Chapter 2
Nanoparticle and Protein Corona

Abstract Nanoparticles and other nanomaterials are increasingly considered for use in biomedical applications such as imaging, drug delivery, and hyperthermic therapies. Thus, understanding the interaction of nanomaterials with biological systems becomes key for their safe and efficient application. It is increasingly being accepted that the surface of nanomaterials would be covered by protein corona upon their entrance to the biological medium. The biological medium will then see the achieved modified surface of nanomaterials, and therefore further cellular/tissue responses depend on the composition of corona. In this chapter, we describe the corona variations according to the physicochemical properties of nanomaterials (e.g., size, shape, surface charge, surface functional groups, and hydrophilicity/hydrophobicity). Besides the nanomaterials' effects, the role of environment factors, such as protein source and slight temperature variations, is discussed in details.

After intravenous administration, blood is the first physiological environment that a nanomaterial "sees." Blood plasma contains several 1,000 different proteins with 12 order of magnitude difference in the concentration of these proteins [1]. In addition to the proteins, lipids are also available in blood plasma. Therefore, upon injection of nanoparticles inside the blood, there is a competition between different biological molecules to adsorb on the surface of the nanoparticles. In the initial stage, most abundant proteins are adsorbed on the surface; however, over the time they will be replaced by higher affinity proteins (Vroman's effect [4]).

The structure and composition of the protein corona depends on the physicochemical properties of the nanomaterial (size, shape, composition, surface functional groups, and surface charges), the nature of the physiological environment (blood, interstitial fluid, cell cytoplasm, etc.), and the duration of exposure. The protein corona alters the size and interfacial composition of a nanomaterial, giving it a new biological identity which is what is seen by cells. The biological identity determines the physiological response including agglomeration, cellular uptake, circulation lifetime, signaling, kinetics, transport, accumulation, and toxicity.

Protein corona is complex and there is no one "universal" plasma protein corona for all nanomaterials and that the relative densities of the adsorbed proteins do not correlate with their relative abundances in plasma. Thus, the composition of the protein corona is unique to each nanomaterial and depends on many parameters.

2.1   Structure and Composition of Corona

The majority of adsorbed biomolecules on the surface of nanoparticles in blood plasma are proteins, and recently some minor traces of lipids have also been reported. The adsorption of proteins on the surface of nanoparticle is governed by protein–nanoparticle binding affinities as well as protein–protein interactions. Proteins that adsorb with high affinity form what is known as the "hard" corona, consisting of tightly bound proteins that do not readily desorb, and proteins that adsorb with low affinity form the "soft" corona, consisting of loosely bound proteins (Fig. 2.1a). Soft and hard corona can also be defined based on their exchange times. Hard corona usually shows much larger exchange times in the order of several hours [1].

A hypothesis is that the hard corona proteins interact directly with the nanomaterial surface, while the soft corona proteins interact with the hard corona via weak protein–protein interactions [2]. There is a general observation that even at low plasma concentrations, there is a complete surface coverage of corona layer [1]. However, the adsorbed corona does not completely mask the surface of nanoparticle or its functional groups. In a study on dextran-coated superparamagnetic iron oxide nanoparticles (SPIONs), the incubation of SPIONs in plasma and formation of the protein corona did not significantly changed the circulation lifetime [3].

The thickness of protein corona can be a factor of many parameters such as protein concentration, particle size, and surface properties of particle. Most plasma proteins present a hydrodynamic diameter of about 3–15 nm; thus, the coronas on these nanoparticles are too thick to be composed of only a single layer of adsorbed protein and are composed of multiple layers. A model for the protein corona has been proposed by Simberg et al. [3]; it consists of "primary binders" that recognize the nanomaterial surface directly and "secondary binders" that associate with the primary binders via protein–protein interactions. Such a multilayered structure is significant for the physiological response as the secondary binders may alter the activity of the primary binders or "mask" them, preventing their interaction with the surrounding environment.

In a recent review, Walkey and Chan [2] summarized a subset of 125 plasma proteins, called adsorbome, that were identified in protein corona of at least one nanomaterial. This list will probably expand due to further studies in the future. Results compiled over many studies since about 20 years ago showed that a "typical" plasma protein corona consists of approximately 2–6 proteins adsorbed with high abundance and many more adsorbed with low abundance. Only a small
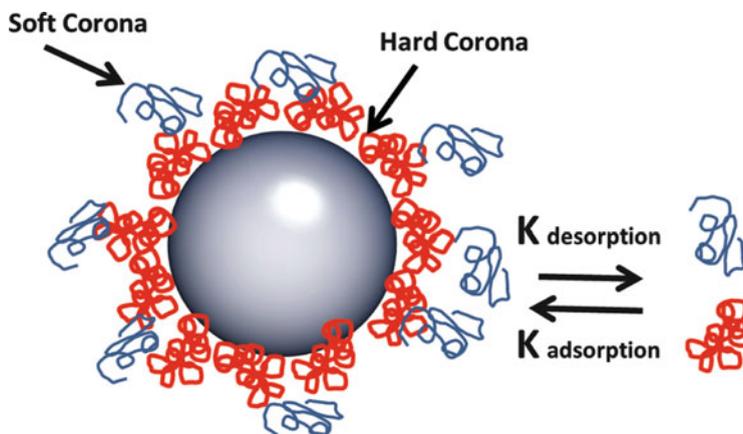
Fig. 2.1 Schematic illustration of soft and hard protein corona and the concept of the rate of adsorption and desorption which determines the exchange time and lifetime of proteins in the protein corona. The hard or soft corona is not composed of only a single protein; in this scheme, the complexity of the presence of different proteins is not shown

subset of the plasma adsorbome binds to most nanomaterials, and only a fraction of the adsorbome is bound to a nanomaterial with high abundance.

The competitive adsorption of proteins on the limited surface of nanoparticles containing the collective effects of incubation time, concentration of protein, and adsorption affinity between protein and nanoparticle surface is called "Vroman effect" [4, 5].

2.1.1   Hard Corona

A review of literature shows that varying nanoparticles with various surface modifications have been studied by different methods to find out the composition of their protein corona. A summary of these studies is provided by Aggarwal et al. [5] which shows that albumin, immunoglobulin G (IgG), fibrinogen, and apolipoproteins are present in the corona of all the studied nanoparticles. These proteins have high abundance in blood plasma, and therefore, at later times, they might be replaced by proteins with lower concentration but higher affinity to the nanoparticle surface. Lundqvist et al. [6] have studied "hard" corona formed around nanoparticles of different materials, including copolymer and polystyrene nanoparticles, of different sizes, and with different surface properties.

One of the mechanisms of adsorption of proteins on the surface of nanoparticles is the entropy-driven binding. The mechanism of entropy-driven-bonded proteins such as fibrinogen, lysozyme, ovalbumin, and human carbonic anhydrase II is the release of bound water from the surface of the nanoparticle. In this case, the increase in entropy of released water molecules is larger than the decrease in

the entropy of adsorbed proteins. It should be noted that the adsorption of proteins by this entropy-driven mechanism usually does not change the conformation of the protein [7]. The change in the conformation of human adult hemoglobin has been reported for bare CdS nanoparticles [7]. The sulfur atoms of cysteine residues are the main linker for attachment of hemoglobin on the surface of CdS nanoparticle which is accompanied by around 10 % decrease in the alpha-helix structure content.

Lundqvist et al. [8] incubated nanoparticles with plasma and then transferred them with their corresponding hard protein corona into cytosolic fluid. Following a second incubation, the hard protein corona is determined and compared to that of incubation in each fluid separately (plasma and cytosolic fluid). Three different nanoparticles (9 nm silica, 50 nm polystyrene, and 50 nm carboxyl-modified polystyrene particles) were incubated in either human plasma, cytosolic fluid, or in plasma followed by cytosolic fluid, and then the bound proteins (hard corona) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results confirm that significant evolution of the corona occurs in the second biological solution but that the final corona contains a "fingerprint" of its history. They concluded that this could be evolved to map the transport pathways utilized by NPs and eventually to predict fate and behavior of nanoparticles in the body.

Karmali and Simberg [9] have reviewed the identification of plasma proteins adhering to different nanoparticles which is summarized below. It is well known that the surface chemistry plays the dominant role in the recognition:

• Apolipoproteins are the main type of proteins which adsorb on liposomes and polymeric nanoparticles, but not inorganic nanoparticles. The exchange of apolipoprotein between lipoproteins and nanoparticles that have hydrophobic domains was suggested to be the main mechanism of adsorption. Using model polymer particles with decreasing hydrophobicity, Gessner et al. [10] demonstrated that ApoA-I, ApoA-IV, ApoC-III, and ApoJ gradually disappear with decreasing hydrophobicity of the nanoparticle.
• The most abundant proteins, albumin and fibrinogen, were found on many types of nanoparticle.
• Cationic lipoplexes and polyplexes show strong albumin binding, probably because albumin is a negatively charged protein. Albumin also shows affinity for hydrophobic surfaces and polyanions.
• Transferrin, haptoglobin, fetuin A (alpha-2-HS-glycoprotein), kininogen, histidine-rich glycoprotein, and contact (intrinsic) clotting pathway factors can be attracted by polymer nanoparticles and nanoparticles with hydrophobic surface component or hydrophilic inorganic nanoparticles. Most of these proteins are able to adhere to the anionic and metal surfaces.
• Presence of hydroxyl groups (e.g., dextran and sugars) promotes the binding of C3 complement through its thioester group. Mannose-binding lectins (MBLs) were shown to bind to sugar moieties of dextran-coated nanoparticles.
• Specific binding of serum mannose-binding protein (MBP) to phosphatidy-linositol (PI) liposomes has been demonstrated.
• Dextran-coated particles appear to be recognized by antibodies.

2.1.2 Soft Corona

The molecules which are loosely bonded to the nanoparticle surface or have weak interaction with the hard corona form the soft corona. In the case of some nanoparticles, especially those with a preformed functional group such as pegylated nanoparticles, there is only a weak corona covering the surface and no hard corona is observed [11].

The theoretical challenge of understanding why certain proteins are adsorbed in a competitive manner is unclear. Certainly there are many hints that this is a collective process, and therefore, it will be difficult to rationalize on the basis of individual protein-binding studies. Thus, while there is growing certainty that the corona is what is "seen" by the cell, there is as yet relatively little progress on why any NP chooses those particular proteins.

2.2 Protein Conformation

During adsorption on the nanoparticles, proteins may undergo structural rearrangements called "conformational changes." These changes are thermodynamically favorable if they allow a hydrophobic or charged sequence within a protein to interact with a hydrophobic or charged nanomaterial surface, respectively. Changes in protein conformation are typically irreversible after desorption. For example, conformational changes in the iron-transport protein transferrin are not recovered after desorption from iron oxide nanoparticles. Conformation of adsorbed proteins is altered more in the presence of charged or hydrophobic nanomaterials. For example, quantum dots grafted with mercaptoundecanoic acid denature and inactivate the enzyme chymotrypsin, while the same particles grafted with a structurally similar but hydrophilic poly(ethylene glycol) (PEG) derivative adsorb the enzyme but do not denature it to the same extent [2].

Binding of proteins to planar surfaces often induces significant changes in secondary structure, but the high curvature of NPs can help proteins to retain their original structure. However, study of a variety of NP surfaces and proteins indicates that the perturbation of protein structure can appear. Lysozyme adsorbed onto silica NPs or bovine serum albumin adsorbed on Au NPs surfaces showed a rapid conformational change at both secondary and tertiary structure levels. Most of the studies have reported that loss of α-helical content occurs as detected by circular dichroism spectroscopy when proteins are adsorbed onto NPs and a significant increase in sheet and turn structures.

2.3   Dynamic of Protein Corona and Its Time Evolution

The attachment of proteins and lipids from the biological environment results in the formation of hard and soft coronas with long and short typical exchange times, respectively. The typical lifetime of hard corona has been shown to be many hours [1]. The hard corona lifetime is long enough for many biological and physiological phenomena, and therefore, this hard corona defines the biological identity of the particle. The competition between more than 3,700 proteins in the blood plasma for adsorption on the surface of the nanoparticle changes the composition of the corona over time [7]. Therefore, corona is not a fix layer, and its composition is determined by the kinetic rate of adsorption and desorption of each protein and lipid (Fig. 2.1). In most of the cases, proteins with high abundance in the plasma are adsorbed on the surface, and over the time, they are replaced by proteins with lower concentration but higher affinity.

Recently the protein corona formation has been studied on FePt and CdSe/ZnS [12] and Au nanoparticles [13]. The protein absorption has been measured after 5–30 min incubation time, showing that the adsorption of blood serum proteins to an inorganic surface is time dependent. The highest mobility proteins arrive first and are later replaced by less mobile proteins that have a higher affinity for the surface. This process may take several hours. As shown by Slack and Horbett, this process is the general phenomenon governing the competitive adsorption of a complex mixture of proteins (as serum) for a given number of surface sites [14].

Cedervall et al. [15] modeled plasma protein adsorption using a bi-exponential function. This model distinguishes protein adsorption and desorption into "fast" and "slow" components. During plasma protein adsorption to copolymer nanoparticles, the fast component (hard corona) is formed in seconds, while the slow component (soft corona) builds on a time scale of minutes to hours. Desorption shows similar behavior with a mean lifetime of about 10 min for the fast component (soft corona) and about 8 h for the slow component (hard corona). Similar kinetic behavior can be applied to plasma protein adsorption to other nanomaterials. The hard corona is probably more important than the soft corona in determining the physiological response. As a result of its long residence time, the hard corona remains adsorbed to a nanomaterial during biophysical events such as endocytosis.

Proteins adsorbed to a nanomaterial are in a continuous state of dynamic exchange. At any time, a protein may desorb, allowing other proteins to interact on the nanoparticle surface. These changes in the composition of the protein corona resulting from desorption/adsorption are known as the "Vroman effect." This effect takes into account that the identities of the adsorbed proteins can change over time even if the total amount of adsorbed protein remains roughly constant. During the initial formation of the protein corona, proteins with the highest association rates adsorb to a nanomaterial. If these proteins have short residence times, they will be replaced with other proteins that may have slower association rates but longer residence times. During plasma protein adsorption, the Vroman effect can be divided into "early" and "late" stages. The early stage involves the rapid adsorption

of albumin, IgG, and fibrinogen, which are replaced in second step by apolipoproteins and coagulation factors [16]. Mathematical modelings suggest that the high abundance and fast dissociation of albumin and fibrinogen coupled with the low abundance and slow dissociation of apolipoproteins accounts for the sequential adsorption. The early stage of the Vroman effect is not observed for every nanomaterial. The late stage of the Vroman effect occurs as proteins having moderate affinities are replaced by those having very high affinities.

2.3.1   Early Stage

As it was mentioned, the early stage involves the rapid adsorption of albumin, IgG, and fibrinogen upon administration of the nanomaterial inside the biological environment. Serum albumin has a high concentration in the blood plasma. Due to exposure of nanoparticles to the blood, a layer of serum albumin is adsorbed on the surface of most nanomaterials in the early stage which over the time is replaced by proteins with higher affinity to adsorb on the surface [11].

It should be noted that due to the change of the protein corona composition from the early stage to the late stage, for investigation of the biological behavior of nanoparticle such as phagocytosis, cellular uptake, and toxicity, the relevant protein corona composition related to the time scale of these processes should be considered.

2.3.2   Late Stage

The evolution of protein corona on solid lipid nanoparticles indicated adsorption of albumin in the early stage which partially was replaced by fibrinogen. The longer incubation time resulted in replacement of fibrinogen with IHRP (inter-α-trypsin inhibitor family heavy chain-related protein) and apolipoproteins [17]. Although the concentration of fibrinogen is substantially higher than that of apolipoproteins, the higher affinity of apolipoproteins to adsorb on hydrophobic surfaces is the main reason for replacement of fibrinogens by apolipoproteins.

Jansch et al. [18] investigated the kinetics of protein adsorption on ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles in order to understand the protein–NP interactions and to clarify if there is a Vroman effect on iron oxide nanoparticles or not. A change in the protein adsorption patterns as a function of time can also change the organ distribution of the nanoparticles. Furthermore, the impact of prolonged incubation times on the protein adsorption pattern of USPIO nanoparticles has been analyzed. The plasma protein adsorption kinetics on USPIO NPs was compared to previously published kinetic studies on polystyrene particles (PS particles) and oil-in-water nanoemulsions and was analyzed by 2D-PAGE. The results indicated that there is no typical Vroman effect on the USPIO NP. No displacement of
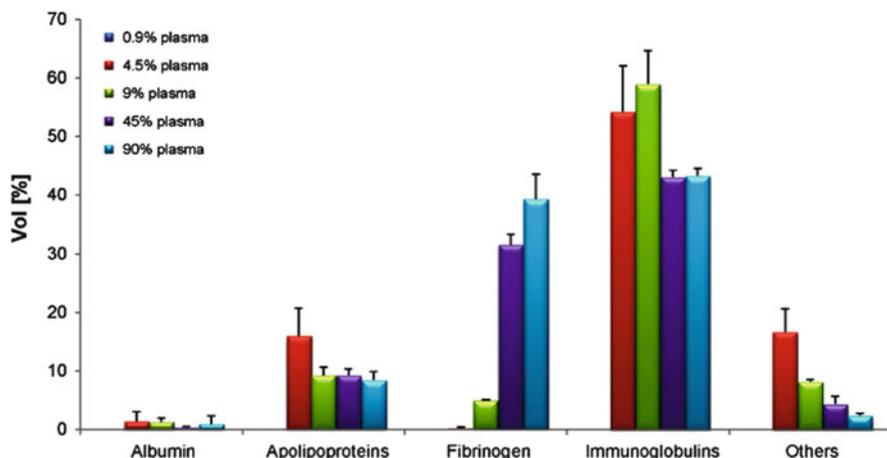
Fig. 2.2 Relative volume of the major proteins adsorbed on the surface of USPIO NP obtained after incubation with different plasma dilutions (adapted with permission from [18])
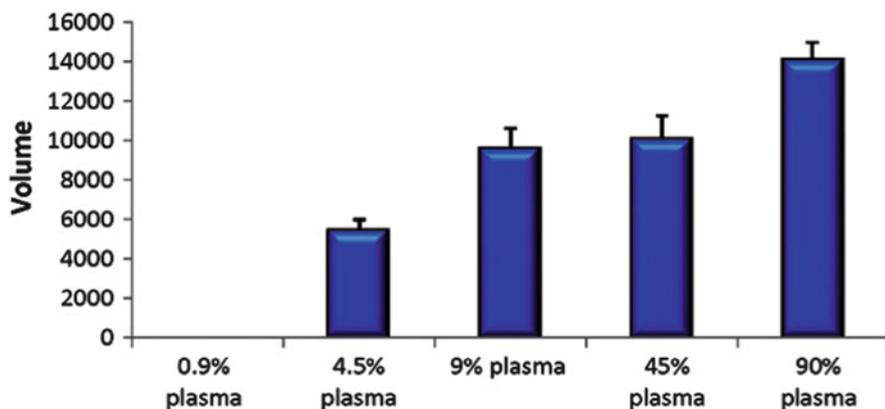

Fig. 2.3 Total amounts of proteins adsorbed on the surface of the USPIO NP after incubation with different plasma dilutions (adapted with permission from [18])

previously adsorbed proteins by other proteins possessing a higher affinity to the particle surface can be determined. Compared to other nanomaterial-based drug delivery systems, similar results have been reported singularly for o/w nanoemulsions, whereas the existence of a Vroman effect has been observed on the surface of polymeric model particles. There are also differences in the protein adsorption patterns received from USPIO compared to nanoemulsions. Immunoglobulins are the dominant protein group during all steps of plasma protein adsorption onto USPIO particles. An increasing amount of fibrinogen with prolonged incubation times has been observed (Figs. 2.2 and 2.3). Low amounts of adsorbed dysopsonic proteins, such as apolipoproteins and albumin, support this prediction. Over a certain period of time,

minutes to hours, more important for the in vivo behavior of intravenously injected particles, the protein adsorption patterns were qualitatively similar to each other. Furthermore, the relative amount of major proteins, such as apolipoproteins, fibrinogen, and albumin, kept constant over time. The amount of adsorbed immunoglobulins increased with incubation time. The knowledge of the protein adsorption patterns and kinetics on USPIO nanoparticle surfaces can be an important step on the way to tailor-made targeted iron oxide nanoparticles. Thus, when processes of protein adsorption and the corresponding body distribution are known, one can design USPIO with optimized physicochemical surface properties, which are expected to automatically adsorb the proteins required for localization in a certain tissue, i.e., these iron oxide NP are "self-targeted" to the desired site of action.

2.4 Parameters Affecting Protein Corona

Although there is a growing agreement that the protein corona is what is seen by cells, yet, more research is required to better understand why any nanomaterial chooses those particular proteins. Various parameters such as nanoparticle size, shape, curvature, surface charge (zeta potential), solubility, surface modification, and route of administration of nanoparticles to the body affect the composition, thickness, and conformation of protein corona. These parameters have been reviewed recently by various groups [6, 7, 11, 19]. Among the nanoparticle (NP) parameters which affect the protein corona, the surface properties such as hydrophobicity and surface charge have more significant role than other parameters [5]. In the following section, the role of each parameter is explained with more details. Better understanding of role of each physicochemical parameter on the protein corona is promising for design of targeting nanomaterial, long-circulating drug carriers, or for decreasing the toxicity.

Casals et al. [20] studied the time evolution of the protein corona in Au NPs. These NPs of different sizes (4–40 nm) stabilized electrostatically with (1) citrate ions and with a self-assembled monolayer (SAM), (2) mercaptoundecanoic acid (negative surface charge), and (3) aminoundecanethiol (positive surface charge). They explored the formation of the protein corona after exposure of Au NP to cell culture media containing 10 % of fetal bovine serum (FBS). Under in vitro cell culture conditions, zeta potential measurements, UV–vis spectroscopy, DLS, and TEM analysis were used to monitor the time evolution of the protein corona. As expected, the redshift of the surface plasmon resonance peak, as well as the drop of surface charge and the increase of the hydrodynamic diameter indicated the conjugation of proteins to NP. An evolution from a loosely attached toward an irreversible attached protein corona over time was observed. Mass spectrometry of the digested protein corona revealed albumin as the most abundant component which suggests an improved biocompatibility.

2.4.1   Surface Charge of Nanoparticle

Nanoparticle surface charge is another important factor in protein interaction. It has been reported that by increasing the surface charge of nanoparticles, the protein adsorption increases. Positively charged nanoparticles prefer to adsorb proteins with isoelectric points (pI) <5.5 such as albumin, while the negative surface charge enhances the adsorption of proteins with pI > 5.5 such as IgG [5]. Using negatively charged polymeric nanoparticles, Gessner et al. [21] observed an increase in plasma protein adsorption with increasing surface charge density. Other studies from the same group with polystyrene nanoparticles reveal that positively charged particles predominantly adsorb proteins with pI < 5.5, such as albumin, whereas negatively charged particles adsorb proteins with pI > 5.5, such as IgG.

Bradley et al. [22] reported binding of complement (C1q) to anionic liposomes. Significant plasma protein binding to vesicles containing cationic lipids has been reported [23]. This may arise from electrostatic interactions between the cationic lipids and most of the negatively charged plasma proteins.

Surface charge can also denature the adsorbed proteins. In a recent study on the gold nanoparticles with positive, negative, and neutral ligands, it was found that proteins denature in the presence of charged ligands, either positive or negative, but the neutral ligands keep the natural structure of proteins [7].

2.4.2   Nanoparticle Material

The study of the plasma proteins bound to single-walled carbon nanotubes (SWCNT) and nano-sized silica indicated different patterns of adsorption. Serum albumin was found to be the most abundant protein coated on SWCNT but not on silica NP. TiO_2, SiO_2, and ZnO NP of similar surface charge bind to different plasma proteins (Table 2.1) [24].

2.4.3   Surface Functionalization and Coatings

Pre-coating and surface functionalization can be employed to decrease the adsorption of proteins or engineer the protein corona composition. Studies on polystyrene nanospheres coated with Poloxamine 908 showed a reduction of fibronectin adsorption. In other studies on functionalization of CNT and SiO_2 nanoparticles with Pluronics F127, a reduction of serum proteins' adsorption was noticed. A summary of the role of various coatings such as PEG, poloxamer, poloxamine, dextran, Pluronic F127, polysorbate, and poly(oxyethylene) on the quantity of adsorbed plasma protein, phagocytic uptake, and biodistribution is tabulated by Aggarwal et al. [5]. It should be mentioned that the available data on the role of functional

Table 2.1 Identification of proteins bound to nanoparticles by gel electrophoresis and mass spectrometry [24]

Nanoparticles	Proteins
TiO$_2$	Albumin, fibrinogen (α and β chains), histidine-rich glycoprotein, kininogen-1, complement C9 and C1q, Ig heavy chain (γ), fetuin A, vitronectin, apolipo-protein A1
SiO$_2$	Albumin, fibrinogen (α, β and γ chains), complement C8, Ig heavy chain (gamma, kappa), apolipoprotein A
ZnO	Albumin, Ig heavy chain (alpha, mu, gamma), apolipoprotein A1, immunoglobulin (J chain), alpha-2-macroglobulin, transferrin, alpha-1-antichymotryspin

group and coatings on the protein corona is not fully developed yet and more studies are still required to let us tailor the composition of the protein corona with surface treatment of nanoparticles.

Surface functionalization with PEG of varying chain length resulted in major changes in organ/tissue-selective biodistribution and clearance from the body, although 2D gel electrophoresis showed that immune-competent proteins (IgG, fibrinogen) bind much more than albumins irrespective of PEG chain length.

Numerous studies established that aqueous suspensions of nonfunctionalized nanoparticles are stabilized against agglomeration by the addition of bovine/human serum albumin (BSA/HSA) and some other proteins. The effect has also been exploited in production for the debundling and dispersion of graphene and CNT material. Especially albumins in water or Dulbecco's Modified Eagle Medium (DMEM) have dispersed and stabilized a wide variety of nanomaterials: CNTs, metal nanoparticles, metal carbide nanoparticles, and metal oxide nanoparticles.

2.4.4 Hydrophilicity/Hydrophobicity

The hydrophobicity affects both the amount of adsorbed protein as well as the composition of protein corona. The enhanced adsorption of proteins on hydrophobic surface in comparison with hydrophilic surface increases the rate of opsonization of hydrophobic nanoparticles [5].

Hydrophobic or charged surfaces tend to adsorb more proteins and denature them with a greater extent than neutral and hydrophilic surfaces. For example, increasing the negative charge density and hydrophobicity of polystyrene nanoparticles increases protein adsorption from plasma, and more hydrophobic copolymer nanoparticles adsorb more protein than their hydrophilic counterparts [15].

Hydrophobic nanoparticles adsorb more albumin molecules than hydrophilic nanoparticles, even though the affinity of the protein to both nanoparticle types is roughly the same [25]. This suggests that hydrophobic copolymer nanoparticles have more protein-binding sites. This may result from "clustering" of the

hydrophobic polymer chains, forming distinct "islands" which act as protein-binding sites.

In an earlier work by Moghimi and Patel, an important observation was made that liposomes rich in cholesterol bind less protein than cholesterol-free liposomes [26]. Liposomes composed of neutral saturated lipids with carbon chains greater than C16 have been reported to bind larger quantities of blood proteins compared with their C14 counterparts [27]. This has been explained by stronger affinities of plasma proteins, especially IgG and albumin for hydrophobic domains. Therefore, it can be concluded that the affinity of proteins to nanomaterials with uniform surface chemistry tends to increase with increasing charge density and hydrophobicity [28].

2.4.5   Nanoparticle Size

Due to surface curvature, protein-binding affinities are different for NPs and flat surfaces. Therefore, the protein adsorption data on flat surface should not be extrapolated for NPs. In addition to protein-binding affinity, the composition of protein corona is different for same NPs but with different sizes [1]. The change of composition and organization of proteins in the corona is very significant when the nanoparticle size is approaching the size of proteins [7]. The highly curved surfaces of nanomaterials decrease protein–protein interactions. Proteins adsorbed to highly curved nanoparticles tend to undergo fewer changes in conformation than those adsorbed to less curved surfaces.

Size and curvature of nanoparticles also appear to affect protein binding. For example, classical IgM-dependent complement activation is most efficient on dextran particles in the optimal size range, ~250 nm, whereas larger particles do not attract as much IgM and therefore do not activate to the same extent. The same phenomenon of size-dependent activation of complement was observed for liposomes. Dobrovolskaia et al. [13] reported that more proteins were adsorbed on 30 nm than on 50 nm gold particles. Lynch et al. [7] studied the role of particle size and surface area on the protein adsorption on NIPAM/BAM (50:50) copolymer nanoparticles. Using nanoparticles varying in size between 70 and 700 nm, they showed that the amount of bound plasma proteins increased with increasing available surface area at a constant particle weight. At a constant weight fraction of nanoparticles, the surface area available for protein binding increases with decreasing particle size. Another study involving the interaction of gold nanoparticles with common plasma proteins suggests that the thickness of the adsorbed protein layer increases progressively with nanoparticle size. Gold nanoparticles can initiate protein aggregation at physiological pH, resulting in the formation of extended, amorphous protein–nanoparticle assemblies, accompanied by large protein aggregates without embedded nanoparticles. Proteins on the Au nanoparticle surface are observed to be partially unfolded; these nanoparticle-induced misfolded proteins likely catalyze the observed aggregate formation and growth.

2.4.6   Biological Environment

Maiorano et al. [29] studied the nano-biointeractions occurring between commonly used cell culture media and differently sized citrate-coated gold nanoparticles (Au NP) by different spectroscopic techniques (DLS, UV-visible, and PRLS). They determined how media composition influences the formation of protein–NP complexes that may affect the cellular response. They demonstrated that protein–NP interactions are differently mediated by two widely used cellular media (DMEM and Roswell Park Memorial Institute medium (RPMI) supplemented with the protein source Fetal bovine serum (FBS)). These media are exploited for most cell cultures and strongly vary in amino acid, glucose, and salt composition. A range of spectroscopic, electrophoretic, and microscopic techniques were applied in order to describe the biomolecular entities formed by dispersing the different sized NP in the cellular culture media. They characterized protein corona composition, exchanging kinetics of different protein classes, along with the physical status of gold NP in terms of agglomeration/aggregation over time. They observe that DMEM elicits the formation of a large time-dependent protein corona and RPMI shows different dynamics with reduced protein coating. Polyacrylamide gel electrophoresis and mass spectroscopy have revealed that the average composition of protein corona does not reflect the relative abundance of serum proteins. To evaluate the biological impact of the new bio-nanostructures, several comparative viability assays onto two cell lines (HeLa (human epithelial cervical cancer cell line) and U937 (human leukemic monocyte lymphoma cell line)) were carried out in the DMEM and RPMI media, in the presence of 15 nm Au NP. Au NP uptake and cellular distribution were addressed by applying a label-free tracking method, based on two-photon confocal microscopy. They observed that the dynamics of protein–NP interactions are differently mediated by the different composition of cellular media. DLS, UV–vis absorption, and PRLS data, obtained by in situ studies, revealed effects on the physical status of the NP mediated by DMEM or RPMI. In particular, DMEM induced a more abundant and quite stable protein corona on different sizes of Au NPs as compared to RPMI. These observations were also confirmed by ex situ analyses, in which the strongly adsorbed proteins onto metal surfaces were analyzed by SDS-PAGE and Mass Spectrometry (MS). The different formation of proteins–NP complexes mediated by liquid environment can impact on cellular response (Fig. 2.4).

These results obtained showed that before cellular experiments, a detailed understanding of the effects elicited by cell culture media on NP is crucial for standardized nanotoxicology tests. Thereby, to evaluate NP dose-dependence toxicity in in vitro tests, all experimental parameters, comprising the choice of the cellular medium, as well as the origin and preparation of serum, should be carefully taken into account with the aim to design standardized protocols.

Monopoli et al. [1] employed differential centrifugal sedimentation and dynamic light-scattering techniques and showed that by decreasing the concentration of plasma, the thickness of hard protein corona around nanoparticles decreases.
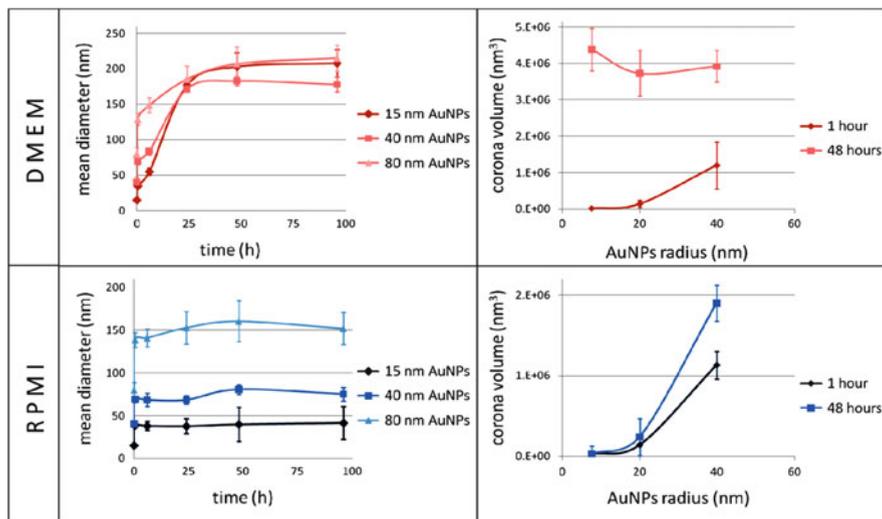
Fig. 2.4 DLS analyses of Au NPs in DMEM (*top*) and RPMI (*bottom*) with 10 % FBS, at 37 °C. *Left panels*: time-dependent evolution of protein corona in the two cellular media, *right panels*: protein corona volume versus Au NP radius at two incubation times (1 and 48 h) (adapted from [29])

Therefore, the protein corona can change significantly between in vitro test (with lower protein concentration) and in vivo tests. This indicates that the in vitro studies of nanoparticles cannot always predict the behavior of nanoparticles in the living biological environments. They studied on the composition of the protein corona at different plasma concentrations with structural data on the complexes both in situ and free. They presented the protein adsorption for two different NPs, sulfonated polystyrene, and silica NP. NP–protein complexes are characterized by differential centrifugal sedimentation, dynamic light scattering (DLS) and zeta potential in situ and once isolated from plasma as a function of the protein–NP surface area ratio. They introduced a semiquantitative determination of the hard corona composition using 1D-PAGE and liquid chromatography (LC)/mass spectrometry (MS) (LC–MS/MS) which allows following the total binding isotherms for the particles, identifying the nature and amount of the most relevant proteins as a function of the plasma concentration. This allows us to illustrate more quantitatively the degree to which the biomolecule corona can change, depending on the biological environment. They found that the hard corona can evolve quite significantly between protein concentrations appropriate to in vitro cell studies to those present in in vivo studies, which has deep implications for in vivo extrapolations and will require more considerations in the future. They have combined studies on the composition of the protein corona at different plasma concentrations with structural data on the complexes. By applying methods of semiquantitative MS, they can create the adsorption isotherms of the different components of the adsorbed layer and relate the amounts bound from MS to those found from

structural studies. Thus, the principal observation is that binding leads to relatively complete surface coverage for even low plasma concentrations. The protein concentration studies suggest a progressive displacement of proteins with lower affinity in favor of those with higher. However, there are significant differences compared to the more usual forms of adsorption: the protein layer is irreversible on the time scales of the experiments. They have interpreted this to mean that the system seeks to lower its surface energy by selecting and exchanging on shorter time scales from the whole set of proteins that diffuse to the surface.

The transfer of nanoparticles from one biological environment to another such as cellular uptake from blood stream or transport from cytosol to nucleus changes the exchange rate and corona composition [7]. One study on the protein corona evolution by transfer from blood plasma to the cytosolic fluid, a process similar to cellular uptake of nanoparticles, showed that a fingerprint of previous environment will be left inside the corona which can be employed for monitoring the transfer pathways of nanoparticles and their fate [11].

2.5   Ignored Issues of Protein Corona

Recent findings proved the fact that there are few additional ignored factors that strongly affect the composition of protein corona and their consequence cellular responses.

2.5.1   Temperature

One of the not investigated but very important influencing factors on the composition of protein corona is the slight changes in incubation temperature of nanoparticles. As the mean body temperature for different individuals is in the range from 35.8 to 37.2 °C [30], this ignored factor is very important for the in vivo applications of nanoparticle. It is noteworthy to mention that the temperature varies for different parts of the body and the body temperature of females is slightly higher than men and can be also influenced by their hormonal cycle (basal body temperature). During sleep, the body temperature decreases and manual work leads to an increase of up to 2 °C. This means that the body temperature for healthy humans varies in the range from 35 to 39 °C and can find a maximum of 41 °C in the case of fever [31]. If the corona formation is influenced by the temperature, then an influence of the body temperature on the cellular uptake of nanoparticles can appear.

Incubation of dextran-coated SPIONs (i.e., Fe_3O_4) with various surface chemistries (e.g., negative, plain, and positive) with FBS, respectively, revealed the fact that slight temperature changes can significantly vary the composition of protein corona (see Fig. 2.5) [32].
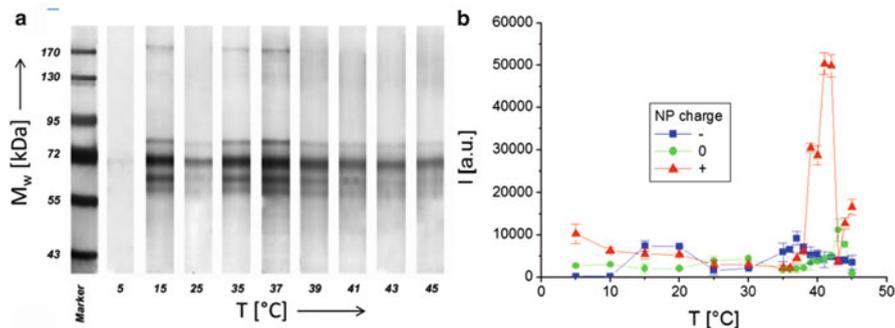
Fig. 2.5 (**a**) SDS-PAGE gel of proteins adsorbed onto the surfaces of negatively charged Fe_3O_4 NPs after 1 h incubation in FBS at different temperatures T. The molecular weights M_w of the proteins in the marker lane on the *left* are reported for reference. (**b**) Quantification of the amount of adsorbed proteins on negatively charged ($-$), neutral (0), and positively charged (+) NPs as derived from the total band intensities of proteins on the SDS-PAGE (one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels (adapted with permission from [32])
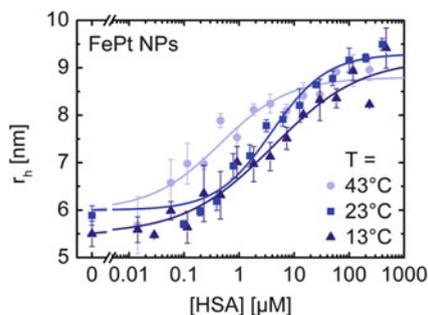

Fig. 2.6 Dependence of the hydrodynamic radius of negatively charged FePt NPs on the concentration of HSA in the solution due to protein adsorption at 13, 23, and 43 °C (adapted with permission from [32])

The fluorescently labelled, negatively charged polymer-coated FePt were also employed for evaluation of the attachment of Human serum albumin (HSA) to their surfaces using fluorescence correlation spectroscopy (FCS) [33]. The HSA were incubated with FePt nanoparticles for 10 min at different adjusted temperatures (T); then, the fluorescence were measured with the FCS setup for 4 min at the same temperature T. Hydrodynamic radii r_h as determined with FCS were plotted versus the HSA concentration in solution, $c(HSA)$ (see Fig. 2.6). N is the number of adsorbed HSA molecules per NP, and N_{max} is the maximum number of adsorbed molecules. At saturation, the hydrodynamic radius of one NP is calculated according to

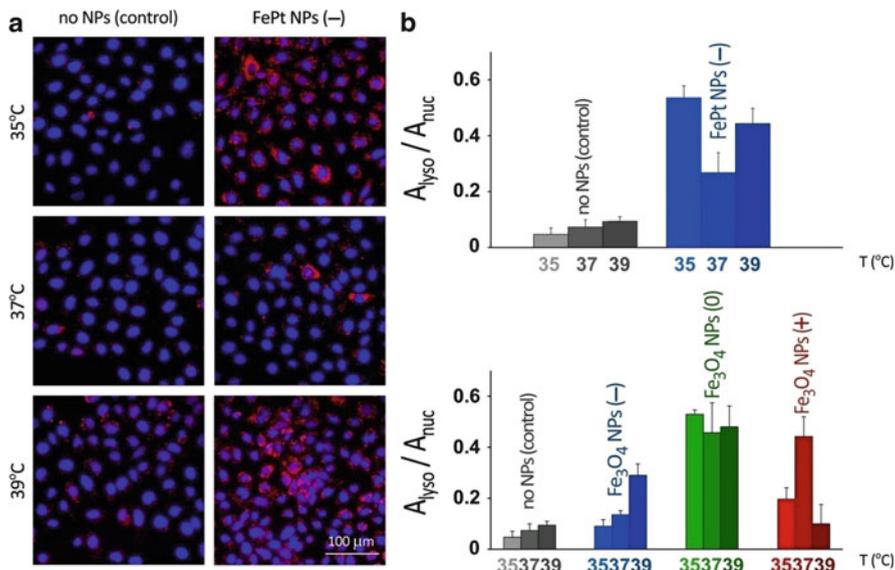$$r_h(N_{max}) = r_h(0) \cdot \sqrt[3]{1 + c \cdot N_{max}} \qquad (2.1)$$

Fig. 2.7 (**a**) Confocal images showing lysosomes (stained in *red*) within epicardial mesothelial cells (nuclei stained *blue*), interacting with Fe₃O₄ NPs at different incubation temperatures. (**b**) Quantification of the lysosome area/nuclear area, calculated by image analysis of confocal images (adapted with permission from [32])

where $c = V_p/V_0$ is the volume ratio of protein molecule to an NP. These volumes are calculated using $V_0 = (4\pi/3)\cdot(r_h(0))^3$ and $V_p = (M_w/N_A)/\rho_p$, with the molecular weight, M_w, of HSA; the Avogadro constant, N_A; and the protein density, $\rho_p = 1.35$ g/cm³ [12]. Concentration-dependent adsorption is described by the Hill equation

$$N = N_{max} \frac{1}{1 + (K'_D/[HSA])^n} \tag{2.2}$$

where K'_D represents the concentration of HSA molecules for half coverage and n is the Hill coefficient which determines the steepness of the binding curve [12].

The cellular uptake results of the various nanoparticles are presented in Fig. 2.7; according to the results, one can conclude that the temperature of the target part of the body should be considered in designing nanoparticles for high-yield biomedical specific applications (e.g., drug delivery and imaging).

In addition to the effect of incubating temperature, the effects of local slight heat induction (by laser activation) have been also investigated [34]. More specifically, cetyltrimethylammonium bromide-stabilized (CTAB-stabilized) gold nanorods (see Fig. 2.8) were incubated with different concentrations of FBS (i.e., 10 and 100 %), and their corona compositions were evaluated before and after laser activation. The compositional changes of the protein corona for the representative
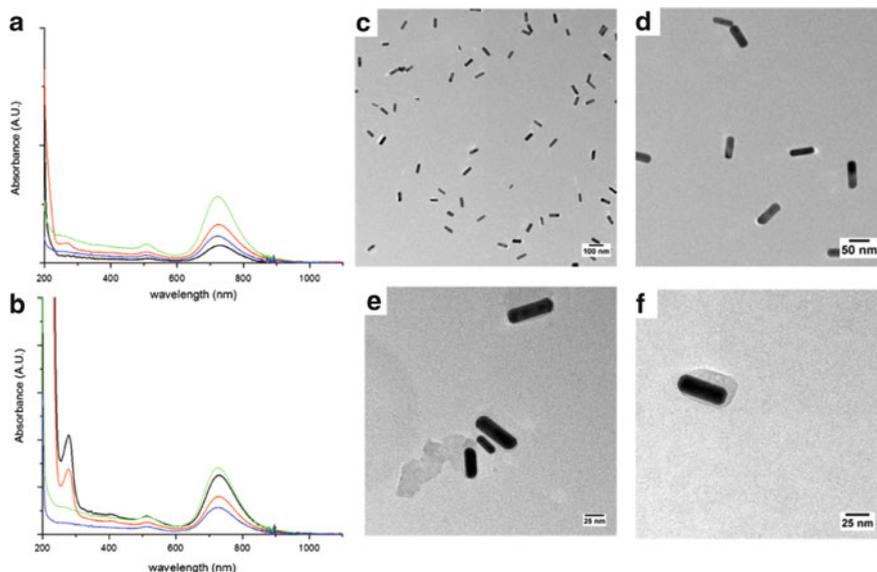
Fig. 2.8 UV–vis absorption spectra and transmission electron micrographs of AuNR–protein complexes before and after laser irradiation or thermal treatment at 45 °C for 55 min. (**a**) UV–vis spectra for 10 % protein–AuNR complexes following hyperthermia treatment. Green spectrum: 45 °C treatment. Blue spectrum: 37 °C treatment. Black spectrum: 27 min laser irradiation. Red spectrum: 55 min laser irradiation. (**b**) UV–vis spectra for 100 % protein–AuNR complexes following treatment. (**c**, **d**) TEM images of CTAB–AuNRs (scale bars 100 nm, 50 nm, respectively). (**e**) TEM image of a protein–AuNR complex (10 % FBS, scale bar 20 nm). (**f**) TEM image of protein–AuNR complex (100 % FBS, scale bar 25 nm) (adapted with permission from [34])

proteins are presented in Tables 2.2 and 2.3. It is notable that the total number of the mass spectroscopy values of the peptides can be analyzed using semiquantitative analysis of the amount of proteins through application of spectral counting method (SpC). The normalized SpC amounts of each protein, identified in the mass spectroscopy study of nanoparticles, would be calculated by applying the following equation:

$$\mathrm{NpSpC_k} = \left(\frac{\left(\frac{\mathrm{SpC}}{(M_\mathrm{w})_k} \right)}{\sum_{i=1}^{n} \left(\frac{\mathrm{SpC}}{(M_\mathrm{w})_i} \right)} \right) \times 100 \qquad (2.3)$$

where $\mathrm{NpSpC_k}$ is the normalized percentage of spectral count for protein k, SpC is the spectral count identified, and M_w is the molecular weight (in kDa) of the protein k. According to the results, one can find that the hyperthermia treatments had modest effects on the overall surface charge of the protein corona associated with the gold nanorods following irradiation but that there were significant changes in the composition of the hard protein corona following irradiation, including

Table 2.2 Representative hard corona proteins associated with AuNRs incubated in 10 % FBS for different thermal and photothermal treatments (incubation at 37, 45 °C, and continuous lasers), as identified by LC–MS/MS; standard deviations were obtained from three individual tests (adapted with permission from [34])

Molecular weight (kDa)	Protein name	NSpC			
		37 °C	Heated at 45 °C	Continuous laser (27.5 min)	Continuous laser (55 min)
69	Serum albumin	5.43 ± 0.10	3.76 ± 1.11	9.75 ± 2.05	9.42 ± 0.70
46	α-1-antiproteinase precursor	4.22 ± 0.24	6.05 ± 3.33	6.37 ± 0.7	7.08 ± 0.59
38	α-2-HS-glycoprotein precursor	7.71 ± 1.57	3.77 ± 1.86	10.02 ± 0.73	14.17 ± 1.79
30	Apolipoprotein A-I precursor	14.95 ± 4.90	9.97 ± 3.29	6.88 ± 2.97	8.40 ± 0.28
16	Hemoglobin fetal subunit beta	9.72 ± 3.85	13.88 ± 0.39	9.51 ± 1.89	13.12 ± 2.06
15	Hemoglobin	5.50 ± 1.59	18.4 ± 5.09	4.84 ± 2.51	5.60 ± 1.63
11	Apolipoprotein A-II precursor	5.89 ± 0.17	7.49 ± 1.08	7.12 ± 0.68	12.51 ± 2.51
11	Apolipoprotein C-III precursor	2.45 ± 0.22	2.90 ± 0.01	4.14 ± 0.02	6.02 ± 1.02

significant changes in the levels of serum albumin associated with the hard corona (see Fig. 2.9). In addition, the time of heat induction during hyperthermia procedure can have a significant effect on the composition of the hard corona, as continuous irradiation with various times (e.g., 27.5 and 55 min) led to different hard corona compositions in the AuNR–protein complexes. The compositional changes observed in the hard corona that are induced specifically by the laser irradiation utilized during hyperthermia treatments are distinct from the changes caused by simple solution heating at 45 °C, and this may reflect relatively high localized temperatures right at the AuNR surface during laser-induced heating.

2.5.2 Gradient Plasma

Although there are too many reports on the protein corona compositions at various circumstances, the interaction between protein concentration gradients and different nanoparticles, which would recapitulate the actual nanoparticle pathways in the human body has been poorly understood [35]. During in vivo journey of nanoparticles, they would be exposed to a variety of biological fluids, according to their administration approaches (e.g., subcutaneous, intradermal, intramuscular,

Table 2.3 Representative hard corona proteins associated with AuNRs incubated in 100 % FBS for different thermal and photothermal treatments (incubation at 37, 45 °C, and continuous lasers), as identified by LC–MS/MS; standard deviations were obtained from three individual tests (adapted with permission from [34])

| Molecular weight (kDa)	Protein name	NSpC			
		37 °C	Heated at 45 °C	Continuous laser (27.5 min)	Continuous laser (55 min)
69	Serum albumin	7.72 ± 3.10	6.69 ± 1.50	9.12 ± 3.05	6.57 ± 1.58
46	α-1-antiproteinase precursor	5.6 ± 1.49	4.76 ± 0.93	4.07 ± 0.33	4.82 ± 0.64
38	α-2-HS-glycopro-tein precursor	14.58 ± 1.22	6.13 ± 0.05	11.06 ± 3.01	11.16 ± 1.39
30	Apolipoprotein A-I precursor	4.15 ± 0.91	8.37 ± 1.96	3.58 ± 0.98	4.21 ± 0.02
16	Hemoglobin fetal subunit beta	16.65 ± 7.04	9.81 ± 1.79	12.31 ± 2.96	9.66 ± 2.44
15	Hemoglobin	7.55 ± 0.70	14.14 ± 4.91	4.78 ± 0.72	3.71 ± 0.70
11	Apolipoprotein A-II precursor	20.95 ± 1.46	3.92 ± 0.57	5.52 ± 0.05	6.01 ± 1.01
11	Apolipoprotein C-III precursor	0.52 ± 0.57	3.12 ± 0.75	2.27 ± 1.18	1.88 ± 0.79
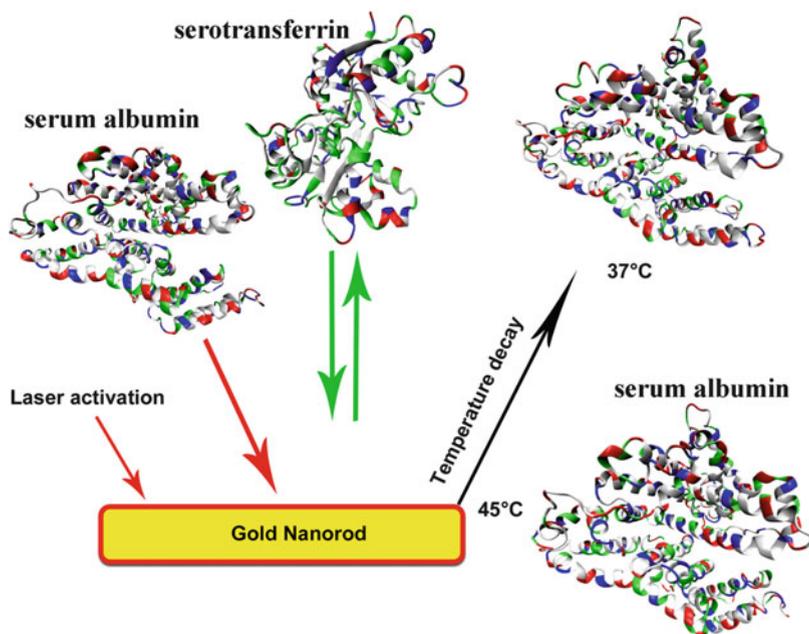
Fig. 2.9 Scheme showing the selective entrance of serum albumin in the composition of protein corona of the laser-activated gold nanorods (adapted with permission from [34])
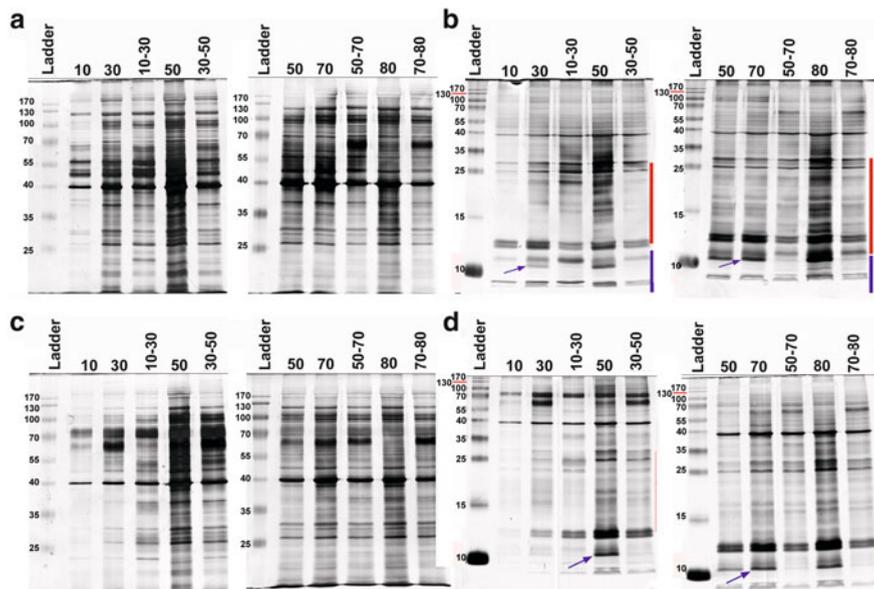
Fig. 2.10 Comparison of the optical intensity across (**a**) 15 % and (**b**) 20 % gel lanes, for silica particles, between non-gradient (*black* and *red*) and gradient (*blue*) coronas; the *x*-axis corresponds to the run length, normalized according to how far different proteins in the molecular weight standards lane had moved in each respective gel; the *y*-axis is the normalized intensity of the lanes (adapted with permission from [35])
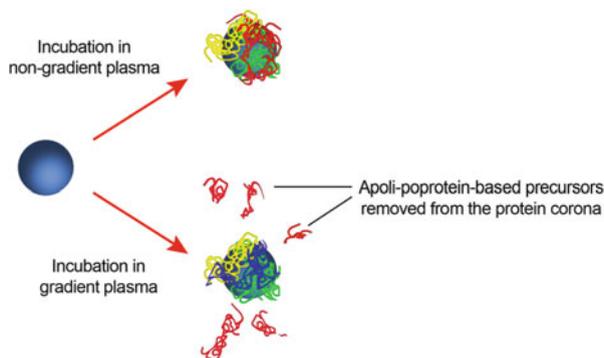

Fig. 2.11 Schematic representation of the importance of NP trafficking on catching apolipoproteins in its corona composition (adapted with permission from [35])

intravenous, intraosseous, intralumbar, and inhalation), which contain different protein compositions and concentrations. For example, the nanoparticle will first "see" the lung cell barrier in the case of being inhaled. Thus, different pathways lead to various corona compositions. In order to show this effect, adsorption of

Table 2.4 The role of nanoparticle's physicochemical and environmental parameters on the protein corona

The parameter	The observed effect
Higher charge density of NP	– Increases density and thickness of corona – Charge particle has higher opsonization rate – Increases protein conformal change
Higher hydrophobicity of NP	– Increases the thickness of protein corona – Increases protein conformal change – Hydrophobicity increases the opsonization rate
Higher curvature of NP	– Increases the corona thickness – Decreases the conformational change – Does not change the identity of adsorbed proteins
Higher protein concentration in environment	– Higher thickness – Change in the identity of adsorbed proteins

Some of the data is adapted from the tabulated data in [2, 5]

plasma protein onto the surface of two commercially available nanoparticles (hydrophobic carboxylated polystyrene (PSOSO$_3$) and hydrophilic silica (SiO$_2$) NPs) were probed. The results showed that apolipoproteins leaved the composition of protein corona following nanoparticle passing low-concentrated proteins to the high-concentrated protein environments (see Figs. 2.10 and 2.11).

2.6   Conclusion

Upon entrance of nanomaterials inside biological environment, proteins start to adsorb on the surface in a competitive manner. The formation of protein layer on the surface is called protein corona which is composed of a hard and a soft region with strong and weak binding to the surface, respectively. Various parameters can affect the composition, thickness, and conformation of these layers which are summarized in Table 2.4. In addition, there are several ignored factored including temperature, protein source pathways, and cell vision, which should be considered in future.

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