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Protein Corona in Nanomedicine

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Highlights

1. Protein corona is a physiological protein layer around nanoparticles, formed by soft and hard corona.
2. Protein corona composition depends by chemical-physical properties of nanoparticles and by composition of environment.
3. Protein corona controls the biological fate of nanoparticles, favouring or preventing their biodistribution and their targeting capacity.
4. The main future challenge is to personalize protein corona around nanoparticles to promote the drug's targeting and delivery.

Abstract

Nanomedicine is an interdisciplinary field of research, comprising science, engineering, and medicine. Many are the clinical applications of nanomedicine, such as molecular imaging, medical diagnostics, targeted therapy and image-guided surgery. Despite major advances during the past 20 years, many efforts must be done to understand the complex behaviour of nanoparticles (NPs) under physiological conditions, the kinetic and thermodynamic principles, involved in the rational design of nanoparticle. Once administrated in physiological environment, NPs interact with biomolecules and they are surrounded by protein corona (PC) or biocorona. PC can trigger an immune response, affecting NPs toxicity and targeting capacity. This review aims to provide a detailed description of biocorona and of parameters, able to control PC formation and composition. Indeed, the review provides an overview about the role of PC in the modulation of both cytotoxicity and immune response as well as in the control of targeting capacity.

KEYWORDS:

Biocompatibility; Cytoxicity; Nanomedicine; Nanoparticles; Nanotherapeutics; Protein Corona.

Abbreviations

MPS: Mononuclear Phagocyte System

PC: Protein Corona

HC: Hard Corona

SC: Soft Corona

NPs: NanoParticles

Au: gold

HAS: Human Serum Albumin

Ab: Antibody

SPIONS: SuperParamagnetic Iron Oxide Nanoparticles

PEG: PolyEthilene Glycol

FDA: Food and Drug Administration

1. Introduction

Over the last 20 years, nanomaterials have been employed in different scientific and industrial fields, like agriculture, textile, cosmetics, packaging and mostly pharmaceutical research for medical purposes (1-3). The use of nanoparticles (NPs) for diagnosis, monitoring, control, prevention and treatment of diseases have contributed to the beginning and the development of Nanomedicine (4-7). Engineering NPs are studying as drug delivery systems in cancer therapy, as vaccine delivery vehicles and as diagnostic tools in medical imaging (8-14). Nevertheless the promising potential of NPs, it is important to consider that their biological identity and, consequently, their fate *in vivo* depend by the type of interactions with biological fluids, cells and all physiological biomolecules, such as proteins (15-17). In fact many physicochemical parameters of NPs, like morphology, size, shape, surface chemistry, are responsible for the creation of a new identity, but also the composition, the pH and the temperature of physiological environment may play a fundamental role (18-23). In biological environments, NPs immediately interact with biomolecules, mostly proteins but also carbohydrates, lipids and peptides, in order to create a layer called protein corona (PC) or biocorona. The composition of protein corona has numerous biological implications like the control of interactions with cell, the induction of cytotoxicity, the achievement of optimal targeting and the possible modulation of drug's pharmacokinetics (24-30). In fact after administration, NPs may adsorb different kinds of proteins: some of them are defined as opsonins, which can be recognized by mononuclear phagocyte system (MPS) avoiding the blood circulation and the targeting, some others are dysopsonins able to improve the biodistribution and the drug's delivery (31-34). Despite the protein corona composition could enhance NPs colloidal stability *in vivo*, remodelling the identity of nanomaterials, some studies reported that NPs targeting ability can be reduced (35, 36). In-depth investigation of biocorona is fundamental to understand its role in blood circulation and to minimize the

adverse effects, but the major limit of PC exploration in vivo is the great individual variability present in biological fluids, that prevent a reliable prediction of corona layer (37, 38).

In this review, the formation of protein corona is deeply described, focusing attention on parameters able to control the adsorption of proteins onto NPs surface. Afterwards, the role of biocorona and NPs parameters, involved in the improvement the biocompatibility and in the minimization of toxicity, will be discussed. The final part of this review will be devoted to an overview of future challenges in nanomedicine.

2. Formation of protein corona and affected parameters

2.1 Protein corona or biocorona

After administration in human body, NPs are immediately in contact with biological fluids, like blood plasma, and the interaction with physiological biomolecules rapidly occurs. The adsorption of plasma proteins allows to form a thick layer on nanoparticles surface, called protein corona or biocorona (39). The formation of biocorona is a dynamic process, explained by "Vroman effect", that involves different forces between nanomaterial and proteins such as H-bonds, Van Der Waals forces, π–π stacking binding, electrostatic and hydrophobic interactions, as reported Table 1 (40-42). During the initial stage, high abundance and low-affinity proteins, present in biological fluids, are rapidly adsorbed onto NPs surface; however, they are replaced by lower abundant and higher-affinity proteins over the time during the nano-bio interaction (43, 44). The adsorbed proteins onto NPs surface form a layer, that could be divided in "hard corona" (HC) and "soft corona" (SC), as shown in Figure 1. Proteins, belonging to HC, directly bind NPs surface with high affinity and they create a stable and non-exchangeable layer, mainly involved in the definition of new biological identity In fact HC is responsible of NPs fate, controlling membrane adhesion, cellular

signaling pathways, biodistribution and interaction with surrounding cells (45-47). The proteins, present in SC, indirectly bind NPs surface because they interact with components of HC, forming a highly exchangeable layer dependent by the type of biological environment (48, 49).

The PC composition and thickness change the properties of NPs, influencing its cellular fate, the biodistribution and the targeting capacity. In general, the formation of nanometric discontinuous monolayer around NPs could improve their new biological identity, conserving the shape and the conformation of proteins (50, 51).

The factors, that play an important role in the formation of PC in vivo and consequently in the cellular fate of NPs, are correlated to nanomaterials, like dimension, morphology, surface properties, but also to environment, like type and composition of biological fluid, pH and temperature, as shown in Figure 2 and deeply discussed below (52, 53).

2.2 Morphology and size of NPs

The type and the shape of NPs influence the interaction with proteins, controlling the absorption of specific biomolecules, the thickness of PC and the possible conformational change of bound biomolecules. The effect of surface curvature may induce different binding affinities in proteins. For example, it has been demonstrated that gold (Au) nanorods have higher binding capacity than Au nanospheres because the linear shape increases the packing concentration of bound proteins (54). Indeed, Au NPs shape has played an important role in the affinity and in the induction of conformational change of adsorbed proteins (55). Indeed, a more recent study has proved that core material of NPs (gold vs. silver) could influence the type and the quantity of proteins, forming the biocorona (56).

Another important NPs physicochemical factor, involved in the influence of PC composition, is the size, strictly related to binding constants. For example, the binding constants of human serum albumin (HSA) and of γ-globulins have been enhanced increasing the size of Au NPs because the hydrophobic interactions between proteins and nanomaterials were promoted (57). Again, small size of PEGylated Au nanoparticles has reduced the adsorption of plasma proteins more than the correspondent big ones (58). The investigation of PC, formed on citrate-stabilized Au nanoparticles, has revealed that small NPs were characterized by thinner protein layer than big ones (59). Another recent study has evaluated the in vivo PC formation after injection of Au nanostars, characterized by dimensions of 40 and 70 nm. The thicker layer of proteins was formed around 70 nm NPs, whereas a more complex biocorona was found onto 40 nm Au nanostars (60).

NPs size contributes not only to control the thickness of PC, favouring the interaction with environmental proteins, but also to change kinetics of adsorption and to modulate biocorona's composition, selecting the components of surface layer.

2.3 Surface properties

The NPs physicochemical properties promote the chemistry modification and the surface functionalization, changing the possibility of interaction with physiological proteins. One possibility is to alter or to neutralize the surface charges of NPs, increasing or decreasing the binding affinity of proteins. Indeed, the presence or the absence of specific charges could drive the formation of specific interactions with proteins, favouring electrostatic and ionic forces or hydrophobic and π–π interactions, respectively. As a consequence, the composition of protein corona depends by the type of weak interactions, performed between selected proteins and NPs surface. When specific bonds are created, they could markedly modify the conformational stability of adsorbed biomolecules. In fact, it was demonstrated that proteins

have changed their structures by interaction with charged surface of NPs, while they maintained their native structure after binding NPs surface covered by neutral ligands (61-62).

Chemistry gives the possibility to improve NPs colloidal stability, modifying their surface with selected chemical groups or coating the surface with specific ligands. One well-known modification of NPs surface, adopted to prevent their aggregation, is the binding of citrate groups as sources of negative charges. The effects were showed in different nanomaterials, for example in Au and Ag NPs (63-65). For example, citrated-capped gold nanoparticles were more abundantly internalized in cells, exerting higher cytotoxic effects (64). The preferred choice to add negative charges than positive ones, onto NPs surface, is suggested by numerous proofs that in biological fluids, NPs coated with positive groups were easly phagocytosed by opsonization, reducing their circulation time (66, 67). In addition, they seemed to promote other uptake mechanisms, like endocytosis (68).

Another strategy in the surface modification is the pre-coating of NPs with biomolecules, like proteins, peptides and polysaccharides, and with polymers, able to modulate PC formation and also to confer a protective layer against uncontrolled protein attachment (69). For example, paclitaxel, anti-cancer chemotherapy drug, was encapsulated into chitosan-coated nanoparticles, designed as oral delivery system. The hydrophilic and positive charged surface have enhanced the intestinal permeability of NPs and the homogenous distribution (70). Indeed, the protein corona, formed around silica NPs coated with gamma-globulin, has reduced the phagocytosis of macrophages: despite the presence of many immunoglobilins, able to favour the opsonization, many other components of biocorona prevented the binding of NPs with macrophages (71).

Also zwitterion biomolecules, such as cysteine, have been used to hinder corona formation. A recent study, performed in cancer cells, has reported the higher targeting efficacy of silica NPs coated with cysteine. These functionalized NPs, conjugated with the biotin-targeting agent, have reduced the proteins adsorption onto surface and increased the targeting capacity, favouring the substrate attachment and cellular uptake (72).

As regards NPs pre-coating surface with polymers, the Poly Ethylene Glycol (PEG) is mostly used for its biocompatibility and its capability to prevent non-specific protein adsorption. Many studies were performed PEGylating NPs of oxides or noble metals (73-76). Moreover, many superparamagnetic iron oxide nanoparticle were PEGylated not only to improve the pharmacokinetics in drug delivery systems, but also to develop new radiolabelling imaging tools. Some researches have proved that the optimum conditions for mitigating the protein corona were due to use PEG chains with densities around 1 nm^{-2} and with a molecular weight between 2000 and 10,000 g mol^{-1} (77-79). Indeed, a monolayer on PEGylated NPs has been measured 1.5 nm thicker than biocorona reproduced in same conditions on bared NPs. This monolayer was found to reduce the intracellular uptake of PEGylated NPs by 10% (80). However, the formed protein corona also contained opsonin proteins in relation with density, thickness and conformation of PEG layer (81, 82).

2.4 Biological environment

The type and the composition of biological environment play an important role in the generation of protein corona: the PC components on the same NPs could change in relation with the type of incubated fluids (83). Despite many studies were performed using simple protein standard solutions, human plasma and serum are preferentially selected as more reliable in vitro models for blood proteins adsorption. The main difference between these two biological fluids is the absence of fibrinogen in serum, due to its conversion in fibrinogen clumps without any anticoagulants. For this reason, the same type of carbon nanotubes has

adsorbed more fibrinogen if incubated in plasma and more complement factors if incubated in serum (84). Another study have demonstrated the different composition of biocorona, formed on silver and silica NPs and incubated in plasma and serum. In detail, the different protein coronas, mainly due to different concentration of apolipoprotein J (clusterin), modified the nanostructures uptake and the cell viability (85).

Other important factors, related to the type of biological fluids, are their composition and their concentration, that could individually change considering sex and health state. Different protein coronas were formed on Fe_3O_4 nanoparticles when incubated in hyperlipidemic serum and in normal one. The biocorona, formed in hyperlipidemic serum, was enriched in cholesterol and proteins associated with inflammation and cell adhesion (86). It was also demonstrated that the concentration of physiological environment has modulated the number of proteins, adsorbed on nanoparticles (87). In detail, the biocorona of NPs, incubated in plasma gradient, was progressively depleted by low molecular weight proteins, like apolipoprotein precursor A-1, A-II, C, well-known as dysopsonins (88).

Finally, the factors and parameters, involved in the formation of biocorona, are more complex in the case of in vivo biological environment and the PC composition has been demonstrated to change in the case of in vitro or in vivo incubation (89). Indeed, in vivo the primary interactions between nanoparticles and biological entities (*e.g.* tissues, cells, fluids) are strongly influenced by the composition of protein source that could change with different diseases and medical conditions. Recently, polystyrene and silica NPs have been incubated in plasma from human subject affected by breast cancer, diabetes, hypercholerolemia, rheumatism, fauvism, smoking, hemodialysis, thalassemia, haemophilia A and B, pregnancy and hypofibrinogenemia. The type of disease had a crucial role in the protein composition of NPs corona, giving rise to the concept of personalized protein corona as a determinant factor in nano-biomedical science. PCs, generated using plasma from patients suffering from the

same disease and with the same lifestyle, were quite similar with only slight differences (90). Also in the case of graphene oxide sheets the corona formation depended on the composition of human plasma used as protein source. Identical sheets were coated with varying PC decorations related to different diseases of donators, exhibiting significantly different cellular toxicity and uptake (91). Moreover, the capability of NPs to adsorb low concentrated proteins present in biological sources may promote the potential use of NPs-PC-based technology in screens for early diagnostic tumor biomarkers (92). Despite PC formation is a dynamic process, the surface layer could act as a "nanoconcentrator" of proteins not easly detectable under physiological conditions. For example, a PC-based assay was designed using gold nanoparticles for early screening of prostate cancer, showing a higher specificity than current standard test for detection of early-stage prostate cancer (93). The concept of personalized protein corona reflects the specific composition of PC on the same nanomaterials, as a consequence of different plasma/serum proteomes expressed during each disease (94). Gold NPs were tested in urine as rapid diagnostic tool for knee osteoarthritis. The comparison with current standard assays has revealed a higher sensitivity, due to low false negative rate, and a lower specificity but in the range accepted for diagnostic objectives (95). Again, gold-coated magnetic nanoparticles were applied in biomarker discovery as tool for pre-concentration and separation of proteins from sera of patients with multiple myeloma (96). These considerations lead to a challenge in the prediction of biocorona composition, strictly related with activity, toxicity and immunogenicity of NPs (97-99).

2.5 Environmental temperature and pH

Considering that physiological temperature can vary between 35 and 41°C under different conditions, some researchers have studied the influence of diverse temperature in the formation and in the composition of biocorona. It was demonstrated how PC thickness, on

polymer coated iron-platinum NPs, changed with temperature: at low temperature (from 13 to 23°C) the adsorption of albumin and apo-transferrin has created a monolayer, while high temperature (43°C) has reduced the thickness of protein shell. Authors have proposed many explanations like the conformational changes of proteins, the reduced number of adsorbed proteins and the increase of flexibility of polymer coating on NPs (100). Changes of temperature probably play a double role in the formation of biocorona: the control of proteins adsorption on NPs surface and the modification in composition of biological fluids. In fact, in human serum, high temperature favoured the depletion of complement proteins and immunoglobulins and for this reason the protein corona on polystyrene NPs of 100nm has appeared a ticker layer if incubated in heated serum. Reducing NPs size up to 20 and 40 nm, the same incubation has revealed a biocorona, able to inhibit cellular uptake of nanostructures (101).

Another environmental parameter, able to affect the conformational changes of proteins and consequently the interaction with NPs, is the pH. It is well known how different pH values could influence the secondary and the tertiary structures of proteins, preventing their biological functions. In addition different biological fluids are characterized by specific pH values: blood has a neutral pH, intracellular matrix a pH= 6.8 and lysosomes an acidic pH (4.5-5) (102). It was demonstrated that environmental pH changed conformational state of proteins and basic pH has mostly promoted the proteins denaturation (103). Indeed, gold and silica NPs have showed major interactions with BSA at acidic pHs than at basic pHs, increasing the dimension of nanomaterials and promoting the reversible unfolding of BSA (104).

3. Effects of protein corona in NPs biocompatibility

3.1 Fate of NPs in systemic circulation

The biocorona composition, thickness and the protein conformation confer a new biological identity to nanoparticles, improving their biocompatibility or inducing toxicity, as summarized in Table 2. In the first case, the aims of NPs could be achieved like drug delivery, in the second case clearance and systemic adverse effects could be enhanced (105). The interactions with specific plasma proteins play a crucial role in the control of their biological fate in vivo (106). For instance, the binding of plasma proteins to graphene oxide nanosheets has decreased their toxic effect, preventing the penetration of cell membranes (107). Again, zinc oxide NPs were able to reduce toxicity in human hepato-carcinoma cell lines, only if covered by a pre-formed corona layer (108). This biocorona was formed by proteins, which inhibited the generation of reactive oxygen species (ROS). ROS production causes oxidative stress and inflammation, for this reason the components of corona layer may affect inflammatory response and also immune system (109). As regards ROS production, many different NPs seemed to induce oxidative stress, showing cytotoxicity by unclear processes (110). For example gold NPs, with 1,4nm diameter, have enhanced mitochondrial damage, amplifying the oxidative stress (111).

Again, the demonstrated binding affinity of zeolite NPs for fibrinogen has induced pro-inflammatory effects (112). If nanomaterials are recognized as “no-self”, they would promote the inflammation through acute phase, due to fibrinogen’s activation, and the immune response, for the release of cytokines by macrophages. The main effects are the alteration of vessels permeability, the activation of immune cells like monocytes and lymphocytes and the final uptake and degradation by macrophages. NPs could modulate and control such defensive mechanisms, binding on their surface physiological molecules able to inhibit them.

For example, the immune response and the cytokine expression have been reported to be dependent by NPs hydrophobicity, eliciting interactions with specific proteins able to activate immune system (113). So it was observed the possibility of NPs to interact with immune system's components, modulating the immune response. In fact, NPs can induce or inhibit the innate immune response, promoting or reducing the recognition and the interaction with cells of innate immune system (114).

Both natural NPs or engineered NPs, like silica NPs, have showed the capacity to directly bind immune receptors (115). The effects of NPs interaction with immune biomolecules seemed to be dual. In fact some NPs have been used as immunosuppressant because they could directly kill immune cells or down-regulate the immune responses (116-119). Some other NPs could activate the production of cytokine with induction of inflammation, leading to adaptive immunity but also side effects like allergy and chronic inflammation (120, 121). Also, polymer coating, like PEGylation, has activated the production of specific antibodies, promoting immune response an immunological memory (122,123). In this contest, NPs have induced strong immune effects and, for this reason, they have been exploited for therapeutic purposes (124). The interaction between engineered NPs and immune system may avoid an immunostimulation or an immunosuppression, depending by the medical applications: while immunostimulation is desiderable in vaccines and in anti-cancer therapy, the immunosuppression is required to treat inflammatory disorders and autoimmune diseases (125).

Another parameter, related to NPs fate in vivo, is the blood circulation time, strictly depended by the composition of protein corona. The NPs interactions with dysopsonins, like albumin and apolipoproteins, inhibit cell membrane adhesion and cellular uptake, increasing the circulation time in bloodstream. On the contrary, the presence of opsonins in biocorona, like complement, immunoglobulins and scavenger receptor, induces internalization mechanisms

and clearance of NPs (126-128). For this reason, a strategy to increase systemic circulation time of NPs is to mask their suface with dysopsonins: recently, pre-coated NPs with apolipoprotein E (ApoE) has shown a reduced liver accumulation, compared with their IgE-coated or pristine NPs. The same ApoE protein has prolonged blood circulation time of gold nanospheres, enhancing their permeabilization and retention effect in tumor tissue (129).

Another possibility to pursue a "stealth effect" is the coating NPs with peptides, designed from human CD47, or with hydrophobin, a fungy secretory protein, in order to reduce the clearance by phagocytes and to inhibit molecules adsorption, respectively (130, 131).

3.2 NPs uptake and cellular targeting

The clearance of NPs from bloodstream may occur by phagocytosis, induced by immune cells after their activation by opsonins, or by macrophages. For example adsorbed complement factors on gold nanoparticles surface has been demonstrated to increase the uptake by macrophages (132). Another protein, responsible to reduce macrophage uptake as component of NPs corona layer, is clusterin (133). Clusterin, or apolipoprotein J, enriched in the corona of PEGylated and PEEP conjugated silica NPs, has provided a general stealth effect. Also the PEG density could play a crucial role in the NPs uptake: increasing PEG density on gold nanoparticles, the protein adsorption was decreased, selecting biomolecules able to prevent macrophage uptake (134). Indeed, another study has reported different cellular uptake of silica NPs in the presence or absence of biocorona (135). The membrane association and the cell's internalization were higher for silica NPs without corona layer and they were localized both in the cytosol and in lysosomes. Instead, the same NPs with biocorona were only found in lysosomal organuli because subjected to a reduced uptake.

The strategy to select and to pre-coat proteins on NPs surface is commonly used to decrease nanomaterials toxicity, to inhibit the cell interaction and uptake. One example were carbon

nanotubes, that have reduced their cytotoxic effects on platelets after binding of blood proteins (136). The choice of proteins, involved in pre-coating of NPs, is crucial because different proteins could show opposite effects. In fact it was demonstrated that cellular uptake of NPs, into mesenchymal stem cells, could be inhibited or enhanced by coating with ApoA4/ApoC3 or with ApoH, respectively (137).

In addiction to the importance of protein corona, it was reported that also physicochemical properties of NPs, like size, shape and composition, have influenced cell autophagy responses: a recent research was performed to guide the design of Fe_3O_4 NPs for further biomedical application (138).

Although the reduction of cellular uptake may increase the blood circulation time, it could be avoided if NPs are used to deliver drug inside cells, as the case of bovine serum albumin coated graphene oxide (GO), able to decrease cellular morphological damage by limiting GO penetration into cell membrane (139). Indeed, in polymer coated superparamagnetic iron oxide NPs (SPIONs) the drug's release has been shown to be reduced by biocorona: both hard and soft shells have prevented the release of paclitaxel from NPs (140).

The specific NPs parameters may also play a crucial role in the targeting capability as a consequence of influence in biodostribution and cellular uptake (141). The targeting capacity of NPs could be defined nonspecific or passive, if drug's release is not directly guided inside tissue or cells, and specific or active, if NPs are guided to achieve and to be recognized by proper receptor present on cellular target. Examples of NPs targeting, described below in detail, are summarized in Table 3.

Considering passive targeting, the destiny of NPs is mainly related to the composition if protein corona, formed after their contact with bloodstream or biological fluids. For this reason, it is important the category of proteins that form the corona layer: opsonins favour the

clearance of exogenous NPs. Examples of opsonisation, with consequent immune/complement activation and macrophages uptake, have been reported for black phosphorus NPs and for dextran-coated superparamagnetic iron oxide (SPIO) core shell nanoworms (142, 143).

As regards the active targeting, the NPs surface is functionalized with specific ligands useful for the selective transport inside target. So the type, the size, the density and the orientation of ligand are fundamental to promote the receptor's affinity and to control the formation of protein corona able to permit the target's achievement. Many are the examples of successful active targeting, mostly in anti-cancer strategies. iRGD peptide has functionalized gold nanoparticles to enhance the tumor penetration efficiency, increasing the permeability of cancer vascular tissue and leading to massive accumulation in tumor target (144). Indeed in chemotherapy, cellular uptake of folic acid functionalized NPs has improved the active targeting effect of docetaxel both in vitro and in vivo (145).

Other researches highlighted that ligand could improve the active targeting for its effect in the formation of biocorona. For example hyaluronic acid has specifically bound to CD44 receptors, overexpressed on cell membrane of tumor: cationic bovine serum albumin-protected gold nanocluster were coated by hyaluronic acid, showing a high accumulation in breast cancer with homogenous intra-tumor distribution (146).

The physicochemical properties of ligand, involved in the control of PC formation and NPs targeting capacity, are dimension and concentration. In fact large size and high density of ligand could prevent some interactions during the formation of protein layer. Indeed, the length of ligand's chain and the type of linker could modify the targeting action (147). It was demonstrated that the use of PEG long chains (5-10KDa), as linker to coat Herceptin to gold NPs, strongly reduced the binding capacity of functionalized nanomaterials (148). Also the type of attachment of ligand onto NPs surface can influence the composition of biocorona

and the targeting. In fact the pre-adsorption of targeting antibodies to surface of nanocarrier has been demonstrated to be a more efficient strategy of active targeting than the correspondent covalent attachment (149). The difference was due to the Ab orientation, able to favour interactions with specific proteins involved to enhance the targeting.

Finally, the in vitro protein corona could completely differ from the in vivo biocorona, resulting in a difficult prediction of NPs targeting capability. The type and the number of corona proteins, involved in the target achievement, are closely related to the type of biological environment and to the fact that protein adsorption in vivo is a dynamic process, hardly predictable (150, 151).

3.3 NPs toxicity

Despite NPs are becoming increasingly promising tools for medical diagnostics and therapeutics, their potential risks to human health, together with environmental issues, has led to increasing concerns regarding their use (152). The first toxicological protocol used for nanoparticles was developed late in comparison with publication about NPs potentials with many difficulties (153). For example, in the MTT assay, a widely used viability assay in nanotoxicology, the presence of nanoparticles can change the structure of dye into crystals which are blue in colour (154). This assay has a major shortcoming in terms of the negative impact of the protein corona in regards to useful assay results.

A comprehensive understanding of NPs toxicity is required in order to design safe, reliable and efficient nanostructures for biomedical applications.

The side effects, induced by NPs, depend by several factors: the way of administration the physicochemical features of NPs and their surface modification. As regards the administration, Figure 3 reports a scheme of advantages and side effects related to the mostly studied routes: oral, intravenous and pulmonary ways. The most tested is the intravenous,

even if several studies are published about the NPs inhalation. In this case, the toxic effects are mainly due to accumulation of NPs in lung cells and to the activation of pulmonary autophagic flux: the toxicological mechanisms have been demonstrated to depend by material of NPs, possible NPs coating and type of cells (155, 156). The oral administration is a modality, mostly used for drugs delivery, and also some NPs have been exploited for a potential oral route (157). The main problems are the evaluation of protein corona formation and the PC role on the delivery. Recently, a study has demonstrated that pepsin at high concentration could favour aggregation of lipid NPs, preventing the release of encapsulated siRNA (158). Many efforts have been done to encourage the development of oral administration in nanomedicine, considering the non-invasive aspect well accepted by patients (159).

On the contrary despite the invasive route, the NPs injection is the most studied for massive effect due to a systemic delivery. Nevertheless, intravenous administration may show induced cytotoxic responses, like activation of inflammation, immune response and macrophages uptake, and indirect toxic effects like induction of fibrillation, denaturation and conformational changes in the adsorbed proteins (160, 161).

One effect of NPs toxicity is the cell membrane damage, enhanced also by the lack of nutrients and pH modification. The NPs pre-coating could favour interaction with specific proteins, able to mitigate such cellular damage, even if specific biocorona would not able to prevent any conformational and pH changes, provoked by imbalance of essential nutrients (162).

Another aspect of NPs toxicity is the possibility to speed up the rate of protein fibrillation, promoting the development of amyloid deseases, as depicted in Figure 4. In fact NPs could induce aggregation of proteins at physiological condition and the formation of unfolded

protein-NPs complexes may generate large protein clusters (163). It was demonstrated that carbon nanotubes has favoured the fibrillation of human beta 2-microglobulin because the interactions of proteins with nanostructures lead to conformational changes, to formation of oligomer and to the final amyloid extension (164). Many parameters, like protein stability, aggregation tendency, NPs types and surface chemistry, may control amylodogenesis even if a crucial role is due by the activation of fibrillation for the high accessible surface area of nanostructures (165). NPs with high OH levels onto surface have favoured protein aggregation, while the presence of cationic groups has inhibited amyloid β–protein aggregation (166). So the nature and the density of surface functionality may control protein aggregation, responsible of both fibrillation and protein's unfolding. Although the precise mechanism of denaturation is not completely clear, one accepted hypothesis is the release of free energy, provoked by contact forces between NPs surface and proteins (167). It is well established that NPs with high surface charge density and hydrophobic NPs may change the conformation of adsorbed proteins more than neutral and hydrophilic nanostructures (168, 169). Indeed, it was demonstrated that conformational changes irreversibly occurred without possibility to refold proteins, after their desorption from NPs (170). As for fibrillation, also the degree of protein denaturation depends by NPs type and surface modification. For example PEGylated gold NPs was able to unfold and to aggregate lysozyme at low PEGylation density, while at high PEGylation density NPs prevented lysozyme adsorption and aggregation (171).

4. Future challenges of protein corona in nanomedicine

Nanomaterials in biomedical applications have to overcome several problems, strictly connected with their toxicity and their medical purposes like diseases diagnosis, biomarker detection and therapy. Protection of human body to NPs is crucial because even very low

concentrations may induce side effects (172, 173). Biocorona formation and composition could represent a promising challenge to reduce NPs cytotoxicity and to govern their fate in vivo, as shown in Figure 5. In fact, a deep knowledge and understanding of corona layer could be fundamental to predict NPs biodistribution and bioavailability responses (174). Hence, NPs may be synthesized and functionalized to interact with selected proteins for drug delivery or for diagnostic analysis (175). The pre-coating nanomaterials with plasma proteins could be one strategy that allows the design of artificial protein coronas with controlled physiochemical properties. In vivo, artificial and customized coronas should be stable to preserve NPs stealth properties and to regulate their interactions in physiological environments, while in the diagnostic field, a customized NPs corona should detect specific biomarkers that are undetectable by conventional methods (176, 177). Recently, polyhedral oligomeric silsesquioxane polymer-caged gold nanoparticles have been developed for the sensitive colorimetric analysis of metallothioneins, biomarkers for the early diagnosis of heavy metal poisoning and malignancies (178).

Moreover, different disease states and medical conditions can change plasma protein concentrations, protein structures and body temperature, modifying the proteins adsorption on NPs surface. For this reason, the composition of protein corona could provide details about the physiological changes in human body, related to protein modulation and to structural deformation during tumorigenesis and disease development.

These new findings have contributed to show a future perspective of nanomedicine: the necessity to design safe and highly efficient NPs for personalized drug delivery system (179, 180). Such challenge could be advantaged by the possibility to use recent automated microfluidic devices for the formulation of pre-coated nanomaterials: they apply standardized incubation protocols and they test the reproducibility of experimental data (181-183).

Indeed, there is a general consensus to designed personalized NPs by pre-coating with specific plasma proteins in order to mask nanostructures and to perform desired biological functions. In NPs pre-coating, important aspects are the structural modification and the approachability of adsorbed proteins because the therapeutic action is often mediated by binding with specific receptors. So the protein directionality as well as the density of adsorption modulate the flexibility and the availability of optimal binding site (184). In fact nanomaterials, that poorly interact with proteins, may increase the blood circulation time, promoting the targeting, but they also reduce the cell uptake, preventing the cellular internalization (185). Recently, gold nanorods were coated only with Apolipoprotein E and they have exhibited high affinity for both hydrophilic and hydrophobic drugs as well as an increased uptake by cancer cells, overexpressing low-density lipoprotein receptors (186). The ApoE corona of gold nanorods was then loaded with hydrophobic photosensitizer chlorin e6 (Ce6): the enhanced uptake of nanomaterials in carcinoma cells has favoured the accumulation in tumor tissue. The combined photothermal therapy by nanorods and the photodynamic therapy by Ce6 have destroyed cancer cells, achieving a complete tumor regression (187). Apolipoproteins are preferentially used to coat nanoparticles and to improve targeted drug delivery because they are dysopsonins able to reduce macrophages action and also the clearance of NPs, prolonging the bloodstream circulation and the target's achievement. Another example was lipid NPs coated with apolipoproteins to target a specific receptor overexpressed in PC3 prostate carcinoma cell lines, increasing NPs internalization by endocytosis mechanism (188). Recently, Apolipoproteins A1, E and J were absorbed onto bio-inspired liposomes by a short nontoxic peptide derived from $A\beta_{1-42}$. Doxorubicin, loaded in nanomaterials, have showed a significant enhancement of brain distribution, due to favoured binding of apolipoprotein with specific brain receptors and a higher anti-brain cancer effect than the same drug loaded plain liposomes (189). Another deep investigation

was performed to examine the effect of protein corona on shielding drugs and disturbing their controlled release. Using various types of synthetic (tamoxifen-loaded crosslinked superparamagnetic iron oxide NPs (SPIONs) and 4-nitroanisole-loaded nanocapsules), and the U.S. Food and Drug Administration (FDA) approved, commercialized drug loaded nanocarriers (Abraxane®, albumin-bound paclitaxel injection), it was demonstrated that the drug release of nanocarriers is significantly attenuated in biological environments. More specifically, the protein corona has showed significant effects on the release profile of the drug loaded SPIONs. The protein corona could reduce the burst effect, however there was no considerable effect of protein corona on the release profile of the payload from the nanocapsules, probably due to the buffer effect able to play a crucial role in the successful application in vivo (190).

In conclusion the main challenge of novel nanoparticles, designed for biomedical applications, would be the preservation of a balance between the biological desired function and the possibility of elimination as "non-self". This harmonization depends by the type of physiological proteins adsorbed on NPs surface: for this reason the protein corona could be considered a milestone in the tailoring and the design of NPs for nanomedicine (174).

5. Conclusions

"I learned to recognise the thorough and primitive duality of man; I saw that, of the two natures that contended in the field of my consciousness, even if I could rightly be said to be either, it was only because I was radically both." Robert Louis Stevenson, *The strange case of Dr. Jeckyll and Mr. Hyde.*

The sentences, from the most famous novel of Stevenson, could also reflect the duality of protein corona: biocorona can control the biological fate of NPs because its components may favour both biodistribution and targeting, the desired action, as well as cellular uptake and toxicity, the side effects. This ambivalent nature is one reason for the limitation of

nanomaterials in current therapies: 51 nanotherapeutics have been approved by FDA and 77 nanomedicines have started clinical trials, of which 40% in 2014 or 2015 (191). Despite the numerous recent progresses in scientific research and in technological field, the protein corona issues represent the actual and future challenge for nanomedicine, aimed to increase the knowledge on nanotoxicology and to reduce the gap between in vitro and in vivo results for clinical investigations (192, 193).

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Conflicts of Interest

Author has declaired no conflicts of interest.

Figure Captions

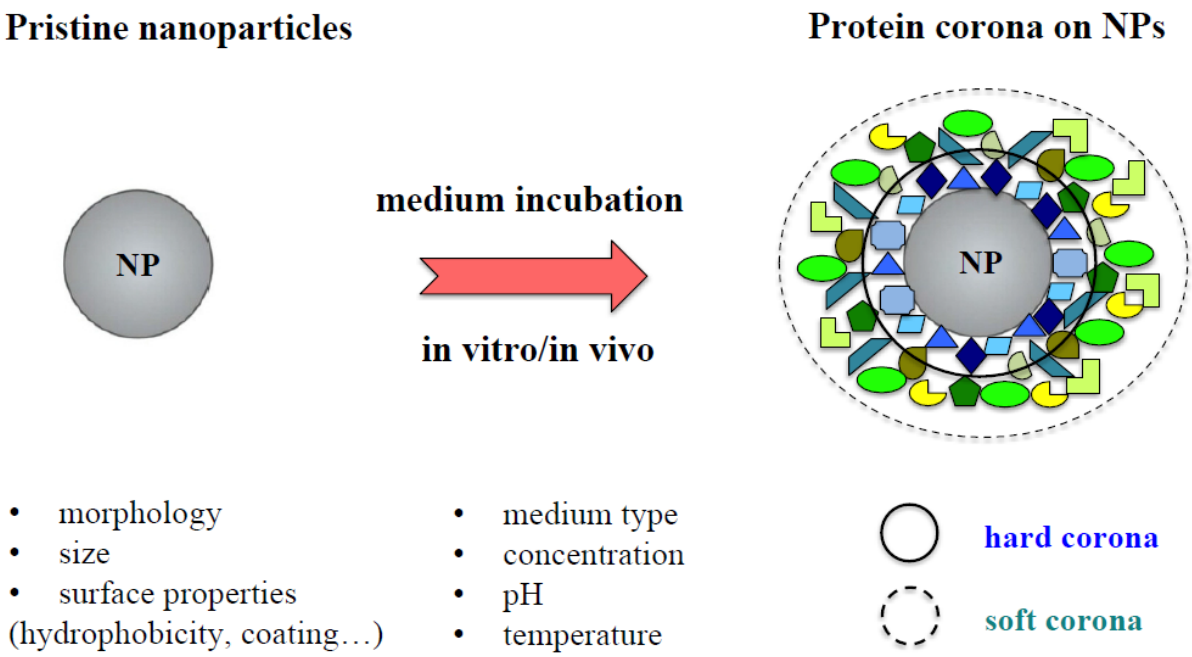

Figure1 Formation of protein corona (PC), in vitro and in vivo, and representation of soft and hard layer. Properties, referred to nanoparticles (NPs) and medium of incubation, were reported as responsible of PC composition and generation.

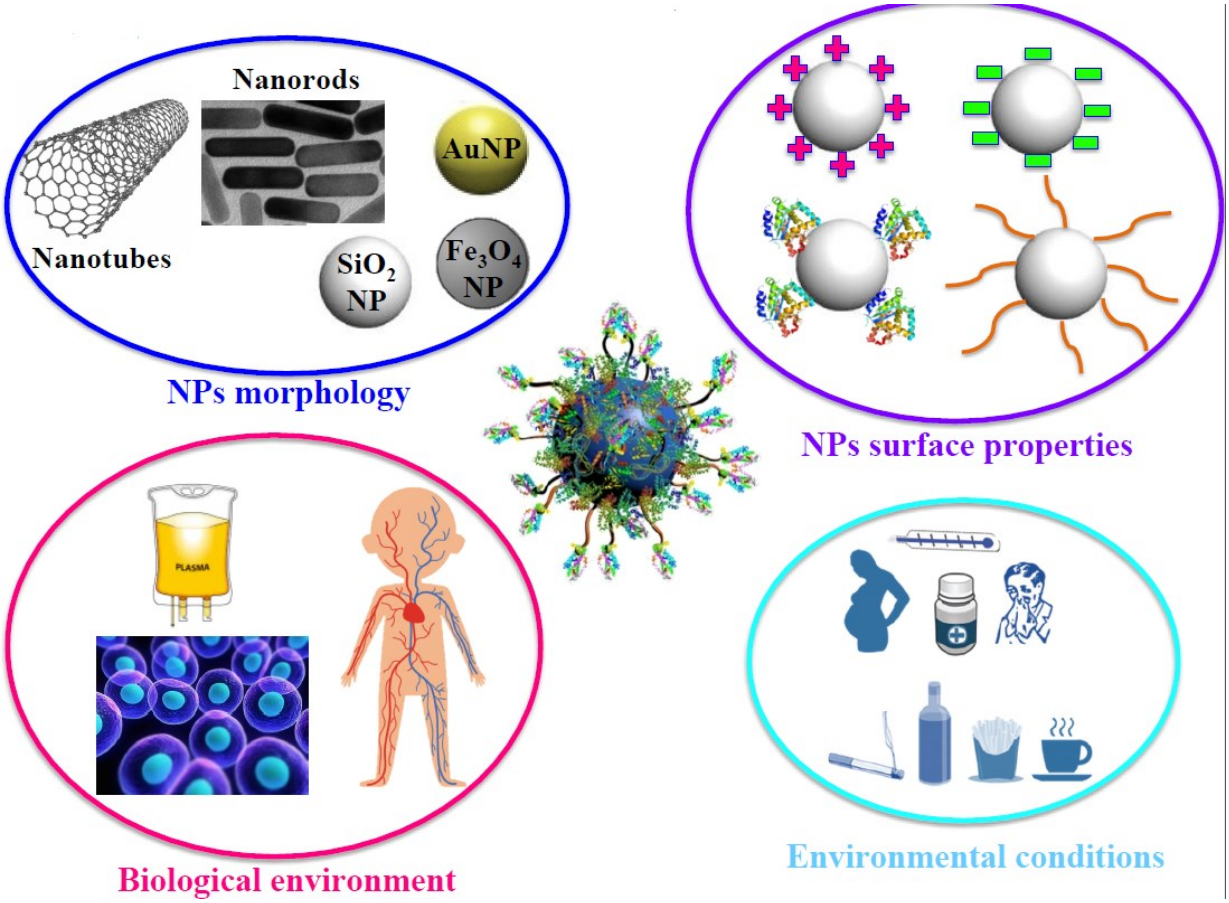

Figure2 Schematic representation of most important parameters, involved in the formation of specific protein corona. They belong both to nanoparticles, like their morphology and functionalization, and to biological environment, such as type of cells or biological fluids used for NPs incubation and the general health conditions in the case of in vivo administration.

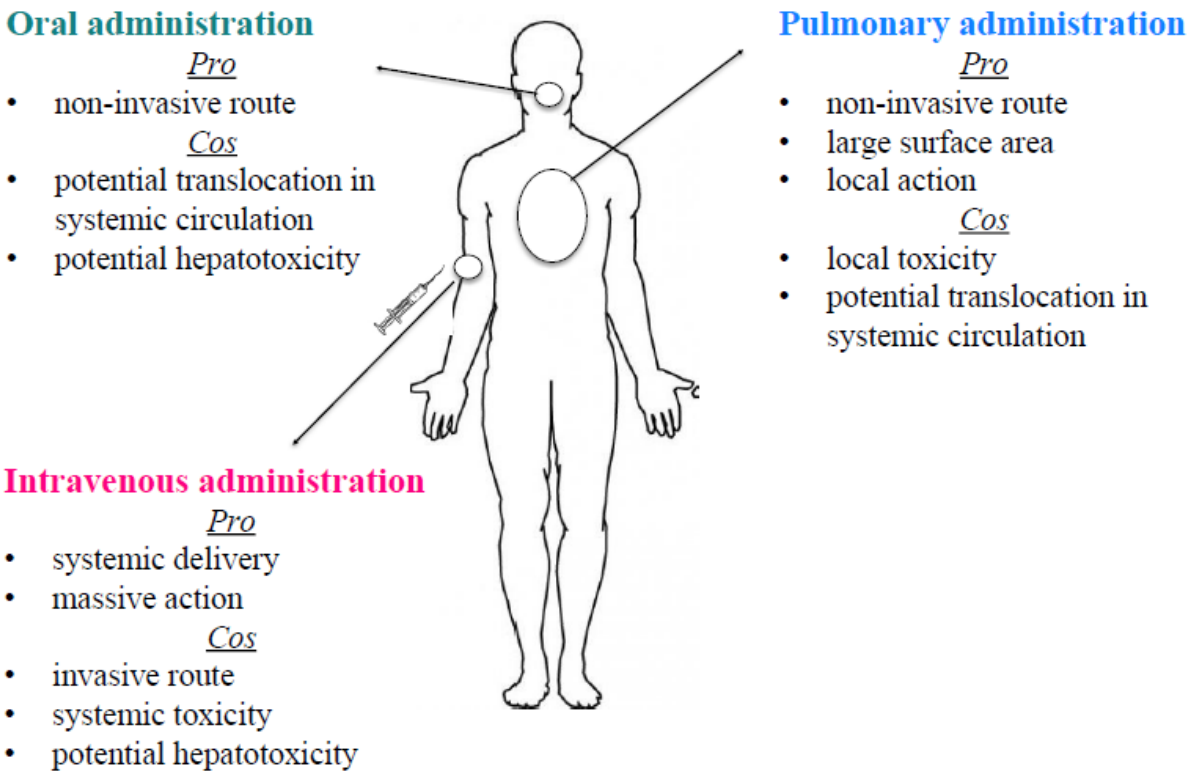

Figure3 Scheme of NPs administration routes, showing specific advantages and disadvantages.

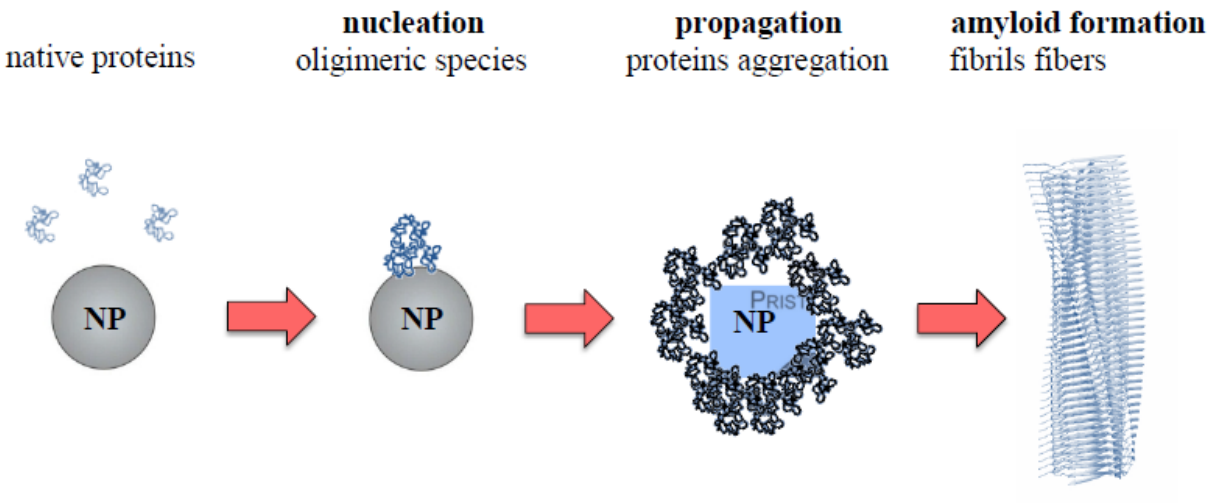

Figure4 Schematic description of fibrillation process and amyloid aggregation, activated by nanoparticles.

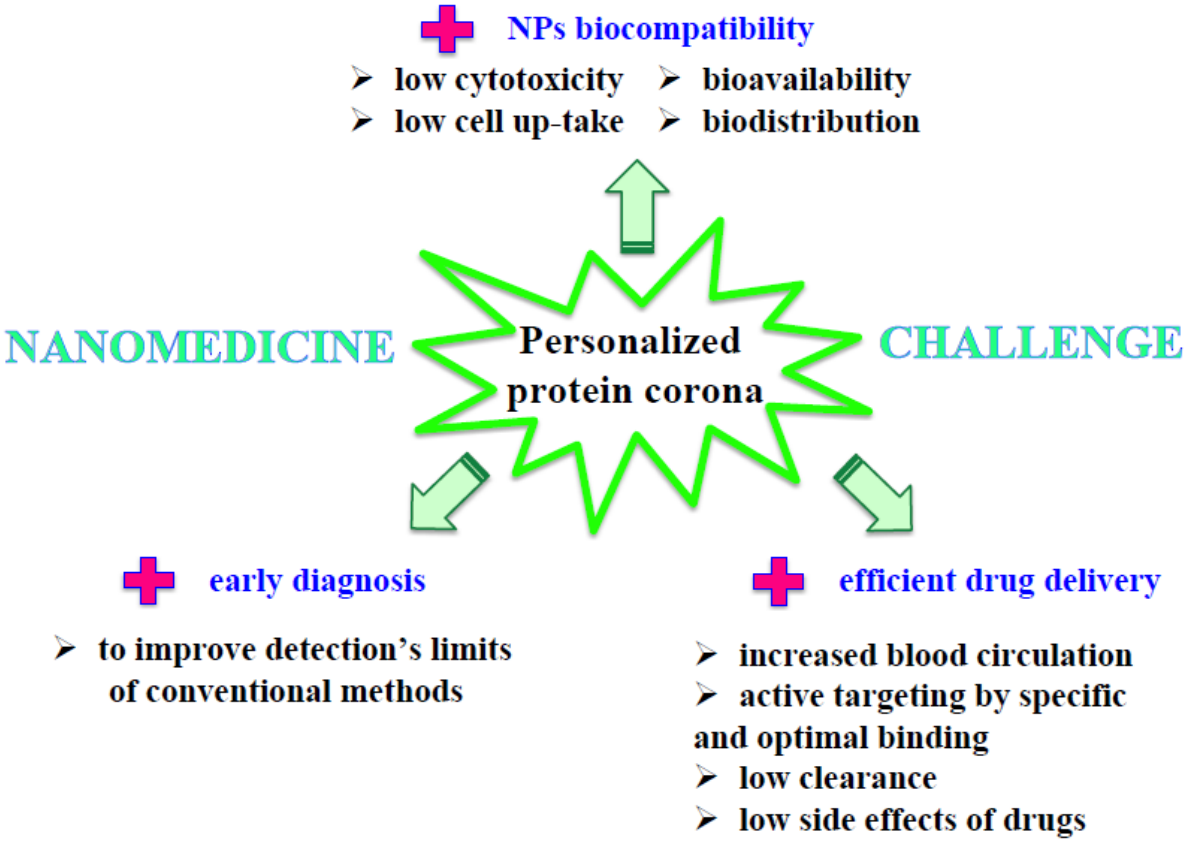

Figure5 Personalized protein corona is the most promising future challenge of nanomedicine research. A specific personalized protein corona may improve the NPs biocompatibility and the drug delivery by active targeting, aimed to develop new therapeutic approaches. Indeed personalized protein corona may have a crucial role in the planning of new efficient and reliable methods for early diagnosis.

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Protein Corona	**Chemical-physical forces**	**Function**
Hard Corona	• Electrostatic interactions • H bonding	creation of *stable and non-exchangeable layer*, responsible of nanoparticle's fate after administration
Soft Corona	• Van Der Waals forces • π–π stacking binding • Hydrophobic interactions	formation of *dynamic and exchangeable layer*, dependent by environmental composition and responsible of nanoparticle targeting capacity

Table 1 Scheme of chemical forces occurred during the formation of protein corona, according to "Vroman effect". Their role is different in the generation of specific protein corona layer.

Nanoparticles (NPs)	NPs parameters	Biological responses	Cellular effect	Reference
Graphene oxide nanosheet	Blood protein coating	Reduced cellular internalization	Biocompatibility	100
ZnO nanoparticles	Pre-formed protein corona	Inhibition ROS production	Biocompatibility	101
TiO_2 nanoparticles	Type of material	Oxidative stress, dysfunction of endothelial cells	Cytotoxicity	103
Gold nanoparticles	Size (1.4nm diameter)	Necrosis and mitochondrial damage	Cytotoxicity	104
Zeolite NPs	Fibrinogen coating	Pro-inflammatory effects	Cytotoxicity	105
Silica NPs	Hydrophobicity	Active immune response	Cytotoxicity	108
Iron oxide NPs	Type of material	Immunosuppression	Biocompatibility	110

Cerium dioxide NPs	Type of material	Production of cytokines	Cytotoxicity	112
Gold nanoparticles	Antibody conjuction	Immune effects in colon cancer cells	Biocompatibility	118
Gold nanospheres	Apolipoprotein E coating	Permeabilization and retention effect in tumor tissue	Biocompatibility	122
PEGylated nanobeads	Peptide coating	Inhibition of clearance by macrophages	Biocompatibility	123
Polystyrene nanoparticles	Hydrophobin coating	Increase of bioavailability	Biocompatibility	124
Silica nanoparticles	PEG and PEEP coating	Stealth effect due to enrichment of clusterin	Biocompatibility	126

Table 2 Influence of NPs type and functionalization in the desired, biocompatible, or adverse, toxic, effects.

Nanoparticles (NPs)	Type of targeting	Mechanism	Effects	Reference
black phosphorous NPs	passive against inflammatory deseases	protein corona enriched in opsonins	immunomodulation effects on macrophages and high cellular uptake efficiency	135
dextran-coated superparamagnetic iron oxide nanoworms	passive	protein corona enriched in opsonins (complement proteins, C3, immunoglobulins and properdin)	activation of alternative pathway and clearance by immune cells	136
gold nanoparticles coated by iRGD peptide	active for human breast carcinoma	recognition and binding to integrins, expressed on cancer endothelial cells	enhanced permeability and retention effect in tumor cells	137
PEGylated polycaprolactone NPs, coated with folic acid	active for human breast and prostate carcinoma, lung cancer	folic acid receptors are overexpressed in many human cancers	increased cellular uptake and drug's accumulation inside tumor	138
gold NPs coated with hyaluronic	active for human breast	hyaluronic acid is recognized by CD44	accumulation in cancer tissue and	139

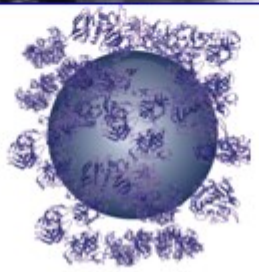
acid	carcinoma, lung cancer	receptors, overexpressed on tumor cell membranes	inhibition of tumor growth	

Table 3 Recent strategies of drug's targeting are obtained by specific functionalized nanoparticles, able to promote different biological mechanisms.

Nanotoxicity

Immune response

Low targeting capability



Personalized protein corona

Biomimetic functionalization

High targeting capacity