

Nanoscopic Agents in a Physiological Environment: The Importance of Understanding Their Characteristics

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Abstract The application of nanotechnology in medicine signifies one of the most exciting developments in science over the last decade. Even though advancement has been made in nanoparticle engineering in terms of size, shape and surface functionalisation, the behaviour in vivo remains poorly characterised and understood. The potential impact of engineered nanomaterials on human health is strictly related to their behaviour in the biological environment. When in contact with biological fluids, nanoparticles spontaneously interact and adsorb proteins to dramatically change their surface properties. Thus, the nanoparticle surface acquires a new biological identity that will influence its stability and interaction with the cellular machinery, thereby affecting the nanoparticle biodistribution in vivo. This protein coating ‘expressed’ at the nanoparticle surface is what is ‘read’ by the cells. Consequently, methods to effectively study the structure and composition of this bio-nano interface have been emerging as key objectives in nanoscience. In this chapter, we discuss the state-of-the-art techniques for the physico-chemical characterisation of nanoparticle-protein complexes in the biological environment with particular emphasis on their impact on the efficiency and safety of a new generation of nanomedicines. We also highlight the barriers faced by nanomedicines for effective targeting and delivery in vivo.

Keywords Engineered nanomaterials, Nanomedicine, Nanoparticles, Nanotoxicology, Protein corona

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Abbreviations

AFM	Atomic force microscopy
AUT	Aminoundecanoic thiol
BAM	<i>N-tert</i> -butylacrylamide
BLM	Bilayer lipid membrane
CD	Circular dichroism
DCS	Differential centrifugal sedimentation
DLS	Dynamic light scattering
ECM	Extracellular matrix
ENM	Engineered nanomaterial
EPR	Enhanced permeability and retention
FCS	Fluorescence correlation spectroscopy
FTIR	Fourier transform infrared spectroscopy
GBM	Glioblastoma
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
i.v.	Intravenous
ICAM-1	Intercellular adhesion molecule-1
<i>ICP-MS/AES</i>	Inductively coupled plasma mass/atomic emission spectroscopy
IFP	Interstitial fluid pressure
k_{off}	Dissociation rate constant
MPS	Mononuclear phagocyte system
MS	Mass spectrometry
NIPAM	N-Isopropylacrylamide
NLS	Nuclear localisation signal
NMR	Nuclear magnetic resonance
NP	Nanoparticle(s)
PC	Protein corona
PEG	Polyethylene glycol
PS	Polystyrene
QCM	Quartz-crystal microbalance

RES	Reticuloendothelial system
SANS	Small angle neutron scattering
SAXS	Small angle X-ray scattering
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SPR	Surface plasmon resonance
SWCNT	Single-walled carbon nanotubes
TEM	Transmission electron microscopy
Tf	Transferrin
TfR	Transferrin receptor

1 Introduction

With the advancement of nanotechnology for a widening spectrum of potential applications comes an ever-increasing production in the number of novel nanomaterials. This has resulted in the increase of nanoparticle (NP) manufacturing in recent years, and by 2020 nanotechnology is forecast to result in the annual production of around 60,000 tons of NPs [1]. Nanotechnology has shown great promise in the area of biomedical science with the development of novel multifunctional nanomaterials possess simultaneous contrast enhancement and drug carrying properties [2]. However, given the application of nanoscaled materials in many different areas such as cosmetics, the food industry, and in high-tech, it becomes crucial to study their interaction with biological substances. The contact of nanomaterials with humans can be intentional (i.e. nanomedicinal application) or unintentional (i.e. environmental exposure), where the latter has been a source of many concerns for unpredictable toxicity. The use of nanomedicines in humans requires further level of confirmation in terms of their safety. However, things are not straightforward due to the lack of proper regulatory safety guidelines for determining the hazard potential of nanoparticles [3]. The increase in number of publications in nanomedicine as well as in nanotoxicology has been exponential in the past decade. However, it is often difficult to attain conclusive results on the toxicity of engineered nanomaterials (ENMs) due to the lack complete understanding of physico-chemical properties [4].

In order to understand the potential impact of ENMs on human health, it is critical to fully characterise them in relevant biological medium [5]. When in contact with biological fluids (serum, plasma, lung lining fluids, etc.), NPs spontaneously interact and adsorb proteins to form what is known as the protein corona (PC), which dramatically changes the surface properties of NPs [6–9]. The NP's surface in a biological milieu is quickly modified by selective adsorption of proteins with the formation of a long-lived biomolecular PC constituting the primary functional interface being processed by the cellular machinery *in vivo* [5, 10]. Interestingly, the PC at the “bio-nano” interface is considerably different from that generated on flat surfaces of the same bulk material under the same experimental conditions [11]. There are various factors that may affect PC formation, such as the physico-chemical properties

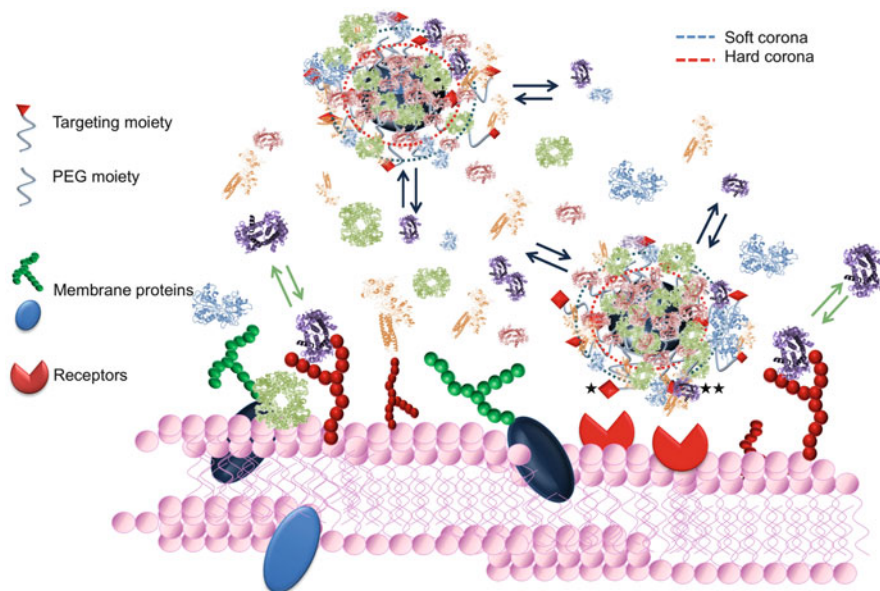


Fig. 1 Schematic drawing of the PC-NPs interacting with the cell membrane; the *red* and the *blue* circles around the NP delimit the hard corona and soft corona protein layers, respectively. The *blue* arrows indicate the dynamic nature of the PC where the adsorbed proteins exchange with the free ones. On the right, the *stars* indicate the NP surface targeting groups interacting with the receptor: (*single star*) the PC does not hinder the targeting group; (*double star*) PC hampers the binding to the receptor

of the NP, the composition of the biological fluid and the time of exposure [12]. The generally accepted view is that the PC is formed by an inner layer of more strongly bound proteins and an outer layer of proteins with less affinity to the NP surface, called the hard corona and soft corona, respectively [10]. It has been shown that the hard corona once formed and isolated from the cognate biological environment is almost irreversibly bound [10, 13], while the outer protein layers can be easily replaced. However, more studies are needed to fully elucidate the soft corona behaviour. While there are several investigations describing the dynamic process of formation of the PC in blood fluids [14, 15], which involves both protein exchange and reorganisation at the “bio-nano” interface over time, the PC evolution upon the NP journey within the body is not elucidated yet. Given that, the chemical nature, the size and shape of the NP as well as the composition of the fluid will dictate the PC characteristics; there are different simultaneous equilibria that compete for making it difficult to predict the fate of the PC in vivo (Fig. 1) [16].

The understanding of the composition, evolution and interactions exerted by PCs in vivo is critical for nanomedicines. It is evident that NPs acquire a new biological identity, which will influence their stability and interaction with living substances, thereby affecting NP bio-distribution. Moreover, their performance might be affected by the PC formation with unforeseen consequences on their efficacy. Finally, as PC is a complex protein mixture (generally between 30 and 50 different

proteins), its formation involves cooperative protein-protein and protein-NP interactions with possible unfolding of one or more proteins upon adsorption with exposure of new epitopes respective to their native structures. These unravelled epitopes may interact with the cellular machinery activating cellular pathways with potential toxicity effects [17]. The latter consequence is very important for NPs that are not designed for biomedical applications as they are generally characterised by a more enhanced protein adsorption and physical instability.

In the following section, we will summarise the methodologies used to characterise the structure, composition and extrinsic function of PC-NPs highlighting the areas that need further elucidation. The biological impact of PC-NPs will be discussed with particular emphasis for drug delivery and biomedical applications. At the end, we will discuss the typical journey of intravenously injected NPs within the body experiencing the biological barriers.

2 Physico-chemical Characterisation of Nanomaterials in a Biological Environment

When NPs come in close proximity to the biological medium, they are immediately coated by a corona of biomolecules, primarily composed of proteins. The formation of this corona is a very complex process, which involves several mechanisms that ultimately cause thermodynamically favourable changes in the total enthalpy and/or entropy of the system [9]. These mechanisms can include formation of new bonds between NP and proteins, modification of the native structure of the proteins, desorption of water molecules and counter ions, and rearrangement at the interface. Finally, protein adsorption reduces the high surface energy of the bare NPs with the formation of PC-NPs whose general structure is shown in Fig. 1. The typical characteristics of PC-NPs will depend on various factors such as the physico-chemical properties of the NPs and the nature and concentration of the protein mixture. There are many experimental and theoretical studies to predict the adsorption of single proteins on nanoscaled surfaces as a function of NP size, surface functionalisation and environmental conditions [18–21]. However, mathematical modelling to predict the formation of the PC from a biological environment is extremely challenging [5].

Recently, Dell’Orco et al. [22] developed a simple mathematical model to predict the evolution of the PC formation on the basis of interaction of co-polymer-based NPs with plasma. This simple dynamical model envisages the instantaneous formation of PC-NPs with the most abundant proteins that are replaced over time by proteins with higher affinity to the NP surface and characterised by slower dissociation rate constants (k_{off}). The model can reproduce well the exchange between HSA (most abundant protein in plasma) and apo-lipoproteins (less abundant but at higher affinity) at the “nano-bio” interface of co-polymeric NPs in plasma over time observed in their experimental data. This model has been extended by Sahneh et al. [23] with the production of explicit analytical formulae to describe the dynamics of the corona

composition at the initial and final states highlighted by Dell'Orco's model. However, no cooperative and/or inter-protein interaction phenomena are considered in these two models, which cannot be excluded to possess an important role in the formation and evolution of the PC-NPs. Moreover, PC-NPs through their journey within the body will encounter not only different protein composition compartments but also more complex biological structures at organ, tissue, cellular and sub-cellular levels with an impact on their structure, composition and dynamics. The implications of the interaction of PC-NPs with those structures will be discussed in the next sections.

Ascertained that ENMs interact with proteins in the biological environment acquiring an altered biological identity, it is of primary importance to determine the structure and composition of these PC-NPs and their evolution over time for understanding and predicting their biological response. Many studies have been done in the past years on the characterisation of PC-NPs in terms of size and composition, and the most conventional methodologies to investigate them are reported in Table 1. Most of the techniques used for determining the size of PC-NPs in aqueous dispersions (i.e. dynamic light scattering [DLS]) and anhydrous state (i.e. atomic force microscopy [AFM]) need to be used in a protein-free environment requiring isolation of the formed PC-NPs from the biological milieu. This involves isolation process usually consisting of centrifugation cycles and washing to obtain either hard corona or soft corona complexes depending on the number of cycles. There are few techniques, which allow the detection of the size of PC-NPs in situ in the biological environment such as differential centrifugal sedimentation (DCS) [10] and fluorescence correlation spectroscopy (FCS) [24]. The possibility to study the structure of PC-NPs in situ is a very powerful tool and permits determination if the isolated PC-NP complexes are representative of those formed in the biological environment. Obviously, any methodology has its advantages and limitations as highlighted in Table 1, and generally it is a good practice to characterise the PC-NPs using multiple techniques. The composition of PC-NPs in terms of proteins enriched in the corona requires the isolation of these complexes and the detachment of the PC from the NP surface by treatment with SDS and high temperature. Then, conventional proteomics methodologies such as SDS-PAGE and mass spectrometry are routinely used to determine the identity of the proteins. The methodologies to isolate and determine the structure and composition of PC-NPs are now well established, but there is still the need to improve in the separation of monomers PC-NPs from dimers, trimers and/or agglomerates as their composition and thereby their biological response might be different. Most of the characterisations are done on mixtures of these PC-NPs, and methodologies to separate and isolate them in sufficient amounts for further investigation are required. Moreover, centrifugation is often less effective and induces agglomeration for low density and small NPs; thus, new less invasive separation methodologies to isolate cognate PC-NPs are also desired. Finally, more sensitive methods to detect the associated proteins are needed for studying the PC of ENMs functionalised with hydrophilic polymers and/or bioactive ligands, which have less affinity to interact with the environment proteins forming a smaller corona (see Sect. 3).

Table 1 Conventional methodologies to study the properties of NP-protein complexes

Methodology	Advantage	Disadvantage	References
<i>Structure</i>			
Dynamic light scattering (DLS) ^a	Fast, accurate, non-perturbative	Monodispersed samples	[18, 29, 111]
Nanosight ^b	Particle tracking analysis, concentration estimation, polydispersed samples	Size limitations, material limitations	[112]
Zeta potential ^a	Fast and easy	Monodispersed samples, ionic strength limitations	[113]
Transmission electron microscopy (TEM) ^a	Small volume, high resolution for small size	Artifacts, sample preparation, electron dense samples	[28]
Atomic force microscopy (AFM) ^a	Anisotropic samples	Artifacts, difficult setting, scanning speed	[114]
Differential centrifugal sedimentation (DCS) ^b	High resolution for polydispersed samples	Density and size limitations	[115]
Fluorescence correlation spectroscopy (FCS) ^b	Low volume, low concentration	Fluorescence labelling	[29, 116]
Size exclusion chromatography (SEC) ^b	Sensitive, low volume	Sample dilution	[12]
Small angle X-ray/neutron scattering (SAXS/SANS) ^b	Shape, local structure	Complex data analysis	[117]
<i>Composition</i>			
Mass spectroscopy (MS) ^a	Protein identification	Expensive, tedious sample preparation	[14, 118]
SDS-PAGE (1D/2D) ^a	Qualitative and semi-quantitative analysis of mixture, cheap, fast, coupling with MS	Sensitivity depends on staining method	[117, 119]
ICP-MS/AES ^a	Low volume, concentration determination	Destructive, tedious sample preparation and calibration	[120]
X-ray absorption spectroscopy ^a	Sensitive, element microenvironment studies, no need of crystalline sample	Data interpretation, sample damage	[26]
HPLC ^a	Coupling with MS	Expensive equipment, possible coelution	[121]

(continued)

Table 1 (continued)

Methodology	Advantage	Disadvantage	References
<i>Conformation</i>			
UV-Vis ^b	Fast and flexible	Not quantitative or conclusive	[28]
NMR ^a	Sensitive, low volume, high resolution	Expensive, not immediate data analysis, protein size	[122]
FTIR-Raman Spectroscopy ^a	Non-destructive, complementary	Difficult data analysis	[123]
Circular Dichroism (CD) ^b	Dilute samples	Qualitative, difficult data analysis	[26]
<i>Extrinsic functions</i>			
Quartz crystal microbalance (QCMD) ^b	Fast, sensitive, label free detection	Complex data interpretation	[48]
Western blotting ^a	High sensitivity	Antibody dependent	[124]
Fluorescence microscopy ^b	Cell studies, specific labelling	Photo-bleaching, photo-toxicity	[125]
Protein microarrays ^b	Fast screening	Expensive, in vitro, not always correlation between activity and protein abundance	[51]
Surface plasmon resonance (SPR) ^b	Sensitivity	Immobilisation required, expensive	[111]

^aAfter complexes isolation

^bIn situ analysis

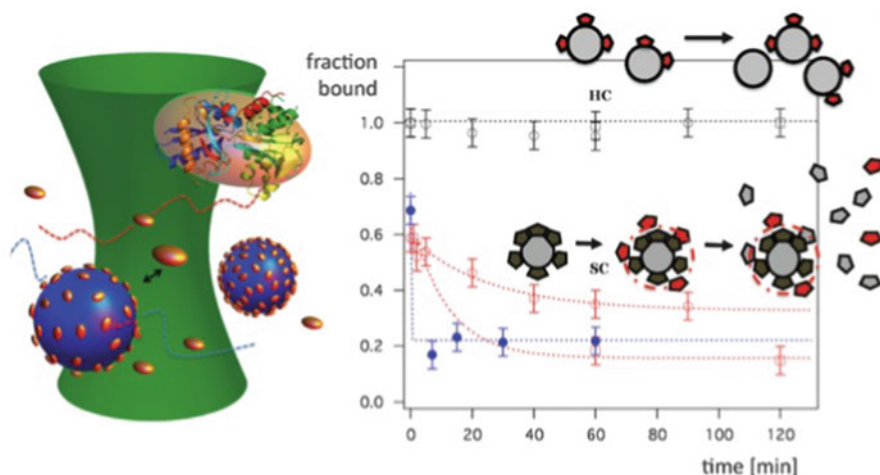


Fig. 2 Schematic representation of transferrin (Tf) PC dissociating from PS NPs measured by FCS. *Left:* Drawing of the FCS measurement volume where PC-NPs and Tf molecules diffuse in and out. *Right:* Graph reporting the bound fraction after addition of fluorescent-labelled Tf molecules to Tf-PC-NPs over time—hard corona Tf-NPs did not show protein exchange while soft corona Tf-NPs showed protein exchange at the first layer level. Reprinted with permission from [13]. Copyright (2012) American Chemical Society

It has been shown for several NPs that the hard corona is strongly bound to the NP surface and proteins do not come away even at high dilution [10]. More recently the structure and dynamics of the PC has been studied by FCS [13], and NP-protein interactions have been successfully modelled for differently functionalised polystyrene (PS) NPs in the size range of 40–100 nm following a Langmuir adsorption behaviour, where NPs and proteins are the substrate and the ligand, respectively. The authors demonstrated that the PC was formed by different layers, with the first being almost irreversibly bound to the NP surface, while the outer layers could be exchanged by competitive binding of the same labelled protein or other plasma proteins (Fig. 2). However, the size and functionalisation of the NP need to be considered in the formation mechanism of the PC, and it is difficult to produce a universal model that predicts PC formation. Generally, protein adsorption on smaller NPs induces less conformational changes in the native structure of the biomolecule than on bigger surfaces, as demonstrated by the several studies carried out on the adsorption of single proteins on nanoscaled surfaces (see Table 1 and associated references). Casals et al. showed that metal and oxide NPs with sizes between 7 and 20 nm form a PC immediately in cell culture medium, which is stable for days even after dilution in water solutions [25]. Recently, Liu et al. [26] reported on the mechanism of formation of the PC on CeO₂ NPs (7 nm) showing that when the size of NPs and proteins are similar, the protein binding to the NP surface was weaker. In fact, PC CeO₂ NPs formed in serum were separated by adequate size exclusion chromatography phases (weakly negative) and showed nearly 94%

retention of the NPs with complete elution of the proteins. This indicated that NP-resin interactions were stronger than NP-protein interactions with the formation of a weaker protein corona (soft) rather than a hard one. If the binding is weak, it is reasonable to speculate a displacement of the pre-existing corona when PC-NPs approach the cell membrane, which can affect the internalisation process.

The surface functionalisation of NPs also exerts an important role on the nature of the PC and its evolution. Many studies have investigated the mechanism of formation of the PC of NPs made of different materials and surface functionalisations in a biological environment. As proteins mainly tend to adsorb on surfaces through electrostatic and hydrophobic interactions [27] with the consequent desorption of water and counter ions (a phenomenon, which is driven by entropy), cationic and more hydrophobic NPs are characterised by a stronger hard corona than negatively charged and/or hydrophilic (i.e. pegylated) NPs. Many studies have been performed on Au NPs functionalised with different ligands bearing negative and positive charges showing conflicting results. For example, it was reported that anionic Au NPs functionalised with mercaptoundecanoic acid form a transient soft PC. Size analogue cationic Au NPs functionalised with aminoundecanethiol form a strong hard corona, which keeps growing over time (rearrangement at the “bio-nano” interface) [28]. Another study showed that positive and negative polymer coated Au NPs demonstrated different biological response although qualitatively characterised by a PC of the same composition [29]. However, the structure of the PC was analysed only in terms of composition and no information about the local conformation of the proteins in the corona was assessed. Moreover, positively and negatively charged NPs exhibited different colloidal stability in the biological milieu, indicating that sometimes it is hard to decouple the diverse factors that contribute to the biological response.

In nanomedicine it is usually desired to prolong the blood circulation time of nanomaterials for increasing their chance to find the required biological target (see Sect. 4). It is known that the interaction with plasma proteins can cause opsonisation with consequent recognition of the opsonised NPs by macrophages and reduction of the blood circulation time. This was first observed for drug nanocarriers such as liposomes, where circulation times were prolonged by adding pegylated groups on the surface of the lipid vesicles, forming a coating now known as stealth corona [30–32]. This approach has been also applied to hybrid ENMs (NPs composed of both organic and inorganic components) for reducing the formation of a PC, but also for linking bioactive ligands indirectly onto the NP surface. The use of a spacer will guarantee a better arrangement of the ligands at the nano-interface [33]. This route has been widely exploited by different chemical strategies with mixed results. Pegylation of a nano-surface strongly changes the physico-chemical properties of the nanomaterial providing the NP with steric colloidal stability and a hydrophilic shell. This shell will interact with the environment proteins in different ways depending on the length and grafting density of the PEG chains. For example, it has been shown that pegylated single-walled carbon nanotubes (SWCNT) form PC complexes in plasma with different patterns as a function of the PEG conformation on the nano-surface, in particular passing from a mushroom to a mushroom-brush state

[34]. While no direct relation has been found between diverse PCs and specific biological responses passing from PEG-SWCNTs with a mushroom to a mushroom-brush conformation, the biodistribution in a murine model considerably changed. Reduced blood circulation times with higher accumulation in the spleen and a faster renal clearance were observed for the mushroom-brush conformation. Several studies have been done on pegylated Au NPs with a size range 20–80 nm, showing that smaller sizes give denser PEG coatings with a reduced uptake by the liver and the spleen *in vivo*, longer circulation time and better extravasation from tumour blood vessels [35, 36].

The development and optimisation of different methodologies for studying PC-NPs has allowed us to extract some general features of these protein-NP complexes.

3 Protein Corona Nanoparticles and Their Biological Function

The interaction of nanomaterials with the biological matter has been widely studied in the last 5 years as reflected from the appearance of large number of publications on this subject [5, 37]. It is now well known that nanomaterials are characterised by specific PCs when in a biological environment, but how this PC mediates the interaction with the cellular machinery still needs to be elucidated.

Generally, nanomaterials are transported into the cells through facilitated passive diffusion and/or energy-active mediated endocytosis as a function of their size and surface functional groups. There are many studies in the literature reporting on this subject and we refer the reader to these papers as a detailed report is beyond the scope of this chapter [38–40]. However, it is worth to note that recently a few groups have reported on surface-modified NPs entering the cell through alternative energy-independent mechanisms without altering irreversibly the structure of the plasma membrane [41–43]. Ultimately, these NPs seem to have cell-penetrating abilities, but the entry mechanism is not completely understood and still under study. Regarding this, it has recently been reported in an investigation on the interaction of Au NPs capped with 11-mercaptoundecane sulphonate and bilayer lipid membranes (BLM) show how the NP ability of penetrating the membrane can be tuned as a function of their size [44]. The authors developed an elegant method to quantify the NP interaction with the lipid bilayer (as model system of the cell membrane) through capacitance measurements using electrophysiology chambers separated by a hydrophobic partition with a micron-sized aperture coated with a BLM. The results were in good agreement with the biological measurements *in vitro* and showed an irreversibly insertion of the NPs into the bilayer, confirming the fusion of these NPs with the cell membrane. The interaction of NPs with lipid bilayers has also been explored using neutron reflectometry, which allows investigations in the local structure of the lipid membrane upon interaction with the NPs at nanometer resolution [45]. This study has shown that while cationic Au NPs tended to pass through the lipid membrane without

destroying it, negative ones did not penetrate the lipid bilayer. While these methods are promising tools to study the interaction of NPs with lipid membranes, more studies are necessary to elucidate the mechanism of cell penetration of these NPs also in the presence of environment proteins whose role in the interaction with the cell surface cannot be ruled out.

A direct relation of the associated PC to the endocytosis pathway exploited by the NPs to enter the cell has not been found yet. However, it is known that the presence of a PC, independently of its specific composition, alters the affinity of the NP to the cell membrane with further consequences on the cellular uptake [46, 47]. The current understanding is that the PC decreases the surface energy of the bare NPs, reducing their association to the cell membrane. Although many studies on the interaction between the NPs and the cell membranes were performed using inhibitors and low temperature (4°C) to deactivate the endocytosis machinery as well as in serum-free conditions to exclude the PC effects, it is hard to extrapolate definitive conclusions as the biological machinery is also affected by these environmental changes. Recently, the NP-cell membrane interaction has been modelled for PS NPs of different sizes with the help of cell membrane model systems such as BLMs [48]. This work combined NP-cell uptake studies with QCM measurements on BLMs in different experimental conditions. The authors found that the NP-cell membrane interaction is a two-step phenomenon, where first NPs quickly adhere to the cell membrane and successively are slowly internalised through different active mechanisms, which make the NPs enter membrane-bound endocytosis compartments. The experiments were performed on PC-NPs isolated from the excess of serum in comparison to the pristine NPs and showed that the PC decreased the non-specific interactions of the bare NPs with the cellular membrane, consequently reducing NP cellular uptake.

While the PC certainly mediates the adhesion of the NPs to the cell membrane by functioning as a coating, it is still debated if it actively promotes cellular pathways due to new and unpredictable interactions between the adsorbed proteins and cell surface receptors. We suggest that this should be evaluated case by case considering the wide range of new ENMs developed for diverse biological applications. There are few examples reporting on this aspect in the literature, though, as it is not trivial to unravel possible new active epitopes disclosed by unfolding of the adsorbed proteins enriched in the PC. An elegant study was performed on Au NPs of different sizes and functionalised with diverse polymers where the authors showed that negatively charged poly(acrylic acid)-conjugated Au NPs bound fibrinogen inducing its unfolding and interaction with the integrin receptor, Mac-1 in cells of hematopoietic lineage [49]. The fibrinogen-associated NPs were found to activate the NF- κ B signalling pathway with release of inflammatory cytokines, and this biological response was tuned by NP size. Indeed, for NP sizes larger than 20 nm, the effect was massively reduced. The study has been further extended to human plasma, and NPs with different surface charges were screened. Although characterised by the same PC in terms of composition (fibrinogen was enriched in all PC-NPs), only negatively charged NPs were found to promote cytokine release from THP cells [50]. These findings demonstrate that it is important to study the extrinsic function of the PC more than its composition, as merely the presence of a protein in the PC does not necessarily mean that it will exert a biological function

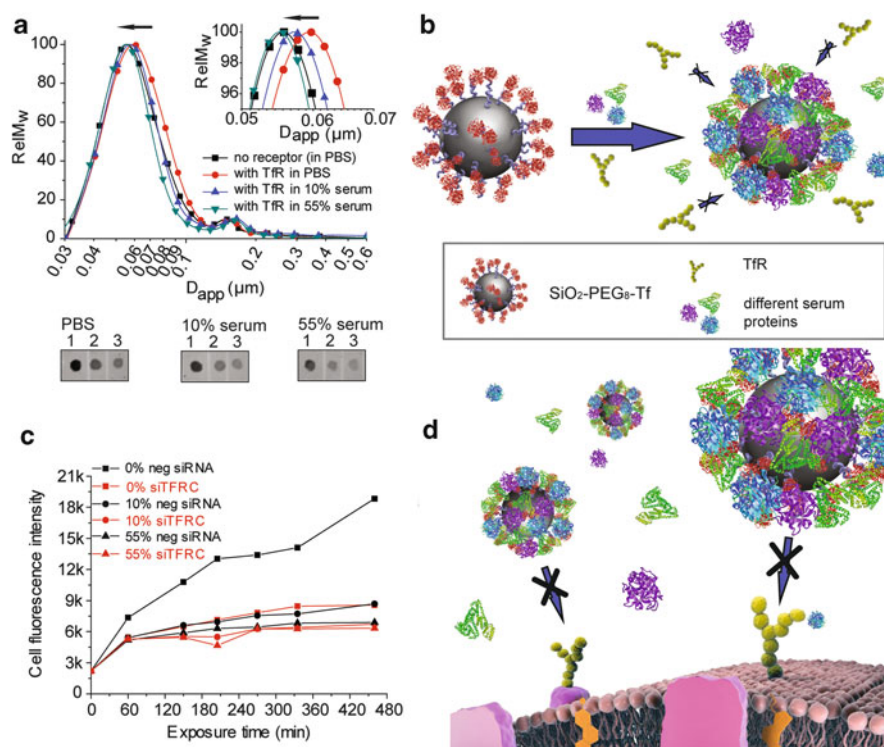


Fig. 3 Schematic drawing of SiO₂-PEG-Tf NPs interacting with the Tf receptor (TfR) in the presence of environmental proteins. **(a)** DCS graph comparing the size of SiO₂-PEG-Tf bound to the receptor in PBS and in serum where the PC hampers the binding (no change in the size). On the neat anti-Tf dot-blot are reported for Tf detection on the recovered NPs from PBS and serum. **(b)** Drawing of SiO₂-PEG-Tf NPs with the PC that hampers the binding with the TfR. **(c)** Cell fluorescence intensity due to NP uptake in serum-free and serum-containing cells in normal and TfR silenced cells. **(d)** Drawing representing the SiO₂-PEG-Tf PC NPs which cannot bind the TfR expressed on the cell membrane. With permission from [52]. Copyright 2013, Nature publishing group

on the cell. In fact, as explained above, the PC is a complex dynamic structure composed of different layers alternately bound to the NP, which likely undergoes molecular rearrangements over time and during circulation in vivo.

Protein microarrays have been used to screen possible unpredictable interactions of PC-NPs in human plasma. Carboxylated and sulphonated PS NPs functionalised with transferrin have been shown to give different binding patterns with the arrayed proteins, but no specific PC-protein interactions have been unveiled [51]. Protein microarrays represent a powerful tool to screen the extrinsic interactions displayed by PC-NPs and may be related to the unfolding of some protein upon adsorption on the NP surface with exposure of new epitopes. One limitation of this technology is the limited repertoire of proteins arrayed currently on commercially available arrays, thus reducing the variety of possible biological targets provided in vivo. The use of custom protein arrays and/or phage-display libraries for exploring

the interaction displayed by PC-NPs could strongly help in unravelling possible extrinsic biological functions of these complexes.

Moreover, it has been shown that the formation of the PC can also affect the desired bioactivity of the ENMs designed for biomedical applications. In fact, pegylated Tf SiO₂ NPs have been shown to lose their targeting ability towards the Tf receptor in the presence of a PC. The loss of targeting capabilities was observed both in solution using a soluble analogue of the TfR and in vitro using cell lines expressing the TfR [52]. Figure 3 is a schematic drawing that represents the speculated mechanism through which the PC might hamper the binding of the PEG-Tf NPs to the receptor. This receptor interaction is the first evidence where the PC is shown to affect the desired biological function of the engineered NP (i.e. targeting), with clear implication for its efficiency in vivo. Further barriers faced by NPs used for biomedical applications are explained in the next section.

4 Nanomaterials' Transport In Vivo: What Barriers to Overcome?

Given that the PC provides the “identity” of the NP in a biological system [9, 10, 53], understanding the evolution or exchange of biomolecules within the PC when NPs are exposed to biological environment, it is critical to understand how NPs interact with cells within different tissues. Clearly this is a key determinant in developing tissue targeted drug nanocarriers or nanosized diagnostic tools that can be used systemically in patients' tumours. Thus, complexity of biological environments has the potential to dramatically alter PC development during targeted delivery of NPs to disseminated tumours. The focus of this section is to examine how the systemic transport of nanomedicines in patients can potentially hinder successful NP targeting in the variety of physiological environments encountered during their delivery, following intravenous (i.v.) administration of nanomedicines, using cancer patients with solid tumours as an example. As summarised in Fig. 4, i.v. administration of tumour cell-targeted NPs in patients will incur the following transport processes: blood-borne transport, transvascular transport, interstitial transport through the extracellular matrix (ECM), target cell uptake and post-delivery clearance. We will discuss each of these processes with the aim to highlight future research efforts that are required to overcome these barriers.

4.1 *Blood-Borne Transport*

i.v. administration of NPs may induce the formation a PC produced from the blood plasma, functionalizing the NPs in response to exposure in the blood vasculature. Within the bloodstream environment, the mononuclear phagocyte system (MPS;

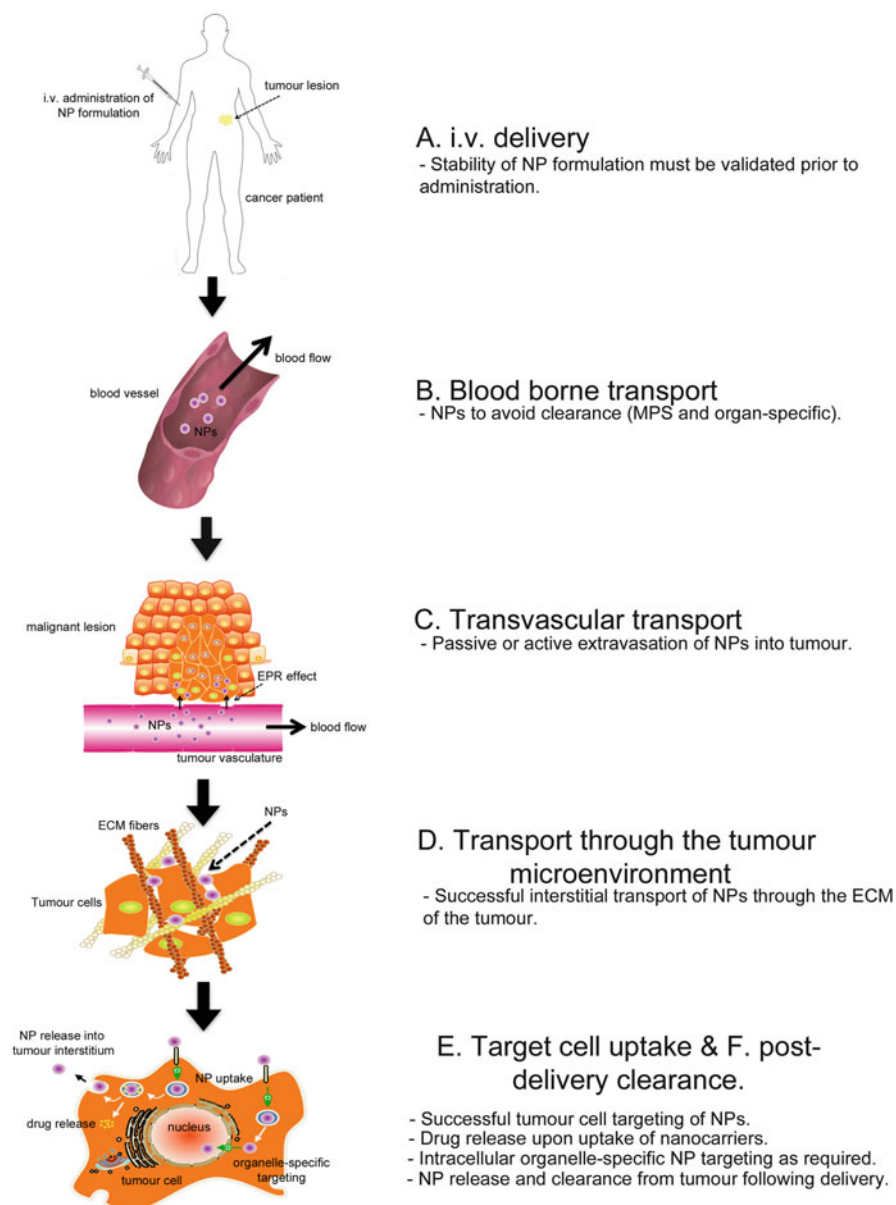


Fig. 4 Possible transport routes of nanomedicines to tumours in cancer patients. Detailed discussion of each of the transport steps is provided in the text. (a) Intravenous (i.v.) administration requires that stable nanoparticle (NP) formulations in the blood plasma are produced. (b) NPs that are not cleared by the mononuclear phagocyte system (MPS) may be delivered to the tumour. (c) Once at the tumour site, NPs can extravasate into the tumour tissue either by targeting the diseased vasculature or through the passive enhanced permeability and retention (EPR) effect. (d) Following accumulation in the tumour, NPs must then penetrate the tumour microenvironment

sometimes also referred to as the reticuloendothelial system, RES) is active. The MPS is a constituent of the immune system primarily comprising of monocytes, macrophages and dendritic cells that are responsible for phagocytosis and removal of blood-borne particulates, including i.v.-delivered NPs. Serum-induced PC formation on NPs (opsonisation) tags the particles for clearance by the MPS. Large, cationic and/or hydrophobic NP preparations are particularly susceptible and are rapidly targeted for removal by the MPS in the blood, with subsequent accumulation in the liver and spleen (see Sect. 2). It has been known as early as the 1990s that functionalizing the surface of liposomal agents with PEG molecules could dramatically enhance prolonged exposure and tumour delivery of the NPs in cancer patients [54–56]. However, pegylation also compromises NP interaction with target cells [57, 58], thus inhibiting the targeted accumulation of nanomedicines in diseased tissues. To address this issue, adaptive NPs capable of depegylation at the desired target tissue, either through external stimulation or over time, have recently been designed [59].

Organs such as the liver and spleen possess phagocytic cell populations that can remove particulates from the blood flowing into them, causing the accumulation of NPs in these organs. Alarming, this accumulation can often be several magnitudes higher than NP accumulation in the desired target site such as the tumour [60–62]. NP size is a critical consideration for overcoming these clearance barriers and increases plasma half-life of nanomedicines. Large NPs (>100 nm) are not only easily detected by the MPS, but can be phagocytosed by splenic macrophages and hepatic Kupffer cells [63]. Small NPs can be easily cleared by alternate mechanisms including glomerular filtration [64, 65] and hepatocyte removal [64]. This suggests that NPs of an intermediate size are favourable for increased circulating half-life, but there is currently no consensus on optimum size scale. Furthermore, NP biodistribution is also dependent on additional factors such as surface charge and composition [66]. As highlighted above, half-life is also likely to be heavily dictated by the composition of the PC.

In addition to pegylation, a number of other stealth factors have been proposed as possibilities to functionalise the surface of NPs to increase plasma half-life. Low molecular weight heparin [67] and glucose [68] have also been used as stealth materials. Another particularly promising approach is the use of a self-recognition peptide derived from CD47. CD47 is a cell surface glycoprotein expressed as a putative marker of ‘self’ that interacts with signal regulatory protein- α (also called CD172a) on macrophages to inhibit phagocytosis [69]. Nanobeads coated with a human CD47-derived, self-recognition peptide demonstrated enhanced circulation

Fig. 4 (continued) to reach the cancer cells. Barriers within this environment include a stiffened extracellular matrix (ECM) and high interstitial fluid pressure (the latter not illustrated here). (e) Target cell uptake can be achieved when NPs interact with the tumour cells. Here the NPs can release their drug cargo if functioning as nanocarriers or can function as contrast agents to provide imaging for the treating clinicians. Organelle-specific targeting can also be engineered into the NPs. (f) Post-delivery clearance of NPs following successful tumour cell targeting is required to avoid unwanted NP accumulation in patients

times, reduced clearance rates and increased drug delivery to cancerous lesions in tumour-bearing mice, highlighting the stealth properties of CD47 [70].

Following i.v. administration, NPs circulate prior to reaching the target tissue (such as the tumour). However, while in circulation, most NPs get cleared from the bloodstream and organs as described. This process may range from several minutes to hours depending on the NP composition and size [71–75]. Ideally i.v.-administered NPs should not aggregate in the blood, resist MPS detection, have prolonged circulation times and should selectively target the desired tissue.

4.2 Transvascular Transport

For access to diseased tissue, intravenously administered NPs must cross the endothelial monolayer. This monolayer functions as a semi-selective barrier that regulates the movement of molecules between the vasculature and the extravascular space within tissues. The pore size within this barrier varies between tissues and is largely dependent on the presence of inter-endothelial structures such as cell-cell junctions and fenestrae. There is strong physiological evidence that a selective size limit exists anywhere between 1 and 12 nm in healthy tissues depending on the anatomical site [76]. Tumours however often lose this selective barrier owing to their abnormal and disorganised vascular architecture [77], which affords the increased accumulation of macro- and nano-sized objects within the tumour tissue compared to healthy tissue. This process is referred to as the enhance permeability and retention (EPR) effect [78].

In addition to the leaky vasculature of tumours, lymphatic drainage within different regions of the tumour is often variable, leading to poor fluid drainage in some areas [79]. This increases the EPR effect by allowing NPs that have extravasated through the leaky blood vessels in tumours to be retained due to poor lymphatic drainage. The EPR effect is a passive targeting process allowing molecules and particulates (including nanomaterials) to accumulate in tissues with disorganised blood vessels and poor fluid drainage. However, exploitation of the EPR effect for NP delivery to tumours is limited by the size of tumours that can be successfully targeted as only large lesions of 100 mm³ or more in volume possess it, making the passive targeting of micrometastases or poorly vascularised tumours impossible [80].

To address this limitation of the EPR effect, NPs can be formulated with ligands specific to receptors over-expressed in the tumour-associated endothelia or stroma. Suggested ligands include RGD, endothelial growth factors and antibodies targeting vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 (ICAM-1), which all increase the tumour-targeting capacity of NPs [81–84]. Recent work has shown that altering the shape of ligand-bearing NPs can affect vasculature binding, where PS nanorods displaying the ICAM-1 antibody exhibit higher selectivity towards the vasculature compared to PS spheres [85]. Finally, another interesting approach taken was to further enhance the EPR effect by using tumour necrosis

factor- α , which can augment tumour vasculature leakiness to increase spaces within the endothelial lining, thereby improving NP extravasation and intra-tumour accumulation [86, 87]. Taken together these studies demonstrate that transvascular transport of tumour-targeting nanomaterials can be substantially enhanced through the manipulation of the tumour vasculature itself.

4.3 Transport Through the ECM

The ECM is an inflexible scaffold formed from the deposition of secreted biological materials that promotes the higher-order organisation of cell structures within tissues. It is comprised in the large part of proteins such as laminins, fibronectins, proteases, proteoglycans, collagens and hyaluronan to name a few, which cells can physically attach to. The ECM of tumours is stiffened compared to healthy tissue due to an abundance of collagen and other ECM proteins [88] and is packed with a variety of cell types in addition to the tumour cells, including fibroblasts and cells of the immune system. Furthermore, factors causing the EPR effect also lead to enhanced interstitial fluid pressure (IFP) within tumours [89]. These factors, coupled together with abnormal metabolic processes leading to reduced pH, create a distinct set of characteristic properties within the tumour compared to healthy tissues that is commonly referred to as the tumour microenvironment.

Transport of NPs through the tumour interstitium is an important consideration for successful targeting of cancer cells. Generally, drug-resistant cancer cells that represent the most favourable tumour cell population for targeting, reside in the hypoxic compartment of the tumor, which is often poorly vascularised. The dense ECM and high IFP inhibit NP penetration to the centre of tumours and ultimately results in their accumulation at the tumour periphery, with their transport through the interstitium reliant on diffusion processes [90, 91].

Various approaches have been proposed to overcome these issues, including remodelling of tumour vasculature to lower the IFP [92], priming with chemotherapeutics prior to NP administration to reduce tumour size [93] and ECM degradation using proteases such as collagenases and matrix metalloproteinases [94–96]. Again NP design such as surface charge and in particular size are also important considerations for penetration of the dense tumour interstitium, where generally smaller NPs (ranging anywhere from 2 to 50 nm depending on NP type) are better suited to penetrate poorly vascularised lesions [83, 97–99]. Such approaches offer significant promise to circumventing the barriers imposed by the impenetrable properties of the tumour microenvironment.

4.4 Target Cell Uptake

A key step in the success of anti-cancer nanotherapies is their ability to mediate effective tumour cell uptake. While free drugs can readily enter cells by either

diffusion or endocytosis, NPs due to their size are more commonly limited to endocytic mechanisms (phagocytosis, macropinocytosis or receptor-mediated endocytosis; either clathrin- or caveolin-dependent). Optimisation of this uptake process in target cells is largely dependent on the cell type, the uptake mechanism employed and the characteristics of the NP. Therefore, in the development of novel NPs for biomedical applications, detailed studies are warranted to analyse uptake mechanisms of NPs to the desired cell type.

Over-expressed cell surface markers on tumour cells can be readily exploited as targeting moieties for NPs that have been functionalised with cognate ligands for these markers. These ligands include antibodies, peptides, aptamers and other molecules such as carbohydrates. Many of the targeting approaches used are directed against highly proliferative cell populations, and as such the targeting moieties are ubiquitously expressed, for example, the transferrin receptor [100, 101]. Perhaps more sophisticated targeting involves the use of moieties that possess a more restricted expression pattern to the target tumour cells. This process is being explored as a possible mechanism to improve target cell uptake of NPs in a variety of cancer types. Indeed for some of the clinically most difficult cancers to treat, nanotechnology offers the promise of highly effective targeted drug delivery. For example, in glioblastoma (GBM) the most common and aggressive primary brain malignancy, one elegant approach has been to use an antibody-conjugated iron oxide NP that targets an epidermal growth factor variant expressed only in malignant gliomas, but not the healthy brain tissue, with effective tumour targeting in a pre-clinical model of GBM [102]. Another promising effective targeting example is the use of melanocortin-1 receptor targeting in the most lethal form of skin cancer, malignant melanoma [103]. These studies highlight that if tumour-specific cell surface markers are identified, NPs can successfully target cancer cells for drug delivery and diagnostic purposes. However, as already mentioned, the ability of the targeted NPs must be optimised using *in vitro* cell lines representative of the tumour tissues for testing the effects of the eventual PC. Indeed pre-screening *in vitro* could strongly help to engineer the NPs with the best targeting properties in the biological environment. Clearly, this will not guarantee that the targeting will work *in vivo* as the PC might be more complex due to the different biological compartments that the NP will encounter within the body. Lundqvist et al. have recently reported that the associated PCs of co-polymeric NPs passing from serum to the cytosol undergo rearrangements in their composition, but a sort of protein fingerprint is kept throughout [104]. Although preliminary and fairly simple, this study is the first investigation on the evolution of the PC of NPs passing through different biological environments.

Another important consideration is the effective targeting of NP cargo to the desired intracellular location, for example, the delivery of nucleic acids (such as plasmids or small-interfering RNA) to the nucleus, or uncouplers, respiratory chain inhibitors and pro-apoptotic drugs to the mitochondria. This is an emerging area of nanoengineering for biomedical applications, which has led to a number of recent technologies being developed to provide intracellular organelle-specific targeting. For example, nuclear targeting can be achieved by attaching a nuclear localisation signal (NLS) peptide to the NP, as the NLS activates the importin protein to facilitate transport across the nuclear pore [105]. For example, 60 nm DNA-polylysine

complexes are able to pass through nuclear pores when attached to a NLS [106]. Mitochondria can be targeted without the need for peptide ligands, by functionalizing the surface of the NP with cationic molecules to facilitate electrostatic interactions with the mitochondrial membrane [107]. Other organelles that can also be targeted for drug delivery include lysosomes [108], peroxisomes [109], and the cytosol [110].

4.5 *Post-delivery Clearance*

Although a variety of NPs can be used for targeted drug delivery, upon reaching the target cell the ideal particle formulation should release its drug contents. Furthermore, the carrier itself should be biodegradable to avoid unwanted accumulation. For nondegradable NPs (e.g. hard crystalline metal nanoparticles), eventual clearance is required to ensure adverse side effects are not triggered by accumulation of NPs in the patient following delivery. The likely route of such clearance is the lymphatic system. Although as this is often perturbed in the tumour tissue, the rate of inorganic NP clearance from the tumour can be unpredictable. More work is required to understand the clearance and potential post-delivery accumulation of inorganic NPs from tumours.

5 **Summary, Conclusions and Outlook**

The large use of nanostructured materials in an ever-increasing number of fields makes it critical to investigate their behaviour and effects on human health in the longer term. These studies should not only be limited to biomaterials designed for biological and/or medical applications, but they should be extended to all types of nano-systems that can be exposed to humans inadvertently. It is known that nanostructured materials interact with biomolecules such as proteins, sugars and hormones dispersed in the biological environment to form dynamic adducts with different composition, size and physico-chemical properties to the cognate synthetic NPs. These PC-NPs represent what interacts with the biological matter, and we have extensively motivated the importance of studying their properties and behaviour. The general structure of these PC-NPs has been modelled as formed of less strongly bound layers of proteins (soft corona) and a more internal protein layer almost irreversibly bound to the NP surface (hard corona). Clearly, this general model is not valid for all types of NPs, and controversial findings have been discussed above. While methodologies for determining the qualitative composition of PC-NPs in terms of protein identities are reasonably advanced, there is a lack of standardised protocols to separate the mixture of different PC-NPs formed by the same NP (monomer complexes from diverse protein-NP agglomerates) in the biological milieu. This would be extremely important as the biological activity of PC-NPs of different structure and potentially composition might be very different. We also stressed the importance of developing new methods to study the extrinsic

functions exerted by these PC-NPs in the perspective to unravel unpredictable interactions due to conformational changes of the proteins upon adsorption on the NP surface. In fact, it is known that proteins can strongly modify their native structure when adsorbed on a surface exposing new epitopes possibly able to activate the cellular machinery. The difficulty resides in finding possible new interactions in such a complex structure as the PC associated to an NP where a large number of proteins are mixed together to form this multi-structured layer. Here we suggested possible approaches to investigate the function of PC-NPs such as the use of protein microarrays and phage-display libraries to find new possible interactions with biological targets, which can be relevant to both medical purposes and toxicological effects. Another aspect on PC-NPs still under debate is their transient nature when encountering different biological compartments within the body. There are a few studies on the role of the PC evolution within the body, but general trends on the effect of NP surface features and size on the biodistribution *in vivo* have been observed. It has also been demonstrated that the functionalisation of NP surface with polymeric hydrophilic chains such as PEG reduces opsonin adsorption prolonging the blood circulation time of the NPs. However, it has also been reported that pegylation does not completely inhibit the formation of the PC with possible unexpected effects such as the loss of targeting ability of the ENM. At the end, in the last section of this chapter, we analysed in detail the ‘journey’ that a targeted NP to a tumour has to face within the circulation. We think that the evolution of PC-NPs should be studied in different biological environments by using cell extracts, lysates and/or *ex vivo* tissues. Such studies involve the use of advanced microscopy and MS technologies, but they will significantly help to design more effective targeted ENMs. Overall, we believe that a better understanding of the evolution of PC-NPs through different biological compartments, which NPs encounter when administered *in vivo*, will strongly contribute to design more effective ENMs for biomedical applications.

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