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Historical Perspective

Insights into colloidal nanoparticle-protein corona interactions for nanomedicine applications

María Martínez-Negro [a,*], Guillermo González-Rubio [b], Emilio Aicart [c], Katharina Landfester [a], Andrés Guerrero-Martínez [c], Elena Junquera [c,*]

[a] Max Planck Institute for Polymer Research, 55128 Mainz, Germany
[b] Physical Chemistry, University of Konstanz, Universitätsstraße 10, Box 714, 78457 Konstanz, Germany
[c] Departamento de Química Física, Universidad Complutense de Madrid, Avenida Complutense s/n, 28040 Madrid, Spain

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ABSTRACT

Colloidal nanoparticles (NPs) have attracted significant attention due to their unique physicochemical properties suitable for diagnosing and treating different human diseases. Nevertheless, the successful implementation of NPs in medicine demands a proper understanding of their interactions with the different proteins found in biological fluids. Once introduced into the body, NPs are covered by a protein corona (PC) that determines the biological behavior of the NPs. The formation of the PC can eventually favor the rapid clearance of the NPs from the body before fulfilling the desired objective or lead to increased cytotoxicity. The PC nature varies as a function of the different repulsive and attractive forces that govern the NP-protein interaction and their colloidal stability. This review focuses on the phenomenon of PC formation on NPs from a physicochemical perspective, aiming to provide a general overview of this critical process. Main issues related to NP toxicity and clearance from the body as a result of protein adsorption are covered, including the most promising strategies to control PC formation and, thereby, ensure the successful application of NPs in nanomedicine.

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* Corresponding authors.
E-mail addresses: martinezm@mpip-mainz.mpg.de (M. Martínez-Negro), junquera@ucm.es (E. Junquera).

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1. Introduction

The application of nanotechnology in biology and medicine has drawn significant interest due to its potential to deal with problems that have traditionally threatened human health, such as cancer or degenerative diseases [1,2]. The so-called nanomedicine relies primarily on the use of nanomaterials for diagnosis (e.g., sensors and imaging) and treatment (e.g., photothermal theraphy) of diseases [3–5]. For instance, nanomaterials can protect insoluble therapeutic drugs and genetic material, allowing them to overcome the physiological barriers and increasing their efficiency [6–10]. In most cases, such processes are carried out through the use of colloidal nanoparticles (NPs), a class of nanomaterials that has been a focus of attention due to their unique and tunable structural, electrical, optical, and magnetic properties [11]. Moreover, the advancement of wet-chemistry methods in the last decades has opened access to an array of NPs with different compositions (e.g., metal, metal oxides and sulfides, polymers, or silica), sizes (from 1 nm to above 200 nm), and shapes (e.g., spheres, cylinders, disks, platelets, hollow spheres, and tubes) [12–17]. Thereby, unprecedented control over the nature and behavior of colloidal NPs is nowadays possible, increasing the potential of such nanoscale materials for applications in nanomedicine.

Nevertheless, the implementation of colloidal NPs in nanomedicine requires control over their physicochemical properties and their interactions with biological fluids (i.e., typically blood). Once the NPs are introduced in the body, the proteins present in the blood plasma (i.e., the acellular fraction of blood) interact with the NP surface, giving rise to a corona of proteins [18–22]. The strong interaction of proteins with surfaces was first recognized by the hematologist Leo Vroman, who investigated the change in the hydrophobic and hydrophilic nature of different materials after exposure to blood plasma [23–25]. Vroman studies revealed that, in the presence of plasma proteins, the surface of hydrophobic materials became hydrophilic. In the case of hydrophilic materials, the surface wettability modification was less evident. The observed effects were attributed to the adsorption of distinct proteins to the investigated surfaces.

In 2007, almost 50 years after Vroman studies, Dawson and coworkers coined the term protein "corona" to describe the shell of proteins formed on NP surfaces [26,27]. Such a phenomenon can cause significant changes in the biological identity of the nanomaterial, as the cells interact directly with the PC rather than with the NP itself. Moreover, NP surfaces can alter the native structure of the adsorbed proteins, thereby inducing undesired effects such as their aggregation [28]. As a result, NPs can be more easily recognized by the immune system and cause harmful biological responses [29–31].

This review provides a brief overview of the complex phenomenon of PC formation on colloidal NPs for nanomedicine. The first section covers main concepts related to the applications of NPs in diagnosis and therapy, as well as those mechanisms involved in their biocompatibility and clearance from the body. The nature and function of the different blood plasma proteins are discussed in the next section, providing some examples related to their influence on the NPs fate. In the following section, the physicochemical aspects of protein-NP interactions are discussed, while the last part of this review deals with those strategies that, from our perspective, hold the most significant potential to control the PC formation.

2. Colloidal nanoparticles in nanomedicine

2.1. Applications of colloidal nanoparticles

The use of colloidal NPs in nanomedicine has typically focused on different aspects of diagnosing and treating diseases. The diverse nature of these applications generally demands the design and synthesis of NPs with different physicochemical properties but meeting essential requirements related to their toxicity and biocompatibility [1–4]:

Diagnosis

The diagnosis of human diseases such as cancer, genetic disorders, or infections is of paramount importance to ensure access to adequate treatment and achieve faster recovery of health conditions. This process often requires the use of technologies and instruments for obtaining information about the causes of health alteration, most typically through the formation of a visual representation of a body, organ, or tissue section, i.e., imaging. Different techniques are currently used for imaging, including m*agnetic resonance imaging (MRI),* positron emission tomography (PET), X-ray computed tomography (CT), ultrasound, optical imaging (OI), and photoacoustic imaging (PAI) [32,33]. In most cases, contrast agents are required to improve the quality of the obtained images. Compared to molecular-based contrast agents, colloidal NPs can be used to get better signal-to-background ratios or reduced cytotoxicity. Due to the unique magnetic (e.g., iron oxide), optical (e.g., gold and quantum dots), and structural (e.g., mesoporous silica, polymeric) properties of colloidal NPs, they can be used directly as contrast agents or carriers of molecular-based ones [4,32–38]. The use of NPs for imaging applications requires an adequate blood half-life, selective accumulation in the desired organs or tissues, effective elimination from the body, and good biocompatibility [39,40].

Treatment

NPs as carriers aim to improve transport and delivery of therapeutic drugs (drug delivery) or genes (transfection) to the pathological location and avoid damaging healthy tissues and organs. Polymeric and mesoporous NPs are usually considered in the design of drug carriers due to their ability to transport an array of drugs (e.g., doxorubicin or paclitaxel.) [29,41–43]. Nevertheless, noble metal, magnetic, and core-shell NPs (i.e., gold-silica core-shell) have also been widely investigated for the design of drug and gene carriers due to their versatile surface functionalization [44–48]. Two different strategies exist depending on the targeting mode: active or passive [49–51]. In those cases where active targeting is desired, the NP surface is functionalized with antibodies, peptides, or molecules that bind to receptors expressed at the targeted cells. In the passive ones, the targeting is mediated by physiological conditions like pH value, temperatures, and molecular sites [52,53]. For instance, active drugs to kill cancer cells can be selectively transported and accumulated at tumors using the enhanced permeability and retention effects. It is worth noting that some classes of NP can be directly used for treatment without loading them with drugs. Plasmonic and magnetic NPs are suitable for thermal therapies owing to their ability to increase their temperature when excited with light or alternating magnetic fields, respectively [54–56].

Moreover, the complex nature of NPs enables them to integrate both diagnosis and therapy. For example, magnetic iron oxide NP can be used as contrast agents for imaging tumors using MRI and as nano heaters to kill cancer cells by hyperthermia (i.e., using an alternating magnetic field) [57,58]. Similarly, the plasmonic properties of gold and silver NPs enable their use as platforms for imaging and photothermal therapy (i.e., by converting light into heat) [59].

2.2. Toxicity and clearance

The PC can strongly alter the NP biodistribution, favor its clearance from the body, induce their accumulation in different locations, or provoke the undesired release of the cargo. Moreover, it can increase NP toxicity or reduce the targeting ability of NPs.

Clearance and bioaccumulation

Elimination of NPs from the body can occur through different pathways (Fig. 1) that include renal filtration of the blood, degradation into to clearable components, excretion through the liver into bile, or uptake in the mononuclear phagocyte system (MPS, i.e., innate immune system) [39,60–62]. The latter is often an undesired pathway as it can result in long term accumulation in the body (i.e., mainly in the liver and spleen). In general, spherical NPs with hydrodynamic diameters

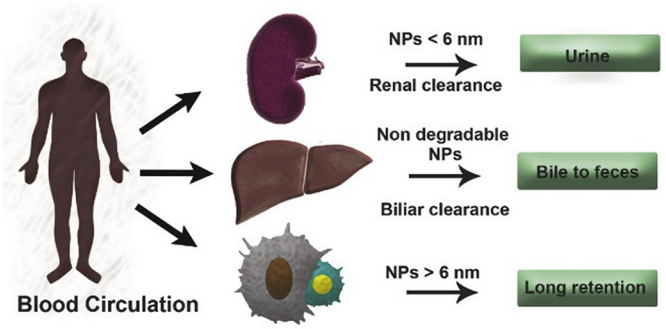

Fig. 1. Different pathways of colloidal nanoparticles clearance and bioaccumulation.

below ca. 6 nm can be filtered by the kidney. However, the adsorption of proteins can modify the hydrodynamic diameter of small particles, impairing its clearance by the kidney [61]. Large NPs can be recognized by MPS, which comprises phagocytic cells (monocytes and macrophages) primarily located in the spleen, liver, and bone marrow. As a result, they tend to bioaccumulate, causing cytotoxic effects in such organs. Moreover, specific proteins known as opsonins can increase the recognition of the NPs by the MPS, which reduces the NP blood half-life [63,64]. Other proteins such as C3 and C4, C-reactive protein, and immunoglobulins act as opsonins. Their presence on the NP surface represents a major obstacle towards the successful use of NPs for diagnosis and treatment [65,66].

Toxicity

The concept of toxicity relates to the ability of a substance to produce harmful effects on a living organism. In the case of NPs, their toxicity typically emerges as a result of (i) their interaction with the cell membrane (inducing cell damage and malfunction), (ii) the release of toxic ions arising from their dissolution (e.g., Ag^{+}, $Cd^{2+,}$ or Zn^{2+} from Ag, CdS and ZnS NPs, respectively) and (iii) oxidative stress due to the generation of reactive oxygen species (e.g., catalytic materials such as TiO_2) [67–71]. The adsorption of proteins can either boost or decrease the intrinsic toxicity of the NPs. For instance, positively charged NPs are highly cytotoxic due to their ability to disrupt cell membranes, which are negatively charged [72]. The formation of a PC can change the NP surface from being positive to negatively charged, thereby reducing their interaction with cell membranes and derived toxicity (i.e., most serum proteins present an isoelectric point < 6, and therefore are negatively charged at physiological pH) [72,73]. Nevertheless, toxic effects can also be caused by NP-induced conformational changes of the protein folding (i.e., denaturation) [74]. For instance, protein fibrillation can be favored, which eventually can lead to neurodegenerative diseases such as Parkinson's and Alzheimer's [74–76].

3. Plasma proteins

Plasma proteins can be classified into three categories: classical plasma proteins, tissue leakage, and cytokines. Classical plasma proteins constitute the majority of the blood proteins (concentrations range from 10^{10} to 10^{6} pg/mL), are produced by the liver and plasma cells (i.e., immunoglobulins), and carry their function in the blood (Fig. 2) [77–80]. Their relative abundance and concentration fluctuate between individuals due to genetics, diseases, infections, stress, or diet. Tissue leakage proteins are proteins released into plasma due to cell death or damage (e.g., myoglobin). Cytokines are small and soluble proteins produced by different cell types in charge of intercellular communication through cell surface receptors (e.g., interleukin 8) [81]. Tissue leakage and cytokines concentrations range from 10^{6} to 10^{3} pg/mL and 10^{2} – a few pg/mL, respectively. The most important classical plasma proteins groups are:

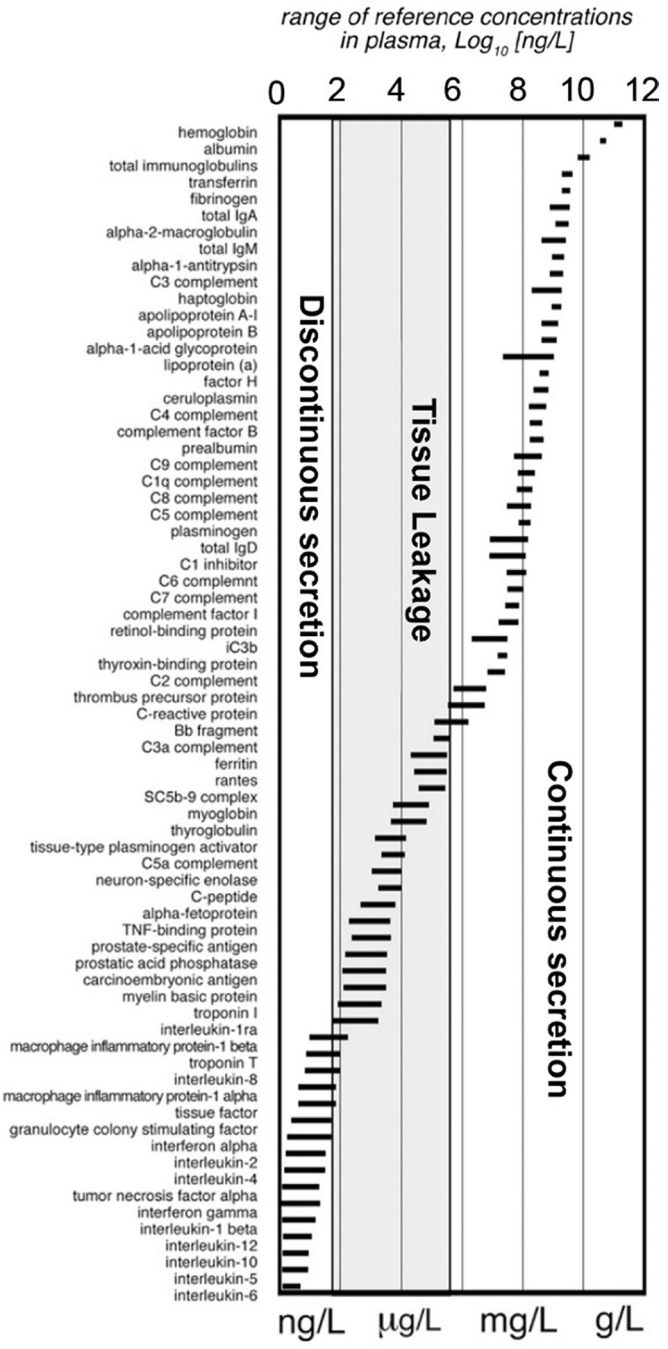

Fig. 2. Abundance of different plasma proteins. Continuously secreted proteins, such as albumin, globulins, coagulation factors, complement, and lipoproteins, constitute most plasma proteins. Those proteins released from the dead cells, known as tissue leakage, form the second largest fraction. Proteins such as cytokines and hormones discontinuously secreted are found at the lowest concentrations.
Reproduced with permission from [80].

Albumin

It belongs to the group of globular proteins, and it is the most abundant one in the blood plasma (more than half of the total plasma protein concentration, Fig. 2) [78]. Human albumin is a highly soluble protein, with a molecular mass of 67 kDa [82]. It plays several different roles, including regulating the osmotic pressure, transport of hormones, metals, drugs, fatty acids, and anti-oxidants. The absorption of albumin on NPs typically contributes to inhibiting their recognition and uptake by macrophages.

Globulins

After albumin, globulins constitute the major fraction of plasma blood proteins [78]. There are four classes: α_1, α_2, β, and γ [83]. The α_1 fraction consists mainly of α_1 antitrypsin, a proteinase inhibitor. In the α_2 class, α_2 macroglobulin and haptoglobin are the main components. The former is a proteinase inhibitor, while the latter is a protein that binds to free hemoglobin and possess different molecular masses depending on the genetic polymorphism. The main component of β globulins is transferrin, a protein in charge of Fe^{3+} transport, while immunoglobulins represent the majority of the γ globulins.

Immunoglobulins

They are produced by plasma cells and involved in the immune response, in charge of recognizing and binding to antigens [63,84]. The most abundant immunoglobulins in plasma are IgGs and IgMs (Fig. 2). IgGs are globular proteins made of four peptide chains (two heavy and two light chains) with a total molecular weight ca. 150 kDa. IgMs are much heavier proteins constituted by one light and one heavy chain with a total molecular weight ca. 900 kDa. IgGs play a crucial role in the defense against infection, participating mainly in secondary immune response, while IgMs are the first produced by the body to fight a new infection. In general, IgGs and IgMs binding to NP surface leads to the rapid uptake of the nanomaterial by MPS [85].

Coagulation factors

They are essential for proper blood clot formation and the cessation of blood loss from damaged tissue and vessels (hemostasis) [86]. The coagulation process involves the aggregation of platelets and the deposition and maturation of fibrin. They also participate in the immune response, as blood clotting can physically trap invading microbes. Moreover, coagulation factors can contribute to the innate immune system by activating the cascade that triggers the subsequent opsonization of pathogenic molecules, microbes, or apoptotic cells. Among the different coagulation proteins present in blood plasma, fibrinogen is the most abundant one (and the fourth most abundant protein, Fig. 2). Fibrinogen is a glycoprotein that, on conversion to fibrin, forms an insoluble gel involved in blood clotting. It is also an acute-phase protein, and its levels can exceed 7 mg/mL during acute inflammation. When it binds to NPs, misfolding, aggregation, and inactivation processes can occur, which can increase NP toxicity [87].

Complement

It is a complex system that forms part of the innate immune system [63,88]. Complement is constituted by many distinct plasma proteins that react with one another to mark pathogens and enable their destruction by phagocytes (increasing the opsonization by antibodies). Complement proteins are also in charge of inducing a series of inflammatory responses to fight infections. The complement system can be activated directly by pathogens or indirectly by pathogen-bound antibody. There are three different pathways for the activation of the complement system: the classical complement pathway (designated with letter and numbers C1-9 and its products C1q, C3b, …), the alternative pathway (capital letters such as factor B and factor D), and the mannose-binding lectin pathway (such as MASP-1 and MASP-2). The activation of the different pathways occurs through the direct or indirect (via antibodies) binding of the complement proteins to antibodies previously attached to pathogens surface or directly to pathogens. In all cases, C3 protein is cleaved into fragments that trigger the complement cascade and, eventually, phagocytes recruiting. The classical and alternative pathways are the most typical routes for complement activation by NPs [89]. The presence of complement C3 in the PC of NPs is believed to increase the phagocytosis of the NPs (i.e., increases opsonization) [66].

Lipoproteins

They are complex particles that play a critical role in the intercellular transport of insoluble lipids. Lipoproteins are constituted by a hydrophobic core of cholesterol esters and triglycerides and a shell of free cholesterol, phospholipids, and apolipoproteins [90]. They are classified as a function of their size, lipid composition, and apolipoproteins type: chylomicrons, chylomicron remnants, very-low-density lipoproteins, low-density lipoproteins, intermediate-density lipoproteins, and high-density lipoproteins. Apolipoproteins contribute to the lipoprotein formation, act as ligands for lipoprotein receptors, and participate in their metabolism by activating or inhibiting enzymes. Among the different apolipoproteins, A-I and J are commonly found in the PC of NPs. Apolipoprotein A-I (Apo A-I) is a small protein that constitutes the major component of high-density lipoproteins in charge of cholesterol transport. Apolipoprotein J (Clusterin) is a highly glycosylated protein that participates in lipid transport, apoptosis, and protein folding, among other processes. The presence of these and other apolipoproteins in the PC of NPs is related to an increased bloodstream time circulation and improved ability to cross the brain barrier of the NPs [91,92].

Acute phase proteins (APPs)

One of the systemic responses to disease is the increment (positive APPs) or decrease (negative APPs) of different plasma proteins, which are known collectively as acute-phase proteins [93]. Positive APPs carry various functions for the immune system that help destroy pathogens, potentiate coagulation, and regulate inflammation responses. Among those that contribute to fighting pathogens, the C-reactive protein (CRP) and mannose-binding protein (MBP) are known to act as opsonins, helping to recognize pathogens by the immune system. CRP binds to phosphorylcholine, thereby activating the complement systems and promoting phagocytosis by macrophages. Its concentration in plasma increases significantly during acute inflammation, up to 50,000 fold. Mannose-binding protein can trigger the complement system by binding to pathogens such as bacteria, yeasts, viruses, and parasites. The presence of APPs on the protein corona could indicate inflammation and promote phagocytosis.

4. Physicochemical aspects of the NP- PC interaction

The PC nature varies as a function of the NP composition, morphology, and stabilizing ligands (chemical composition, molecular mass, and hydrophobicity/hydrophilicity) required for maintaining the NPs colloidal stability (via electrostatic and steric repulsions). These parameters affect mostly the thermodynamic of the NP-protein interactions occurring under given experimental conditions (i.e., the state with the lowest Gibbs free energy). Kinetics also play an essential role due to the dynamic nature of the PC (i.e., kinetically trap states with Gibbs free energy higher than of the thermodynamic one) [20,23,94–96]. Therefore, the identity acquired by NPs results from the complex interplay between distinct thermodynamic and kinetic parameters.

4.1. Main forces driving the NP-PC formation

The adsorption of proteins on the NPs surface is a process that develops spontaneously when the variation of the Gibbs free energy of the system is negative. While the experimental observation indicates that NP-protein interactions are generally favored, the exact driving forces might differ depending on the nature of the NPs and proteins and the experimental conditions. On one side, NPs are colloidally stable due to the larger magnitude of the repulsive forces than those of the attractive ones between them [97,98]. Van der Waals interactions are generally responsible for the attractive forces, while electrostatic, steric, or electrosteric forces constitute the repulsive side (Fig. 3). Other important interactions arise from solvation/solvophobic effects or osmotic depletion [95]. In aqueous media of high ionic strength, such as blood (i.e., ca. 150 mM), the NP-water (and ions) interaction is greater than between water-water and NP-NP. As proteins can also be considered as colloidal systems, the formation of the PC can only occur if the magnitude of the NP-protein attractive forces overcomes those of NP-water and protein-water. Finally, the repulsive forces between proteins such

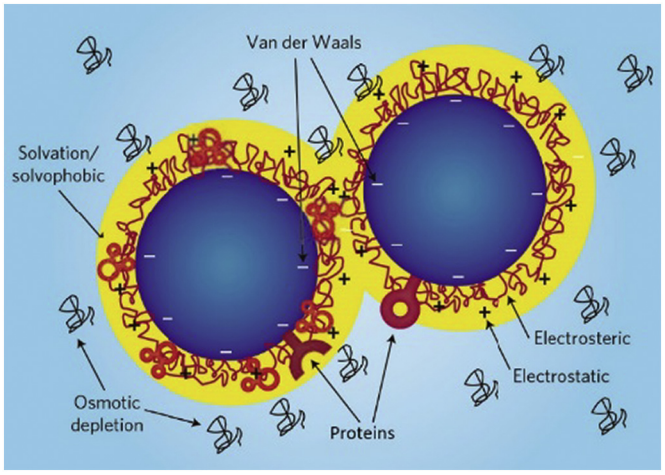

Fig. 3. Schematic draw of main forces acting on colloidal NPs, such as electrostatics, Van der Waals, steric, electrosteric, osmotic, and solvation/solvophobic effects. Modified with permission from [95].

as electrostatic or steric (i.e., increased local osmotic pressure at short distances and or excluded volume effects) help keep the colloidal stability of NP in solution after the PC formation [99].

On the other side, the complexity and variability of protein composition imply that favorable NP-protein interactions can occur in different and multiple manners [100,101]. The activity of proteins is directly related to its amino acid sequence and 3D structure. Among the 20 proteinogenic amino acids, 9 are hydrophobic (formed by short alkyl chains or aromatic rings), and 11 are hydrophilic (constituted by hydroxyl, amine, ammonium, and carboxylic functionalities) [102]. The eventual reduction of the interaction between water (and ions) and the hydrophobic residues is responsible for the 3D structure adopted by the protein. Thus, the hydrophilic residues are typically exposed at the surfaces, thereby ensuring the colloidal stability through electrostatic repulsions. In some cases, glycosylation also contributes to stability via steric repulsions [103,104]. As a result, the surface Gibbs free energy of the protein is minimized, giving rise to the most thermodynamically stable conformation (the native state) [100,101].

However, the interaction of proteins with the NP surfaces (i.e., via Van der Waals, hydrophobic, electrostatic interactions, and hydrogen bonding) can result in the emergence of potential states with lower free energy than that of the protein in its native biological environment. This phenomenon can occur via reducing the surface energy and/or a rearrangement of the 3D structure [100,101]. For instance, hydrophobic NPs can strongly interact with hydrophobic residues of the protein, subsequently leading to a reordering of the 3D structure [105–107]. Moreover, the Gibbs free energy of the NPs can also be reduced during this process, thereby leading to an even more stable NP-protein system. In general, all these phenomena are difficult to predict due to the vast array of distinct physicochemical features displayed by NPs and proteins.

4.2. The effect of NP properties

From the NP perspective, the main parameters determining PC formation are the composition, curvature, and nature of the surface ligands.

Composition

The interaction of proteins with a specific NP surface is strongly dependent on the chemical composition (Fig. 4). For instance, NPs constituted by hydrophobic materials such as polystyrene can interact with proteins through hydrophobic forces [108]. In transition metal oxides NPs (e.g., iron oxide), carboxylic groups present in the protein surface can efficiently interact with the surface metal atoms [109]. Similarly, protein thiol groups (i.e., cysteine) can bind to noble metal NPs surfaces (e.g., gold or silver) [109–111]. For example, it has been observed that Au NPs and Ag NPs of similar dimensions and surface functionalization adsorb different amounts of kininogen-1 when incubated in plasma [112]. Thus, the composition of the NPs determines which moieties of the protein interact preferentially with the nanosurface and the strength and extent of such interaction.

Curvature

The NP curvature impacts the protein adsorption on the surface and the lateral interaction between adjacent proteins [113,114]. At higher curvatures, the NP-protein contact area decreases, as well as the magnitude of their interactions. Moreover, the curvature can affect the acidity and hydrophilicity of NPs, which impact the nature of the NP-protein interactions [115,116]. The main factors governing the NP curvature are the size and shape (Fig. 4). For instance, small NPs possess higher curvatures than larger ones, which is experimentally related to suppressing the protein adsorption [113,117,118]. In general, NPs with larger dimension contains more developed PC. Thus, the amount of proteins and the relative abundance is strongly influenced by the NP size. Moreover, protein-protein interactions are favored in planar surfaces, which provides an additional mechanism for PC stabilization (e.g., via avidity

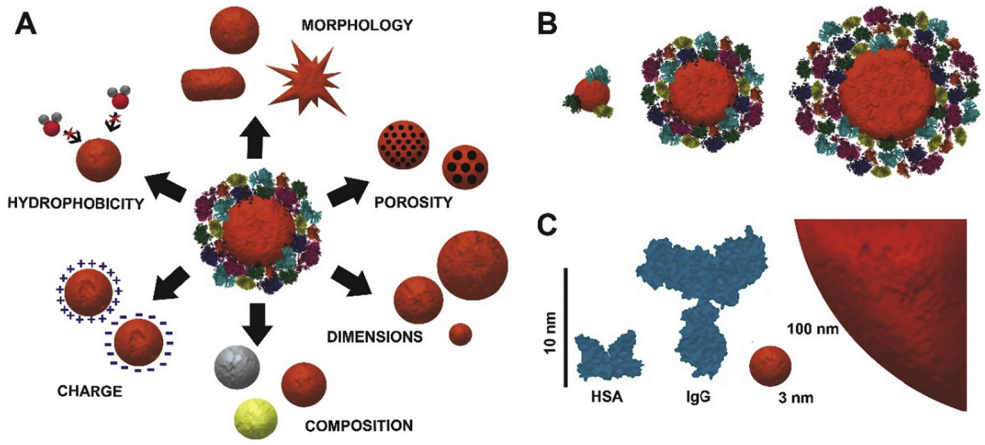

Fig. 4. (A) Main physicochemical characteristics of NPs that govern the PC formation: composition, charge, hydrophobicity, morphology, porosity, dimensions, and composition. (B) As the size of the NPs increases, the accompanied reduction of the curvature favors the formation of thicker PC. (C) Relative dimension of important proteins, such as HSA and IgG, compared with 3 and 100 nm spherical NPs.

effects) [114]. Besides, the native structure of the proteins is subjected to different tensions depending on the NP curvature, which may result in denaturation or further stabilization of the functional structure [119–122].

The curvature also varies with the NP shape, as it increases with the degree of anisotropy. Anisotropic NPs possess larger surface areas than isotropic ones, therefore providing greater support for the adsorption of proteins. For example, 70 and 40 nm gold nanostars attach significantly higher amounts of proteins than their respective 70 and 40 gold nanorods [123]. This fact can be explained by the larger surface area of the gold nanostars. In both cases, smaller NPs showed a lower amount of adsorbed proteins, an expected consequence of their higher curvature. Similarly, a recent investigation focused on mesoporous silica nanospheres and nanorods revealed the presence of a higher amount of proteins on the anisotropic species [124].

Surface ligands

This is probably the most critical parameter, as most NPs are covered by a shell of surface ligands that maintain NP colloidal stability by providing sufficient electrostatic, steric, or electrosteric repulsions [97]. Surface ligands determine aspects such as the NP charge and the hydrophobicity, thereby affecting the proteins binding affinity and specificity [20,21,95]. Due to the negative surface charge presented by most of the proteins at physiological pH, NP stabilized by positively charged ligands (e.g., bearing amino and ammonium moieties) tend to adsorb a higher number of proteins [112,125–127]. Cationic ligands are also found to increase conformational changes in the adsorbed proteins [76,128]. Moreover, the magnitude of the surface charge is also relevant, as thicker PCs are often observed at higher ζ potentials [129]. Related to the nature of the adsorbed proteins, neutral NPs generally show a narrower range of different proteins respective to the charged ones [130]. Besides the surface charge, the hydrophobic/hydrophilic nature of the ligands also plays a key role in the PC formation. Hydrophobic surfaces tend to provide more stable protein adsorption and more significant modifications of the protein folding (via interactions with the hydrophobic residues located in the interior of the protein 3D structure) [105–107]. The latter can result in the loss of protein functionality or protein aggregation, eventually, lead to toxic effects. For these reasons, the design and study of surface ligands with specific composition and structures have been a focus of intense research to control PC formation. In the following section, we will discuss relevant strategies related to the surface functionalization of NP to avoid or control the adsorption of proteins.

Other parameters, such as the surface roughness, porosity, and crystallinity of the nanoparticles, also contribute to PC formation [131]. For instance, in a recent investigation carried out on mesoporous silica NPs, the PC was enriched with proteins of low molecular weights (<50 kDa, 60, and 80% of the total mass of adsorbed protein) when compared to non-porous NPs. These differences were attributed to size-exclusion effects arising from the porous structure.

4.3. Dynamics and structure of PC

In practice, NPs introduced in biological fluids can contact hundreds of different proteins, having different affinities towards the NP surface. For instance, plasma blood contains more than 3500 different proteins, with concentrations that can differ several orders of magnitude. Therefore, such plasma proteins can interact with the NP surface in a complex, dynamic, and competitive process [27,132–134]. This phenomenon was first described by Leo Vroman and Ann Adams in the late 1960s [135], and it is nowadays known as the "Vroman effect" [136,137]. Initially, proteins with higher mobility (smaller proteins) and concentration, such as albumin, are adsorbed on the surface. Over time, they are replaced by those proteins with higher affinity, e.g., fibrinogen or fibronectin [138].

In general, it is possible to differentiate two different coronas: tightly bound proteins form the so-called "hard corona," while loosely bound proteins interacting with the hard corona proteins compose the "soft corona" [94,139–144]. Some authors also refer to the hard and soft corona with their spatial arrangement, i.e., the hard corona is constituted by proteins directly adsorbed on the particle surfaces, and the soft corona corresponds to the outer layer. Therefore, the PC can be seen as a multilayered structure of proteins with different affinities for the nanomaterial surface. Nevertheless, as the PC formation is a very dynamic process (i.e., closely related to the Vroman effect where different proteins adsorb and desorbed at different rates), the boundaries between "soft" and "hard" corona are often very diffuse. Thus, it is extremely complicated to define a transition between them. For these reasons, the characterization of the PC using different methods can provide distinct results and represents one of the most challenging aspects of the PC formation on nanoparticles.

A recent investigation focused on the PC structure has shown that not all proteins in the PC can bind to their biological targets, which can be a consequence of their organization around the NP [145]. It was observed that protein-protein interactions were responsible for the organization of the PC in an assembly-like structure, constituted by multilayers of protein. It was then proposed the existence of three different layers: the foundational, assembly, and binding layer (Fig. 5). The foundational shell is formed by those proteins directly bound to the NP surface, while the assembly layer was constituted with the cognate proteins. Additional proteins can bind to the cognate ones, giving rise to the binding layer, which links to cellular receptors.

Additionally, the route of administrations is also an essential factor that affects the PC structure due to the different composition of body fluids and organs [146]. For instance, when NPs are introduced through the lungs (inhalation), they are first exposed to a fluid that is rich in lipids and surfactant proteins (80% and 20%, respectively) [147]. The blood proteins then replace the PC formed in the lung once the NPs reach the bloodstream. A similar phenomenon occurs for ingested NPs that are in contact with saliva and gastrointestinal fluids, constituted primarily by enzymes.

It is worth noting that the characterization of such complex and dynamic protein assemblies in the PC has been afforded thanks to the rational combination of different methods. For high-affinity proteins, techniques such as protein quantification assays (protein amount), gel electrophoresis (protein pattern), liquid chromatography-mass spectrometry (LC-MS, protein pattern), Fourier transform infrared (FTIR)

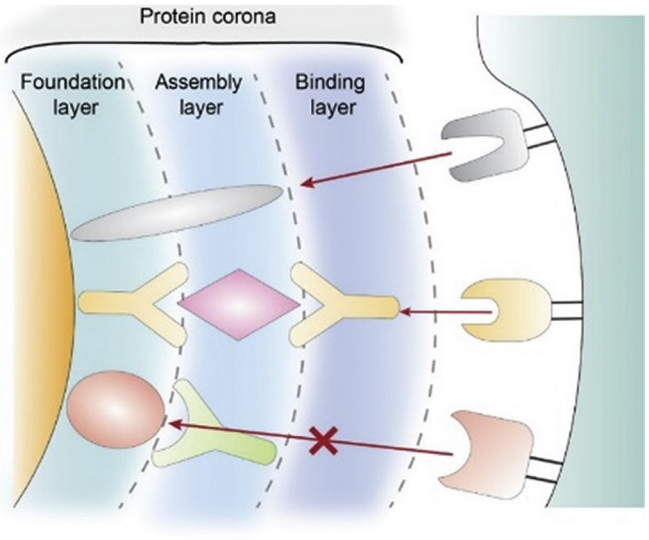

Fig. 5. Proposed structuration of the protein corona where it is possible to find at least three different layers: the foundation, assembly, and binding layers, which result from NP-protein and protein-protein interaction.
Modified with permission from [145].

and circular dichroism (CD) spectroscopies (protein secondary structure), sum frequency generation and second harmonic generation (SFG and SHG, respectively; orientation, hydration and secondary structure of the protein), X-ray photoelectron spectroscopy and time-of-flight secondary ion mass spectrometry (XPS and Tof-SIMS, respectively; protein amount, secondary structure on flat surfaces and orientation) and scanning force microscopy (SFM, surface topography, dynamics of proteins). For low-affinity proteins, other techniques are typically employed: dynamic light scattering (DLS, size changes, aggregation detection, intensity fractions), fluorescence correlation spectroscopy (FCS, size, concentration, binding affinity), isothermal titration calorimetry (ITC, protein binding affinity, stoichiometry, enthalpy, and entropy) and quartz crystal microbalance with dissipation monitoring (QCM-D, the hydrated mass of protein films, properties of viscoelastic films) [148]. The hard and soft corona composition analysis has provided essential information to the engineered specific functional systems in an independent manner.

5. Modulation of nanoparticle-protein corona interactions

As discussed in previous sections, important issues encountered with the formation of PC on NPs relates to their rapid clearance from the body and loss of the targeting properties. Most strategies explored in the last decade tackle such issues by increasing the ability of the NPs to avoid the uptake by the immune system and increase recognition by the targeted cells and organs [29,31,149]. While parameters such as composition, shape, and size of the NPs need to be considered, the nature of the surface often plays a critical role in determining the clearance of the NP. The use of highly hydrophilic and neutral polymers such as polyethylene glycol (PEG), zwitterionic ligands, and the preformation of a personalized hard corona can be considered as the most relevant strategies.

5.1. Neutral hydrophilic polymers

Highly hydrophilic non-charged polymers are widely used for industrial and medical purposes due to their chemical stability, high biocompatibility, and low toxicity, being polyethylene glycol (PEG) the most investigated one [150–152]. In the field of NP synthesis and applications, PEG is probably one of the most researched ligands due to its ability to provide colloidal stability in a wide range of solvents (e.g., water, chloroform, or toluene) and medium with high ionic strength, including biological fluids [153,154]. Moreover, in the presence of blood plasma, NPs functionalized with PEG present a significant reduction of the non-specific attachment of proteins, thereby emerging as one of the first materials explored to avoid the PC formation and as antibiofouling agents [155–158]. Such properties of PEG might be ascribed to a water solvation effect, which makes energetically unfavorable the exchange of the proteins with water molecules of the hydrated polymer chain [159–161]. Entropic contributions to the protein repellent properties arise from an increase of the osmotic pressure (due to water displacement by the proteins) and steric interactions (loss of PEG chain configurational entropy when compressed by the adsorbed protein). The dehydration of ethylene glycol units and the increase of interfacial free energy of the polymer with protein are the main enthalpic contributions [162–164].

Although the influence of PEG on the adsorption of proteins can vary as a function of the molecular weight and polymer chain architecture, the surface density of PEG is typically the most critical parameter. In general, a more efficient reduction of PC formation is achieved at increased grafting densities (Fig. 6A) [107,165,166]. The magnitude of the protein adsorption may arise from the distinct conformation acquired by the PEG chains on the NP surface. At low grafting densities, the polymer chain adopts a “mushroom” conformation due to collapse on the surface. A “brush” conformation is observed at high grafting densities when the chains interact with each other and completely extend away from the NPs surface. However, the grafting density effect can be entangled with the impact of the molecular weight, as revealed by computational experiments. Molecular dynamic investigations performed on the adsorption of human serum albumin showed an increased stealth effect with the length of the chain [167]. Thus, PEG of higher molecular weight might repel more effectively the adsorption of proteins. In practice, the longer PEG chains are also more advantageous to maintain the colloidal stability of NPs. In this context, the NP size effect was found to affect the PEG graft density required to acquire the brush conformation. Smaller NPs possess larger curvature that reduces the lateral interaction of PEG chains and therefore favors the” mushroom” conformation [165]. Aiming to determine the most efficient PEG configuration on NPs surfaces, other strategies have focused on using looped or crosslinked PEGs, probing their enhanced stealth effect compared with linear PEGs [168,169].

Nevertheless, the most significant contribution to the stealth effect of PEG arises from its ability to enrich the corona with proteins such as clusterin and apolipoprotein, which act as dyopsonin and avoid the recognition of the NP by the immune system. For instance, polystyrene NP functionalized with PEG were found to possess PCs constituted by more than 70% of lipoproteins, which was correlated with a low macrophage (Fig. 6B) [155].

Unfortunately, in recent years it has been observed that the continued administration of PEGylated NPs can induce the development of anti-PEG antibodies [170]. In this case, the clearance of the NPs from the body was accelerated [171]. For these reasons, the search for polymers with similar stealth properties as PEG but with lower activation of the immune system is nowadays attracting significant interest. The

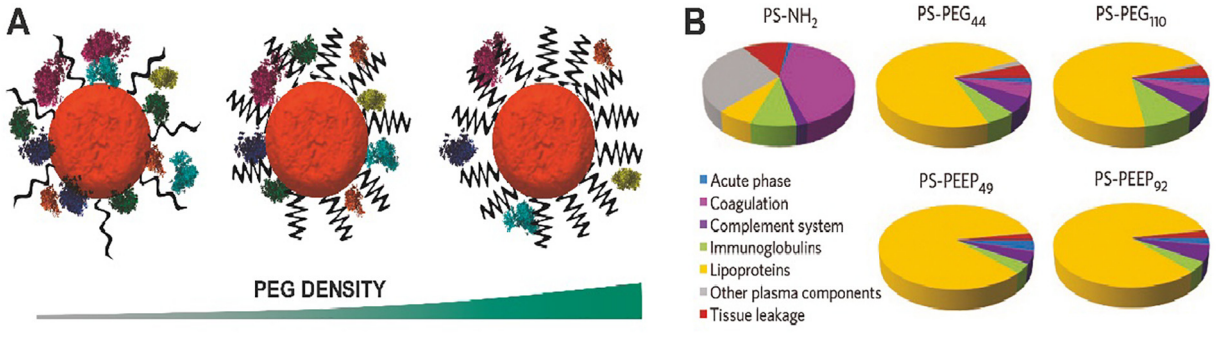

Fig. 6. (A) Schematic draw that shows the effect of PEG density on the protein adsorption. At low PEG density, the polymer chains adopt mushroom conformation, which flexibility allows the adsorption of proteins. However, increasing PEG density gives rise to a change in polymer chains to a more rigid state, i.e., brush conformation, which impairs the protein adsorption. (B) The protein patterns of nanoparticles functionalized with PEG and PEEP show a marked increase of apolipoproteins adsorption than the amino functionalize NP. Modified with permission from [155].

most promising ones are based on polyphosphoesters, polyglycerol, and hydroxyethyl starch due to their probed biocompatibility and low toxicity [155,172,173]. For further information about the use of PEG for biomedical applications of NPs rising concern about its immunogenicity, we recommend interested readers different recent publications dedicated to this topic [174–176].

5.2. Zwitterions

One of the most important functions of mammalian cell membranes is to avoid the non-specific interaction with surrounding proteins. It is worth noting than 50% of mammalian cells membrane is constituted with lipids such as phosphatidylcholine and phosphatidylserine, which contain cationic and anionic moieties in equal proportions [177]. These lipids, known as zwitterions, are recognized as powerful antifouling agents. As for PEG polymers, such an effect is most probably caused by their strong interaction with water molecules [164,178,179]. In the case of zwitterions, the presence of two functional groups with opposite charges in the same molecule can induce the water solvation molecules to organize in a similar fashion as that found in the bulk water (Fig. 7) [180]. This hydration arrangement around the zwitterions results in no free energy gain during their replacement by proteins (i.e., adsorption driven by interfacial energy change is not favored) [164,178,179]. Indeed, the interaction with water molecules in zwitterions is stronger than in neutral hydrophilic polymers, where it is formed through hydrogen bonding instead of ionic solvation.

An additional entropic contribution to the antifouling effect of zwitterionic systems has been recently proposed [181]. For charged surfaces, the adsorption of charged proteins gives rise to the formation of ion pairs accompanied by the release of counterions (i.e., two counterions per ion pair). During this process, water molecules are released from the hydrated counter ions giving rise to a favorable entropy increase. For attractive interaction between the charged surface and protein, where the enthalpy variations is closed to zero, the association process is then entropically driven. In the case of zwitterions, the absence of ions impairs the release of counterions, and the process is entropically unfavorable, thereby giving rise to an antifouling effect. It is worth noting that in PEG and other hydrophilic neutral polymers, the same principle applies [181].

However, zwitterions become more hydrated as the concentration of NaCl or other salts increases (i.e., they screen the electrostatic attractions between zwitterions) while PEG chains collapse [182]. Thus, the antifouling effect in a biological medium such as blood (ionic strength ca. 150 mM) is enhanced for zwitterionic molecules and polymers while it decreases for PEG. This phenomenon might also contribute to explain the ability of zwitterions to avoid the adsorption of proteins.

The nature of the water interactions with zwitterions and zwitterionic polymers, and its effect on the adsorption of the PC, depends on: the zwitterions molecular weight, the nature and distance between the ions, the chain conformation in the case of zwitterionic polymers, and the concentration of salt [154,173–175]. The most common cations are quaternary ammonium salts, while sulfite (sulfobetaines), carboxylates (carboxybetaines), and phosphates (phosphorylcholines) are typically used as anions [154,173]. Moreover, zwitterions are known to improve the stability of proteins, as they can enhance the interactions (i.e., hydrophobic mainly) responsible for the protein folding into the functional conformation [178,179].

Overall, the ability of zwitterions to avoid interactions with proteins and other biological molecules and structures such as cell membranes might thus explain the low toxicity and long blood circulation time of NPs stabilized by zwitterionic ligands. Moreover, the colloidal stability of NPs in high salt concentration increases with the use of such a class of ligands. A representative example of the effect of zwitterionic molecules on NPs stability, protein adsorption and cytotoxicity was recently reported [183]. They observed that when the surface of silica NPs were solely functionalized with amine moieties, protein adsorption was pronounced and its hemotoxicity and cytotoxicity. However, partial or total replacement of the amine groups by zwitterionic ligands resulted in little or no PC formation. Moreover, minimal hemotoxicity and cytotoxicity were observed regardless of the NPs concentration.

5.3. Predesigned hard corona

While the search of methods for avoiding PC formation centered most efforts during the last decade, recently reported strategies strongly suggest that it is possible to control the fate of NP in biological media through the PC formation. The critical aspect enabling to design NP

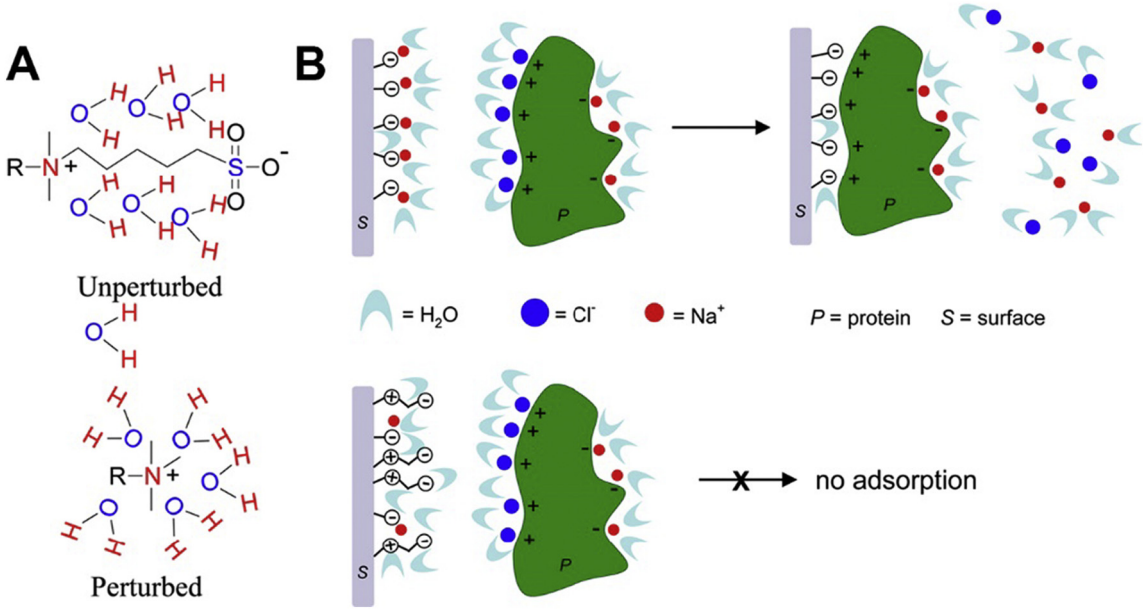

Fig. 7. Schematic draws of (A) the arrangement of water molecules around zwitterionic (upper) and ionic molecules (lower), and (B) the protein adsorption process to zwitterionic and ionic molecules. The former is facilitated by the thermodynamically facilitated releasing of ion pair.
Modified with permission of [164].

with efficient targeting capabilities is the assumption that the PC formation is typically an avoidable process, and we should take advantage of it to obtain the desired NPs functionality. In practice, this means the fabrication of NP functionalized with a predesigned hard corona.

For example, the over-expression of the HER2 plays a vital role in certain types of breast cancers, and therefore the development of NP capable of targeting such receptor might open access to selective delivery of chemo-drugs. Towards this aim, a recently reported approach focused on the functionalization of mesoporous silica NPs with a human epidermal growth factor receptor HER2-binding affibody [184]. By controlling the HER2-binding affibody orientation on the NP surface, it was possible to reduce the number of serum proteins bound to the NP surface. Compared with NPs functionalized only with PEG, the amount of adsorbed proteins was more than an order of magnitude lower, demonstrating the effectiveness of predesigning the hard corona (Fig. 8A). Moreover, the avoidance of phagocytosis by macrophages was also observed, while the up-take by the HER2-receptor-overexpressing cancer cells was enhanced. In accordance, improved targeting efficiency and prolonged half-life in the body compared with PEG were noticed in the in vivo experiments.

In a different approach, it was aimed at altering the IgG-binding properties in the PC to reduce the recognition by macrophages [145]. To demonstrate the suitability of this idea, it was proposed a workflow termed knockout assisted binding activity modification where 60 nm gold nanospheres were incubated with depleted plasmas for C3, C4, Antithrombin III, Fibronectin, Factor V, or Fibrinogen, i.e., the main proteins that bind IgG. In all cases, the strategy was able to tune the functionality of IgGs due to depleting its cognate protein (or binding pair) and thereby rearrange the corona. This strategy relies on the idea that the PC is constituted by different proteins organized in different layers due to protein-protein interactions. In the absence of Factor V, fibrinogen, antithrombin III, and fibronectin, the number of IgGs capable of binding to their target was decreased. These results suggest that such proteins support the IgG-binding during the PC formation. However, when C3 and C4 were depleted, a larger fraction of active IgG was noticed, suggesting that both C3 and C4 block the binding ability of IgGs in the PC.

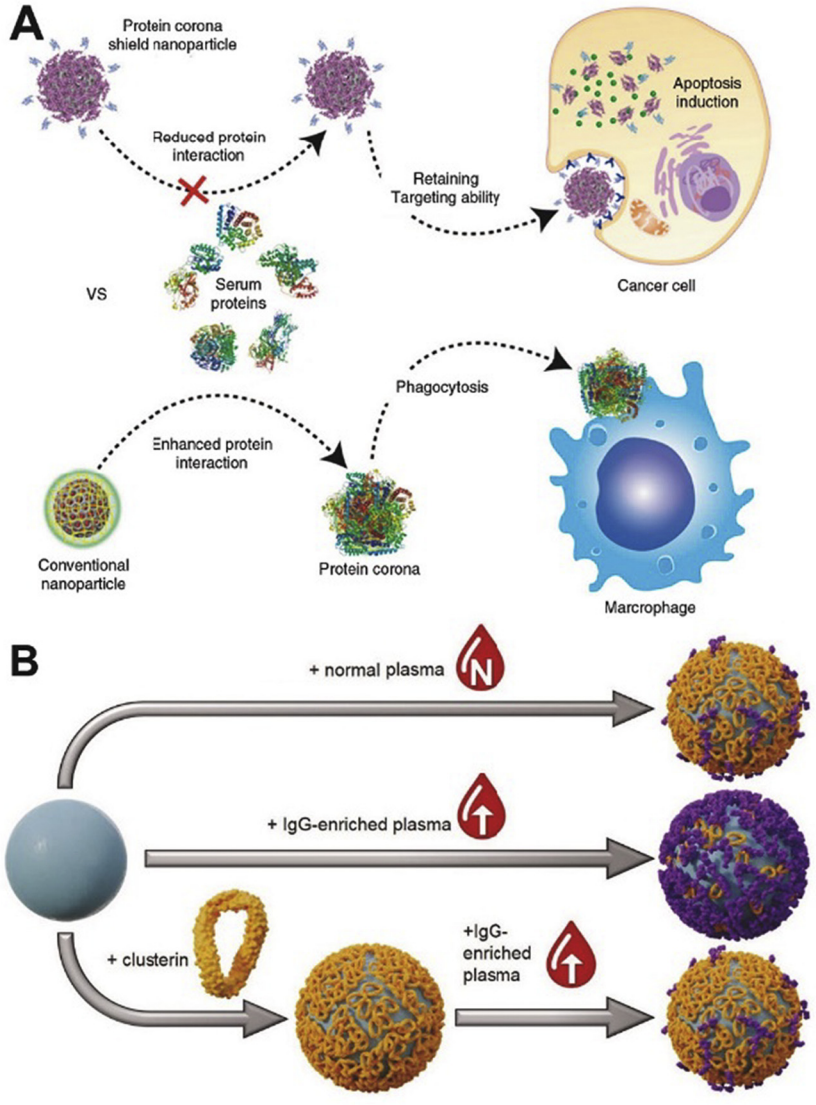

Fig. 8. (A) Schematic draw of NPs with a preformed hard corona made of HER2 affibody, which helps maintain the targeting while reducing the PC formation and avoiding macrophage uptake compared with conventional NPs. Modified with permission from [184]. (B) Schematic overview of the NPs incubated with normal plasma (top) and IgG-enriched plasma (middle), and the reduction of the IgG adsorption in IgG enriched plasma of NPs preincubated with clusterin (bottom).
Modified with permission from [22].

In the same perspective, the stealth effect of the clusterin was used to reduce the IgG adsorption on NP [22]. PEGylated polystyrene NPs (PS-NPs) and hydroxyethyl starch nanocapsules (HES-NCs) were first exposed to two different human plasmas containing the standard and double concentration of IgG (Fig. 8B). The amount of IgG was up to 40 times higher when the IgG concentration was doubled for both types of nanoparticles PS-NPs and HES-NCs, while clusterin was predominantly found in the case of the normal plasma. These results suggest that elevated IgG levels suppress clusterin-surface interactions. As a result, a significantly increased uptake of the NPs was observed in human and murine macrophages. Based on the noticed experimental results, precoating with clusterin was hypothesized to reduce the IgG adsorption. The protein corona composition found on NCs preincubated with clusterin and incubated with enriched IgG plasma was similar to those previously observed for normal plasma experiments. Therefore, the precoating by proteins providing stealth effects such as clusterin was successfully revealed as a strategy to avoid the adsorption of IgG and thereby reduce the NP uptake by macrophages [22].

Another appealing approach to avoid recognition of NPs (in this case, SNAs, i.e., gold NPs functionalized with dense DNA shell) by the immune system was based on the incubation with antihuman epidermal growth factor receptor 2 (anti-HER2), IgG, or HSA. Here, simple electrostatic NP-protein interactions were responsible for the formation of the hard corona. To determine the targeting ability of this approach, NPs covered by an anti-HER2 corona were incubated with a mixture of HER2+ and HER2− cells. The observed results revealed that the uptake by the HER2+ cells was comparatively higher than that in the HER2− cell populations. Moreover, the ability of SNAs to hybridize with complementary nucleic acid oligomers was maintained regardless of the PC. The suitability of this approach to target or avoid macrophage cells was investigated by coating the NPs with IgG and HSA, an opsonin, and a dyopsonin, respectively. As expected, the uptake by macrophages in the case of HSA corona was reduced compared to bare NPs. However, the IgG corona also reduced the up-take, contrary to what is expected for IgGs.

6. Conclusions and outlook

The advancement of chemical methods for the synthesis of colloidal NPs have provided new platforms for novel imaging and therapy technologies in nanomedicine. However, the functionality of NPs can completely be altered after exposition to biological fluids such as blood due to the formation of a corona principally composed of surrounding proteins. This review looks into the PC formation process and its impact on colloidal NPs for application in diagnosis and therapy. The PC modifies the NP behavior, leading in most cases to their rapid clearance from the body, cytotoxic effect, and loss of the targeting ability. Here, the characterization of the driving forces involved in the PC formation is of foremost importance to engineering NP with desired and specific functionalities suitable for application in nanomedicine. Nevertheless, accomplishing such a task represents a multifaceted challenge due to the complex composition of biological fluids and the wide variety of available NPs.

The distribution of NPs in the body typically occurs through the bloodstream, which contains more than 3500 proteins. This implies that NPs are in contact with complex media, containing various proteins in different concentrations and possessing different compositions, molecular masses, and 3D conformations. Moreover, they carry different functions in the immune system, transport of lipids or maintaining the osmotic equilibria, among others. In this sense, the preferential adsorption of certain proteins and their biological function radically impact the NP fate. In general, preferential attachment of proteins that activate the immune system leads to a rapid clearance of the NPs from the bloodstream, thereby impairing them to reach the target. Nevertheless, for a given blood composition, the amount of adsorbed proteins and their arrangement in the PC are eventually determined by the NPs nature. The complex interplay between the NP composition, curvature (dimensions and morphology), and surface ligands likely govern the binding affinities of the blood proteins to the NPs. While the PC formation occurs immediately after NP introduction in the biological fluid, the most thermodynamically stable PC is formed after some hours, once the most abundant proteins are replaced by those with higher NP surface affinity but found in lower concentrations.

Among all the different parameters that determine the PC formation, it seems that the key for successfully controlling the fate of NPs relies on their surface chemistry. It is reported that the use of appropriate surface ligands enables suppression of the PC formation partially. That is the case of neutral hydrophilic polymers, such as PEG and polyphosphoesters. Besides, these polymers enrich the PC with "stealth" proteins such as clusterin. Although PEG is probably one of the most widely used polymers to design efficient NPs for biomedicine, it presents important drawbacks, such as the potential to induce the formation of anti-PEG antibodies and activate the immune system. Moreover, it can reduce the cell uptake and the release of drugs, which impair their desired delivery (i.e., this issue is known as the PEG-dilemma). Complete suppression of the protein adsorption and issues related with PEG can be achieved with the use of zwitterionic ligands due to their ability to interact with water molecules strongly. More recent strategies have focused on the predesign and, therefore, engineering of hard coronas capable of avoiding immune system recognition while maintaining the targeting capabilities. Either by directed attachment or simple electrostatic interaction, NP can be functionalized with different proteins that eventually determines the PC. Such strategy relies on simple concepts that open access to more personalized NPs, for instance, by functionalization of the NPs with selected proteins previously extracted from the patient. The formation of a predesigned and personalized corona is probably the strategy holding the most potential, as it should enable more precise control of the NP biological behavior. Thereby, the targeting efficiency can be enhanced while the immune response is minimized. This represents a tremendous advantage compared with other strategies based on neutral hydrophilic polymers or zwitterions, mainly focused on avoiding the immune response and toxic effects via impairing the PC formation.

Unfortunately, essential issues remain and need to be overcome to develop effective and safe technologies for medicine based on colloidal NPs. One of the most critical drawbacks existing in the analysis and comparison of PC formation on distinct NPs is the variability of the incubation conditions and the composition of the biological medium. While the effect of the NP properties has attracted most of the attention, the biological environment has often been overlooked. These facts might explain the low success rate of NPs in clinical trials, despite the promising results previously obtained during in vitro and in vivo experiments. Understanding the effect of the physiological conditions of the patients on the biological fluids such as blood plasma used to investigate the phenomenon of PC formation might provide better fundaments for the design of better NP systems for nanomedicine.

Declaration of Competing Interest

The authors declare no conflict of interest.

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