughput techniques [122] will permit to obtain a sort of molecular signature of different pathologies with the potential of revolutionizing

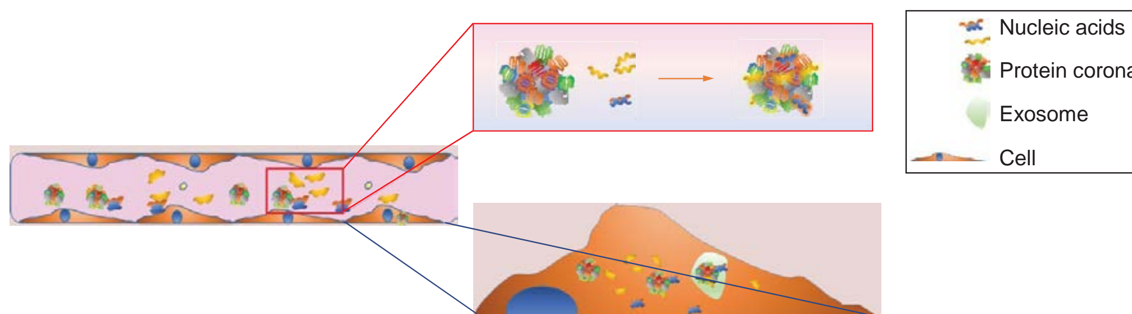

Figure 7. Exploiting protein corona for harvesting nucleic acids. Protein corona can potentially harvest nucleic acids secreted by cells in the blood stream (top of the picture) or through exosomes (bottom of the picture) following the cellular exocytic pathway.

the clinical practices to cure diseases [123]. For going in this direction one however has to get a better understanding of the physics, chemistry and biology behind the NP surface, and to accurately predict PC formation and evolution [124]. The systematic investigation carried out so far has allowed for understanding the basis of the PC formation under laboratory conditions as well as partly *in vitro*. Extending this knowledge to *in vivo* conditions, for example predicting the PC *in situ* in a specific organism district will allow for a better control on its targeting ability. Just after having the full comprehension of the dynamics characterizing the PC formation it will be possible to obtain smart PCs combining different exploiting strategies in a single nanotherapeutic formulation [125].

Conclusion & future perspective

The increasing ability of chemists to engineer novel nanotherapeutics tuning their synthetic identity has produced a plethora of nano-objects of different material, shape, size and surface properties [126]. Particularly, much effort has been dedicated to develop new strategies to endow NPs with specific molecular recognition functions exploitable for *in vivo* targeting. We discussed on how attempted targeting strategies often gave vexing results due to the lack of control over the biological fate of NPs *in vivo* (i.e., presence of a dynamic PC). The lesson that we learned from the PC is that, as it usually happens in a biological context for molecular therapeutics, there is not always an obvious connection between the synthetic identity of a NP and its *in situ* behavior. Thus, engineered NPs for biomedical applications must be characterized in relevant biological environments where they are supposed to function. Therefore, we think that prerequisite for developing efficient nanotherapeutics must be to set standardized protocols for the characterization of the cognate PCs in the development of a nanoformulation. In this regard, the advent of new technologies such as tumor-on-a-chip models and microfluidic systems has provided the possibility to extensively screen NPs properties in environmental conditions very similar to those existing *in situ* allowing a more efficient prediction of their behavior *in vivo*.

In the last decades different challenges have been stimulating nanoscientists and one of these has been PC avoidance in the perspective of both optimizing the targeting ability of the NPs and reducing their escape through MPS. With this contribute we also highlight how recently the nanoscience community has started to see at the PC not only as something to avoid but also as a resource. In this regard, it is sometimes possible to overcome the drawbacks imposed by the PC formation, transforming what has been seen as an unspecific and unwanted effect into an advantage that allows for adding to the intrinsic features of an NP the extrinsic functions of the PC. For example, the formation of the PC has been used to promote controlled drug release, to quantify exosomes as well as to target specific cells. This PC redirection to the desired target is leading to one of the central goal of nanomedicine that is to fill the existing gaps in the prognosis of many untreatable diseases, improving their diagnosis as well as their cure.

Overall, the recent awareness that the biological identity of a NP *in situ* is associated to its PC has brought to unveil many aspects of the NP-cell interactions improving the *in vivo* performance of many nanotherapeutics. In particular, this acquired knowledge has recently revealed that not always the PC routes the NPs off road but it can provide additional properties that make the nanotherapeutics more effective in their function [54].

Executive summary

- The protein corona (PC) is the biological identity of the nanoparticles (NPs) *in vivo*.
- The PC is immediately formed when the NPs enter in contact with the biological fluids, but it can evolve in response to new environments:
  - The molecular structure of the PC is still debated;
  - The impact of the soft moiety of the PC on NPs biological fate is still unclear. Future studies to shed light on these topics are crucial to develop safer and more efficient nanotherapeutics.
- PC formation does not represent only a limit but it can be also an advantage for the development of new nanotherapeutics. PC can potentially be endowed with extrinsic functions giving to the NP new theranostic features. PCs exploitation can contribute to the following processes:
  - Control and tuning payload drug release;
  - Stabilizing nanoparticle;
  - Gauge extracellular vesicles or tumor markers;
  - Improve the molecular recognition functions of nanoparticles.

Conclusion

- PC can be seen to have Janus behavior. Sometimes it routes NPs off road, but it can also provide additional properties that can make nanotherapeutics more effective in their function. More studies on the kinetics of formation and evolution of the PC *in vivo* are needed to effectively exploit its functionalities.

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