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Nanoparticle interactions with blood proteins and what it means: a tutorial review

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ABSTRACT

Nanoparticles find many uses in medicine and biomedical technology. Such applications imply that they must be colloidally stable and do not interact with proteins in the blood or blood serum. A nanoparticle put into the blood will instantaneously be covered by a protein corona that compromises the function of the nanoparticle core, changes the effective size of the nanoparticle, and determines its biological fate. Strategies developed to gain control over nanoparticles in biological fluids, particular in blood, heavily rely on creating a hydrated polymer shell that sterically and osmotically prevents a protein corona from forming. In this tutorial review, we provide an overview of factors that affect the formation of the protein corona in blood and how to prevent it forming. We focus on describing the latest advances in our understanding of how small core-shell nanoparticles (core diameter 4–20 nm in diameter) with a shell of densely grafted polymer chains, a so-called polymer brush, interact with proteins and cells *in vitro*. Such nanoparticles are among the most well-defined and well-characterized colloids used for biomedical applications, from which an improved understanding of how nanoparticle architecture influences their biological fate can be obtained in detail.

Keywords: nanoparticle; interaction; blood protein

INTRODUCTION-ADVANTAGES OF NANOPARTICLES *IN VIVO*

Nanoparticles find many uses in medicine and biomedical technology[1–3], with the number of applications increasing continuously,leading to demands for newer and more versatile nanoparticle designs. The functions of nanoparticles, even within the limited areas of medicine and biotechnology, strongly depend on their intended use. Broadly speaking,these can be categorized as traffic cargo, i.e., to act as drug delivery vehicles[4–7];to extract biomolecules or cells, as adjuvants; to present biological cues; to serve as markers for enhanced contrast in medical imaging[8–10]; or to directly combine with therapies such as photothermic therapy[11–14].The human body provides several opportunities for the application of nanoparticles. In addition to their unique functional properties, nanoparticles can be transported throughout the body via the blood vessels, the lymphatic system, and the nerve system[15].

Cells are around 10 μm in diameter. Organelles and proteins are even smaller, making them similar in size to nanoparticles. Thus, nanoparticles are great candidates for detection, imaging, and delivery, as well as for subcellular environments[1]. In theory,they should be able to navigate such spaces without being excluded or causing significant interruption, due to their

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small size, providing they exhibit similar properties to biological transporters of material and information, such as proteins and vesicles. At sizes below 100–200 nm, nanoparticles have shown to be uniquely suited to deliver anti-cancer drugs[16]. The enhanced permeation and retention effect[16–17] allows nanoparticles in this size range to penetrate through the leaky blood vessels of certain cancers while being unable to do so through the blood vessels of healthy tissue.

All the above applications are strongly dependent on the nanoparticles´ ability to keep their nanoscale size. Like all particles, nanoparticles can aggregate with each other and other particles that they encounter. Aggregation changes their effective size and size–dependent properties. For example, if they aggregate, they lose their ability to diffuse through blood vessels, tissue, enter cells, or efficiently mix with proteins and cells. In addition, they also lose special functions that are strongly dependent on size, such as superpara–magnetism, quantum dot fluorescence, and nanoplas–monic extinction spectra.

Targeting

For all afore mentioned applications it is a require–ment that we target the particles to specific biomark–ers on cells or in tissue, or to the analyte (protein) that should be detected or extracted out of a complex mix–ture. For *in vivo* targeting of drug delivery vehicles or contrast agents, proteins or sugars expressed on the surface of cells in the tissue are of interest. Tradition–ally in biology and medicine, targeting is thought of as achieving a high-affinity interaction with molecules of interest. An acknowledged, but underappreciated fact of biomolecular affinity targeting is that nanopar–ticles must achieve negligible non-specific binding to all other particles, proteins, and cells. If this is not the case, even a high affinity to the target molecule will be outcompeted by weaker attractive interactions with other molecules that are more abundant. This includes non-specific interactions such as protein physisorption to the particle surface. A nanoparticle in the blood is in a medium that is dense with other colloids, i.e.,in particular when the concentration of proteins is very high. It, therefore, undergoes frequent collisions with blood proteins and the interactions between nanopar–ticles and these proteins become of utmost impor–tance; they determine whether the nanoparticles can continue to perform their function or whether they ag–gregate and lose their function, including their ability to target.

Nonspecific interactions of nanoparticles

Nonspecific binding between nanoparticles and proteins (or other particles and molecules) means at–tractive physical interactions must bestronger than the thermal energy, k_BT. Physical interactions that can reach this strength on the nanoscale can be summa–rized as the dominating van der Waals and double–layer interactions of the Derjaguin-Landau-Verwey–Overbeek (DLVO) theory, complemented by entropic interactions such as hydrophobic and depletion inter–actions, as well as specific interactions such as mag–netic forces [18–19].

The basisof van der Waals interaction is that every atom or molecule in a particle contributes an attractive permanent or induced dipolar interaction with those in other particles. The sum of these attractive interac–tions between particles is strong and leads to nanopar–ticles irreversibly binding together, providing there are nolong-range repulsive interactions present. Thermal energy alone is generally not enough to prevent nano–particles from aggregating by van der Waals interac–tions.

Double-layer interaction soccur between particles in electrolytes that have a permanent surface charge. Counter-ions in asolution accumulate to screen the surface charge giving rise to an electroosmotic inter–action between two charged particles. If they have the same sign of the surface charge, the interaction will be repulsive, while if they are opposite signs the force will be attractive.

Entropic interactions arise from the number of pos–sible conformations and distributions of molecules in the medium. The most well-known such interaction is the hydrophobic interaction, by which the reduced entropy of water at non-polar interfaces leads to a short-range but powerful driving force for aggregation between non-polar molecules and colloids in aqueous suspensions. Another such interaction is the depletion interaction that forms anattractive long-range interac–tion, originating in dispersions of colloids that possess very different sizes. Entropic interactions also exist, with the abilityto repel particles from each other,which will be discussed below.

Nanoparticle interaction with proteins and formation of the protein corona

There is an abundance of studies on the fate of na–noparticles in in vitro cell culture and also *in vivo*[18]. The fates of nanoparticles are stongly demonstrated in the body to be determined by: size [9,20], shape [20], surface charge [21], and surface chemistry [22]. With the exception of size, these properties are mainly relevant for how they affect the adsorption of proteins on the surface of the nanomaterial. In terms of size, particles larger than 200 nm are rapidly cleared by the spleen,

NPs smaller than 10–50 nm are generally removed from the body through extravasation and renal clearance. Therefore, the optimal NP size range for *in vivo* applications of intravenously injected NPs that require prolonged blood half-life times is 10–100 nm[9,23]. This is a size range that is not only suitable for nanomaterials with unique nanoscale properties, but also at which frequent and strong interactions with proteins in the blood can occur.

As already mentioned, biological fluids, such as blood are full of cells and proteins especially. These proteins have dimensionsin the order of 10 nm, which technically make them nanoparticles. They exhibit all varieties of surface charges, so the combination of double-layer and van der Waals interactions, therefore,on average, tend to be strongly attractive for any artificial nanoparticle in blood. In other words, proteins will adsorb on nanoparticles that have not been engineered to repel protein adsorption. As they adsorb, they also partially denature to expose internal hydrophobic residues that further increase the strength of the adhesive interaction. The resulting layer of proteins adsorbed on the surface of nanoparticles is referred to as the protein corona[18,24]. Other biomolecules, such as lipids and saccharides, are present in the blood. However,owing to the sheer abundance of proteins, their small hydrodynamic size, and their diverse physicochemical properties, it is these that dominate the adsorption of biomolecules onto nanoparticles in the blood.

The formation of the protein corona occurs almost immediately due to the high concentration of protein in the blood, and is the result of the minimization of free energy from the energetic contributions of the van der Waals, double layer and entropic hydration (i.e.,hydrophobic) interactions between the particles and proteins in the biological fluid that were described above (***Fig.1***)[19,25–26].

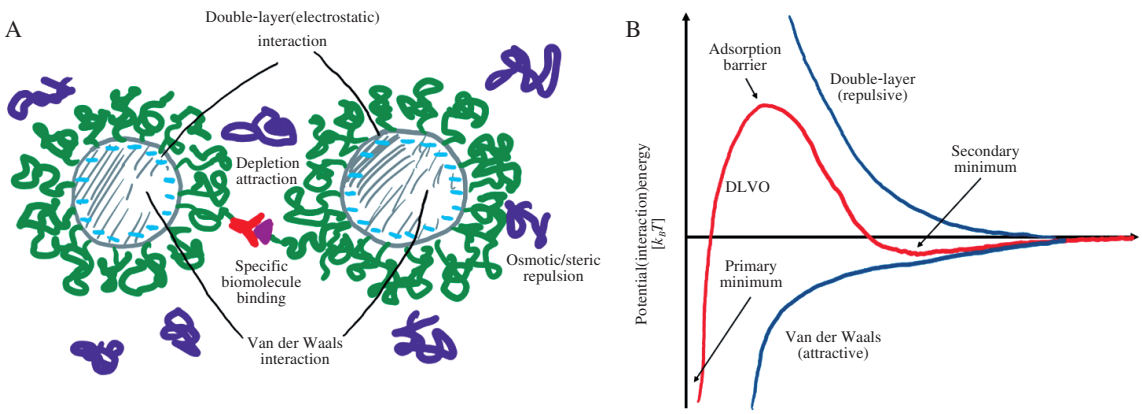

***Fig.1* Colloidal interactions between nanoparticles.** A: The dominant forces between dispersed nanoparticles are the double-layer and van der Waals forces (the DLVO forces), which are complemented by osmotic polymer and depletion interactions as well as specific biomolecule binding. B: Schematic representation of a typical DLVO potential resulting when a strong double-layer repulsion, due to the same surface charge is present. Other contributions to the total potential are ignored. The length-scale of the interaction potentials are many times the protein size.

Weak attachment of abundant proteins will take place first when a nanomaterial without a protein-repulsive surface modification is inserted into the blood. This first formed protein layer is known as the "soft corona" [27]. The soft corona is defined by the reversible binding and relatively fast exchange of the proteins in this shell. Due to this dynamic nature of the soft corona, proteins at lower concentrations are able to continue to form stronger bonds to the surface, resulting in the formation of a more permanent layer of tightly bound proteins on the nanoparticle. This layer is known as a "hard corona" [27]. This time-dependent dynamic is in accordance with the model proposed by Vroman for the evolution of the relative and absolute composition of adsorbed protein layers on surfaces[28], from initially containing primarily abundant weakly adsorbed proteins to finally containing mostly large proteins adsorbing with high affinity [26,28]. Usually, the hard corona will be covered with loosely bound proteins that result in an additional soft corona. An illustration of the soft and hard corona is given in ***Fig.2***.

In conclusion, in whole blood or blood serum, every nanoparticle that has not been specifically engineered to control its interactions with proteins will acquire a protein corona right after injection. While this adsorption of proteins onto nanoparticles has been known for decades [29], its importance and effect on the design of nanomaterials for medicine and biotechnological applications have only been addressed in detailed studies during the last decade[24–25]. So it is only relatively recently that extensive research has been performed to understand the complex interplay of colloidal interac–

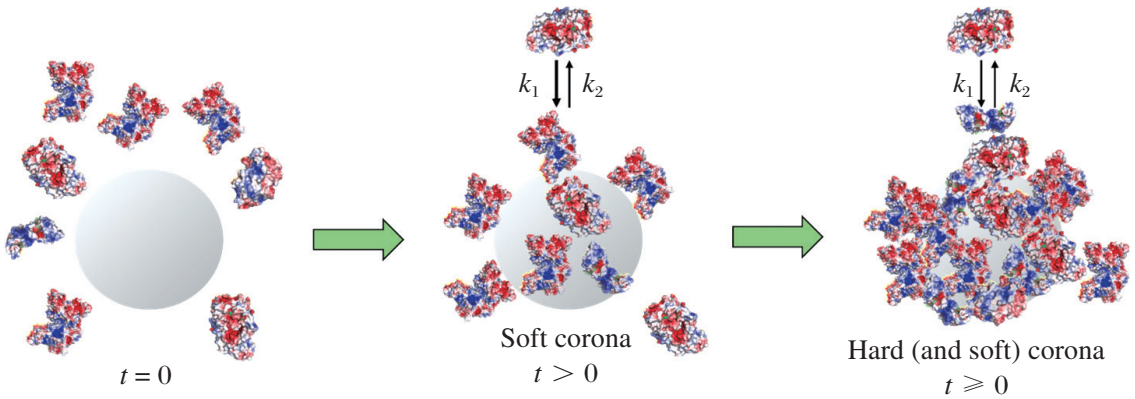

***Fig.2* Illustration of the stages of protein adsorption on a nanoparticle.** Initial protein adsorption leads to the formation of a soft protein corona. Conformational changes of the adsorbed proteins combined with the adsorption of stickier but less abundant proteins lead to the creation of a hard corona. On the hard corona, a new soft corona can form in dynamic equilibrium with the pool of proteins in the blood.

tions in the dynamic formation of the protein corona, as well as its implication on the use of nanoparticles in biomedicine and biotechnology.

The term "protein corona" was initially coined for the adsorption of a layer of proteins onto nanoparticles, which go on to determine further interactions of the nanoparticle in a biological environment [25]. Chithrani *et al.* [30]were the first to suggest that the adsorption of serum proteins on nanoparticles could affect their fate in interactions with cells. They showed that gold nanoparticles introduced into serum-containing media are coated with proteins. The term "protein corona" for the nonspecific coating of nanoparticles with a diverse combination of proteins was subsequently introduced by Cedervall *et al.*[31]

Acellular plasma is composed of 91% water, over 1 100 unique proteins and a small percentage of other biomolecules[32]. Thus,blood plasma contains a high volume of protein of great variety, which makes it hard to predict the corona´s exact composition[33]. However, the abundance of blood proteins is very skewed towards a few classes, with around 55% being albumins, 38% being globulins and fibrinogen comprising 7%[34]. One can, therefore,assume that albumins and globulins dominate the protein corona of most particles introduced into the blood, however two similar particles can end up having different coronas in the same system, reflecting the statistical nature of their formation[19]. It has also been shown that their absolute,and not only their relative abundance affects the composition of the protein corona. The relative amounts of some abundant proteins adsorbed on the surfaces of both inorganic and organic nanoparticles varied with the concentration of blood plasma that they were exposed to[35].

Since the corona is formed by nonspecific colloidal interactions, the physicochemical characteristics of nanoparticle will strongly affect the composition of corona[36]. The corona will also be different from one biological fluid to the other, reflecting that not only the surface properties of the nanoparticles, but also the composition of the biological fluid influences the result. This makes the type of administration of a nanomaterial to the body important. For example, inhalation, intravenous injection,and oral ingestion all lead to different first encounters in terms of environment and protein composition for the nanoparticles [25]. Although the so-called "hard corona" has a long life span, it will eventually dissolve and re-equilibrate when the environment is changed[37]. Thus, the path of the particles through the body, i.e., the environments they encounter, can also strongly influence their functionality over time[38]. The primary determinant of the hard (and soft) corona for injected nanoparticles as well as particles for diagnostics or in longtime circulation is, therefore, the protein composition of blood. Also, for interactions with blood, the composition of the protein corona has been reported to change with time due to continuous adsorption and desorption of proteins with different affinities to the surface[27]. For example, albumin from plasma was shown to be displaced by other proteins from cell lysate on nanoparticles[39]. However, the formation of the first hard corona is critical due to its longevity. For example, both silica and polystyrene nanoparticles continue to display their original plasma protein compositions even after subsequent exposure to other biological fluids[37]. Finally, it might also be interesting to consider that blood physiology can be altered when certain nanoparticles are present in the blood. If nanoparticles present the blood with a high total area for protein adsorption, or if they preferentially bind less abundant proteins, they can change the biochemical balance of the blood in a physiologically relevant way[40–41]. Proteins that are

sequestered by nanoparticles are no longer active, and the absolute and relative amounts of the proteins are changed.

The state of the displayed protein corona is decisive for all applications. Even if adsorption of protein on the particle surface does not lead directly to aggregation, loss of properties,or the ability to circulate or diffuse, it will still play a decisive role in determining the biodistribution of the particlein vivo[35]. Adsorbed proteins can,for e.g., initiate an immune response, which leads to rapid clearance from the body. This may be caused due to size increase, or in a more sophisticated way, for e.g., if the adsorbed protein undergoes misfolding or aggregation. Misfolding can lead to the exposure of new epitopes, which increases cell recognition or enhances the immune response; or it may alter their function and/or affect their avidity[31,42]. Opsonins, i.e., proteins that trigger receptor–mediated phagocytosis, are abundant in blood. When adsorbed to foreign materials such as nanoparticles, they directly signal that an antigen is present that should be cleared. Examples of this effect are,exposure to serum, complement protein or immunoglobulin, which leads to drastically different uptake of nanoparticles, or a change from endocytosis to triggered phagocytosis by macrophages [43] and / or Kupffer cells [44]. However, it is challenging to design experiments that entirely separate effects, due to an increase in size and aggregation from specific biological cues provided by proteins adsorbed on the surface.

Furthermore, a protein corona can severely affect nanoparticles that are supposed to display a specific biological function on their surface. A thick protein layer may mask the functionalization [45] and replace it with rapid recognition and clearance from the body. However, there are also reports suggesting that a protein corona can provide higher stability, lower toxicity, and lower unspecific uptake of nanoparticles [46–48]. This is mostly true for nanoparticles, which on their own are not colloidally stable under physiological conditions. Fast adsorption of a tight shell of proteins on the surfaces of the nanoparticles can lead to a water-soluble layer that sterically stabilizes the cores in the biological environment. The most abundant protein in blood is albumin, which, when tightly adsorbed to a surface, is known to prevent further adsorption of proteins, thereby acting as a bioinert coating. However, surfaces covered with albumin are also known to trigger the complement system and blood clotting. It is not well understood under what conditions these protein coronas are beneficial, and when they,fore.g., trigger recognition by the immune system[47].

Surface modification of nanoparticles with polymers for controlled protein interaction

Leaving the biologically relevant surface properties of nanoparticles and their biodistribution to chance encounters with proteins in the environment, for e.g., in the blood, is not acceptable for the development of any useful applications. Therefore, strategies have been developed to control protein interactions and, thereby,the biological fate of nanoparticles in biological fluids by modifying their surface properties. The objectiveof such surface modifications is to suppress nonspecific adsorption of proteins to the nanoparticles and, when required, to introduce specific protein interactions by the addition of ligands to the particle surface. We described above the strong and prevalent attractive interactions that drive the adsorption of proteins to nanoparticles. Hence, there are not many strategies available, which are able to successfully reduce the adsorption energy sufficiently to suppress the formation of a protein corona. The most common and probably the most successful approach has been to modify the particle surface with a hydrophilic polymer coating. In this way, a highly hydrated coating of a biocompatible and flexible polymer creates a steric–osmotic repulsion that prevents proteins from getting close enough to the particle surface to bind strongly. The polymer shell creates a shell around the core that excludes the formation of a protein corona and protects the function of the core. If proteins cannot adsorb to the nanoparticle surface, there is also no driving force for aggregation, and the size-dependent biodistribution and nanoscale properties remain unchanged.

Methods to create such polymer shells and thereby their structure vary in sophistication. As an example, super paramagnetic iron oxide nanoparticles introduced for clinical use as magnetic resonance contrast agents were typically coated with high-molecular–weight sugars such as dextran or synthetic polymers such as silicone[49], where the affinity of the polymer itself to the particle surface was used for the coating. The inherent compromise in letting the biocompatible and strongly hydrated polymer itself bind to the nanoparticle surface leads to reversible adsorption of these dispersants. The coating around the particle will remain if the polymer is of sufficiently high molecular weight due to the multivalent interactions of the polymer to the particle surface. However, the surface and structure of the shell are ill-defined, and over time, proteins will bind to the particle surface and cause aggregation. For example, the weak affinity of the repeat units of dextran to iron oxide[50] leads to particle aggregation and phagocytosis over a relatively short time-span, even in cell culture media[51]. Additionally, these dispersants typically enwrap multiple iron oxide

cores due to their affinity to all nanoparticle surfaces (***Fig.3A***), which leads to loss of control over the effective size and properties of the resulting nanoparticle clusters[52–53]. Methods to improve the uniformity of the size distribution and to crosslink the shell preferentially around single cores were introduced [54], but the toxicity of chemicals used for crosslinking as well as the limited control over cluster size reduced the attractiveness of this approach.

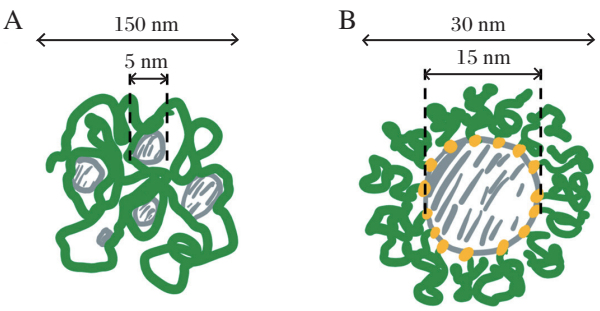

Fig.3 **Schematics of nanoparticles sterically and osmotically stabilized by hydrophilic polymers in clinical applications.** A: Commercially available inorganic nanoparticles in clinical use, fore.g., iron oxide-based magnetic resonance contrast agents are enwrapped in dextran or poly(vinyl alcohol) with an affinity for the nanoparticle surface. The low affinity of the physisorbed polymer leads to a dynamically rearranging and colloidally metastable nanoparticle. Multiple cores are enwrapped by the polymer leading to poor control of overall size. B: Current state-of-the-art research on nanoparticles for biomedical applications emphasizes controlled size and stable surface properties achieved by grafting the polymer around a single larger core. Colloidal stability in biofluids is obtained by the osmotic-steric repulsion of the dense polymer shells.

The core-shell nanoparticle concept for controlled nanoparticle-protein interactions

In nanoparticle research, the dominating approach to controlled structure synthesizing with a polymer shell has become the grafting of a polymer brush to the core surface. Often, the term ´core–shell´nanoparticle in the field of biomedical nanoparticles refers specifically to polymer-brush-grafted nanoparticles. This long established technique was the first patented and most successful nano-container for cancer drug delivery, namely PEG isolated drug delivery liposomes [55]. In this method, poly-ethylene glycol is grafted to the head groups of a fraction of the lipids, which is said to reduce the direct interaction of proteins, in particular lipases and opsonins, with the lipid membrane capsule. State-of-the-art inorganic nanoparticles are almost exclusively grafted with polymer brushes when a controlled structure and reduced protein interactions are desired. The ability to avoid detection by the immune system by preventing protein adsorption is referred to as "stealth", in analogy with the ability of modern military aircraft to avoid detection by radar. The schematic illustration of an inorganic core-polymer brush shell nanoparticle below in ***Fig.4*** can thus be interpreted as a general representation.

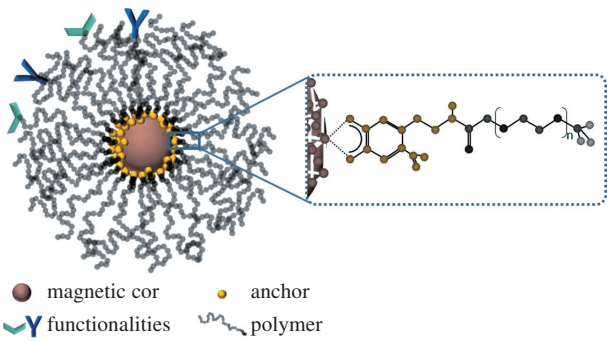

Fig.4 S**chematic representation of a core–shell nanoparticle.** The core functionality, such as drug encapsulation, magnetic, or plasmonic contrast enhancer, is protected by a polymer shell that provides colloidal stability. The shell is linked to the nanoparticle core by an anchor forming an irreversible chemical bond between core and shell. Additional functionalities can be linked to the shell. Reprinted with permission from the American Chemical Society (https://pubs.acs.org/doi/10.1021/acs.langmuir.9b00665)[56].

The hierarchical structure of this type of core–shell nanoparticle allows for the defining of the core´s properties independently of the environment and design the polymer brush to mediate all interactions with the environment, such as solubility and colloidal interactions with proteins and cells in the blood[57–58]. This hydrated shell thus provides an essential function in composite nanoparticles with an inorganic core by providing an osmotically repulsive chemical potential to balance out van der Waals and electric double-layer effects and other long-range attractive interactions of the core[19,56,59]. The van der Waals interaction especially provides a long-range interaction strong enough for nanoparticles to flocculate as well as modulate specific particle interactions even in the presence of a polymer shell [60]. The polymer shell also provides a steric shield that prevents direct physical and chemical interactions of molecules in solution with the core[59].

The repulsive interaction of a hydrated polymer brush shell can either completely neutralize or partially suppress the attractive interactions. If only the latter, the result is weak flocculation or increased probability of protein localization at the nanoparticle shell interface [60–61]. The repulsive osmotic potential of the polymer brush,and therefore the brush itself, must extend over the entire distance over which the attractive potentials are in the order of k_BT[58]. If it doesn´t, blood proteins can bind weakly, i.e., flocculate, on the sur–

face on top of the polymer brush. For a grafted polymer brush to achieve this, it must have high solvation, flexibility, dense grafting, and high molecular weight of the polymer chains[62]. There are today quite a few biocompatible polymers that show suitably high hydration and flexibility, and which can be synthesized using controlled polymerization methods to reach a defined molecular weight. Some of them are also thermoresponsive, which allow advanced smart materials functions for drug delivery and therapeutics [56]. However, despite the fact that arguments can be made for other polymers such as peptoids[63–65], polyoxazolines[66,67], polyoxazines[68] and various zwitterionic polymers [69], the gold standard for applications has remained poly-ethylene glycol (PEG).

Calculating the strengths and extensions of both the attractive interactions of the core and the repulsive potential of the polymer brush are challenging. The challenges in terms of quantifying the DLVO interactions that dominate the attraction to the core are multiple[26,70]. The surface charge or zeta potential of the core surface after modification is difficult to determine, and the polymer brush can also change the screening contribution of ions, due to preferential interactions, like that of the so-called crown ether effect of PEG. Furthermore, the Hamaker constant of a surface, and in this case a nanoparticle, isnotoriously difficult to calculate or even predict reproducibly from experimental data. Likewise, the highly curved geometry of nanoparticles with a diameter <30 nm leads to a strong dependence of brush structure and segment density distribution based on curvature and grafting density[71–74]. Attempts to formulate scaling and self-consistent field models of brush structures have been presented [71], and verified by computer modeling[75–76], but uncertainties in physical parameters such as grafting density, excluded volume and persistence length of the grafted polymers make any direct application of these models impractical. Instead, each system should be characterized carefully, in order to estimate whether aparticle design fulfills the criteria of the brush, preventing the adsorption of proteins to the core or onto the shell.

The relative influence of the DLVO interactions to the extension of the repulsive polymer shell can be estimated by performing experiments that scale the attractive potential contributions differently, with respect to each experimental parameter. For example, the screening of the electric double-layer interaction is strongly dependent on ion concentration while the van der Waals interactions are not. The ionic strength can, therefore, be sequentially changed in a set of experiments to investigate whether the electric double-layer interaction is responsible for aggregating polymer-coated particles. The binding of the particles to a charged interface can then be measured as a function of ionic strength[60–61], which is less challenging than directly measuring the binding of proteins in bulk. Similarly, the van der Waals interaction can be investigated by varying the size of the core particle, if the synthesis method and thereby the particle bulk and surface properties can be kept the same. The dependence of the van der Waals interaction on the radius is known, so if the polymer shells of various particles are identical and the double-layer interaction screened by performing the measurements at high ionic strength[60–61,77], the contribution to the attractive interaction can be measured.

The steric and osmotic repulsion created by a polymer brush is a result of polymer chains being densely grafted on the particle surface that they are forced to stretch in the direction out from the surface. It is this stretching that has given rise to the name brush to describe this type of polymer layers. The stretching is a result of the entropy of the brush and the excluded volume of each chain segment. When the equilibrium volume of free chains overlap, they will regain a part of that volume at the expense of chain entropy by expanding in a direction out from the surface [78]. This is practically achieved by spacing the grafting sites of the polymer chains much closer than their radius of gyration, which defines the equilibrium size of the free chains [79]. The distance that the brush extends is strongly affected by curvature on the same length scale as the polymer chain size. Polymer chains used to graft polymer brushes have radii of gyration close to the size of nanoparticles; thus, this consideration applies [71]. The rapidly expanding free volume available to the polymers in the radial direction leads to a rapidly decreasing polymer segment density, which is different from the more well-researched planar polymer brushes that have an almost constant segment density profile. The consequence is that the osmotic repulsion gets weaker radially from the surface, and in particular that the steric hindrance to protein adsorption might be significantly reduced at a quite short distance from the core surface. This increases the risk of protein adsorption onto and into the polymer shell. In terms of design, these considerations suggest that extremely high grafting densities of relatively high-molecular-weight flexible polymer chains are required.

Achieving polymer brush grafted core-shell nanoparticles that prevent protein adsorption in blood

A highgrafting density means a high energy state

for the polymer chains, which requires a non-reversible bond to the nanoparticle surface to keep the stretched polymers within the brush from cleaving off the surface and dispersing. The binding of the polymer in a polymer brush, therefore, does not rely on physisorption of polymer segments to the nanoparticle core, which would also make the polymer chains susceptible to displacement by proteins adsorbed from the blood. Instead, a chemical moiety with an affinity for the surface, called an anchor, is used as an end-group on the chain (***Fig.4***). We exemplified the importance of using anchor chemistry that irreversibly binds the polymer chains to the nanoparticle core surface in our work on super paramagnetic iron oxide nanoparticles used as contrast agents in magnetic resonance imaging. During experimentation, it was demonstrated that only ligands that strongly coordinate to surface iron ions, such as nitrodopamide-functionalized polymer dispersants, can prevent nanoparticle aggregation under the challenging conditions that are common for in vivo conditions, such as continuous removal of excess dispersants,increased temperature or high ionic strength [80–81]. That the stability of the anchor must be investigated under relevant conditions was demonstrated by that iron oxide nanoparticles, when grafted with hydroxy dopamine-poly(ethylene glycol) (hydroxydopamine-PEG) were able to be dispersed for years at room temperature[82]. In contrast, if they were filtered to remove excess dispersants, they precipitated [80]. This result showed that polymer dispersants in solution can exist in equilibrium with polymers on the surface or in a dynamic equilibrium which provides the appearance of colloidal stability. However, the equilibrium is shifted if the free dispersant is continuously removed and the polymer will be removed from the particle surface accordingly. It is the latter situation that corresponds to the *in vivo* situation in the blood circulatory system, where nanoparticles arepresent at extreme dilutions and are subject to continuous filtration. If the anchor chemistry is inappropriately weak, the shell will be removed, which causes aggregation with proteins and other colloids. In contrast, nitrodopamide provides a much stronger complex with the iron ions on the core surface and withstands not only stringent purification but also thermal actuation, high dilution, and the presence of other dispersants[67,80,83–84]. To further complicate matters, the instability of most nanoparticle surfaces, especially those reliant on ionic bonds, means that a too strongly complexing anchor also can lead to the dissolution of the nanoparticles. An illustration of this, on the same PEG-isolated iron oxide nanoparticles described above, was provided by using mimosine to anchor PEG[80]. The dissolution of the cores during grafting led to only a fraction of the expected colloidally stable core-shell nanoparticles to be formed.

Using a strongly binding anchor is even more critical if a ligand, such as oleic acid or oleylamine, is already present on the nanoparticle surface after core synthesis. Such dispersants are used in almost all methods for synthesizing monodisperse nanoparticles with controlled diameter and shape. They are used to control the growth of the particles after burst nucleation, which has been explained by extensions of the LaMer burst nucleation and growth model by Talapin and others [85–86]. An example is the thermal decomposition of organometallic precursors, such as iron oleate or iron pentacarbonyl to synthesize iron oxide nanoparticles in the presence of oleic acid [87–88], which leaves a strongly complexed shell the ligand on the core surface [87–89]. When the synthesis method results in a strongly bound ligand shell, it is also critical to optimize the protocol, to replace the original ligand with its polymer replacement to achieve a high grafting density of the polymer dispersant[90–91]. Finding such optimized protocols is especially challenging[90,92], while the original ligands are usually nonpolar, and the polymer ligand and final particles are hydrophilic. Thus, the solubilities of the different grafting states vary tremendously, and a protocol for efficient ligand replacement ensuring a uniform distribution on the surface of all nanoparticles requires that the particles at all times during the process are well dispersed[84,93]. The affinity of ligands complexed to the core surface can also be sensitive to the history of the ligand-coated nanoparticle, such as the aging of oleic acid capped iron oxide nanoparticles, which changes the core oxide as well as the dentate binding mode of the oleic acid [94].

Quantitative testing of polymer brush grafted nanoparticles for applications in blood

As explained above, the structure of a core-shell nanoparticle directly translates into the strength and type of interactions, it has with proteins and cells in its surroundings. This structure is sensitive to the environment, especially to factors such as temperature, the concentration of other colloids (particles, polymers, and proteins), ionic strength, and composition. Core-shell nanoparticles should, therefore, be tested under conditions that correspond to the environments relevant to biomedical and biotechnological applications. However, checking them directly in, for e.g., blood, makes it impossible to perform quantitative measurements that are able to implicate improvements in design, in additionthe *in vivo* testing of materials

under development raises ethical concerns. Thus, the emphasis is on trials under conditions that mimic the challenges in blood *in vivo* but that are compatible with quantitative colloidal measurement techniques. These should also enable systematic variations of experimental variables that make it possible to pinpoint the interactions at play and guide improvements in design.

Being able to vary the experimental variables entails performing complete purification of free dispersants, performing experiments at high dilution, using different salts that are present *in vivo* over a range of concentrations including physiological strength, as well as performing tests over a broad range of temperatures from lab temperatures to above body temperature, to name but afew. Achieving the complete purification of excess dispersants after nanoparticle synthesis is a very demanding and often underestimated task[95]. This challenge is posed by the fact that a nanoparticle with a stable shell of polymer shares almost all of its physicochemical properties with the free polymer dispersant in asolution. As PEG-brushes are the most widely used shells for clinical applications, we recently investigated the pros and cons of different methods of purifying PEG-grafted iron oxide nanoparticles[84]. Dialysis through high molecular weight dialysis membranes is a slow but efficient way to remove excess dispersants. Dialysis also risks destabilizing densely grafted nanoparticles, possibly due to the long purification times and highosmotic stress on dense polymer brushes that can take place at the dialysis membrane, which also has an affinity for e.g., iron oxide nanoparticles. Wherever possible, as for magnetic nanoparticles, solvent extraction can be combined with magnetic decantation, to accelerate the extraction of nanoparticles that are too small to sediment or individually to respond to the application of a magnetic field. Free polymer in asolution is not affected by the magnetic field and is extracted more slowly. As a result, particle dispersions can be thoroughly purified substantially more quickly, gently and in larger batches than when using dialysis [84]. Filter centrifugation to remove free dispersant through a filter also tends to cause aggregation of the nanoparticles into the types of mesh filters that are commercially available, which leads to a loss of sample and small batches[84].

Testing forcolloidal stability is often performed using dynamic light scattering (DLS), which tracks the nanoparticles´ hydrodynamic size and their aggregates in a dispersion [96]. Aggregation and lack of sufficient stability are detected if the size increases above the expected size of the single particles, including the strongly hydrated and extended shell. In severe cases, the aggregates become large enough to sediment, resulting in a loss of scattered photon count rate[97–98]. Thus, both the hydrodynamic size and count rate should be monitored. DLS can also be performed on nanoparticles subjected to diluted protein solutions, including blood serum[97,99]. This provides an estimate of the colloidal stability in the presence of proteins. Again, aggregation is monitored, and if different solutions of defined protein compositions are used, problematic protein interactions can be elucidated. One limitation is that the standard commercial DLS instruments available to most labs cannot be applied to blood or protein concentrations at the level of blood or other body fluids. Such protein suspensions scatter and absorb too much light for reliable measurements.

A combination of transmission electron microscopy (TEM) and DLS is usually applied to understand the structure of core-shell nanoparticles. TEM provides an accurate assessment of the size of materials with high electron density, for e.g., inorganic particle cores or heavy-metal stained proteins, while providing almost no contrast for the polymer shell. The measurements are performed in an ultra-high vacuum, which means that even when sufficient contrast is achieved, for e.g., through negative background staining, the size of the shell or the aggregation state of the nanoparticles cannot be estimated correctly. The hydrodynamic size of the particles approximates the size corresponding to the steric-osmotic interactions of the particle, including the shell. Comparing the hydrodynamic diameter obtained by DLS with the core diameter obtained by TEM, therefore, is a powerful way to get a first understanding of the dimensions and structure of a nanoparticle that will correlate with its biomolecular and biological interactions. Advanced techniques such as small–angle neutron [100–101] and X-ray[72] scattering can be used to obtain more detailed insights into the core and shell structures, respectively. Such measurements are currently the only way to compare simulations and theoretical models of the shell directly to measurements.

The current standing of investigations of core-shell nanoparticle interactions with blood proteins and cells

Using these and additional methods, we have, in recent years, performed a range of detailed studies on the interactions of nanoparticle cores in the 4–25 nm size range protected by grafted polymer brush shells. Using an exquisitely well-controlled and monodisperse platform of super paramagnetic iron oxide nanoparticles [100,101] and nitrocatechol anchor chemistry

for grafting ligands irreversibly to the surface of the cores through ligand replacement [90,101,102]. These investigations first showed that a proper characterization and long-term stability without a change in properties requires strongly binding anchor chemistry[102] and a thorough removal of excess dispersants [101].

As described above, for nanoparticles in this size range, the high curvature leads to a rapidly decreasing segment density of the polymer shell. Thus, a combination of very high grafting density, close to 1 chain nm^{-2}, of polymers with a degree of polymerization of at least 50 seem to be required to stabilize nanoparticles at the lower end of this size range[101,103–104]. As a comparison, this corresponds to PEG polymer chains of a molecular weight higher than 2 500 g /mol. While for interactions, which preferably is on the order of a degree of polymerization of 100, a molecular weight of 5 000 g/mol is required for PEG when the colloidal stability should be assured in a complex protein suspension such as blood. As long as the polymer is strongly hydrated, highly flexible and uncharged, results indicate that moredetailed chemistry is less critical. Similar degrees of polymerization also led to results equivalent to those of PEG for different kinds of thermoresponsive poly(2–alkyl–2–oxazoline) and polysarcosineusing the same platform[64,67]. This is in general agreement with research on other platforms. Grafting densities that were even higher (~3 chains nm^{-2}) were achieved for nanoparticles that were less spherical and synthesized without oleic acid ligands [102,105] or alternatively synthesized by a two-step grafting method that first replaced the oleic acid with a small reactive nitrodopamine molecule followed by the grafting of an aldehyde-terminated polymer chain from a melt[97]. Nanoparticles with such high polymer grafting densities are stable even under conditions that denature serum proteins and cause them to precipitate [97].

Observations on how the detailed core-shell structure influences interactions have been made in the following way. Stronger double-layer interactions were observed for core-shell nanoparticles that had a higher curvature (smaller size) when the grafting density and polymer molecular weight was held approximately constant. This observation was made for core iron oxide nanoparticles smaller than 10 nm at negligible ionic strength. Under these conditions the charge on the nanoparticle surface was sufficient for the small nanoparticles to have attractive interactions with negatively charged membranes and other colloids. This is because the segment density profile of a polymer shell for nanoparticles with a lower curvature is more uniform and the shell extends farther from the core surface. Therefore, they were colloidally more stable at these extreme conditions. However, interestingly a tentative correlation with slightly elevated cell uptake was observed [106].

However, importantly, it was shown that core-shell nanoparticles that are stable in blood serum protein suspensions also avoid phagocytosis when tested on in vitro cell cultures[67,103,106,107]. This was shown to be true for a broad range of cell lines, relevant to the environments that nanoparticles will encounter in vivo. Thus, in vitro, the design of nanoparticles that seem to suppress blood protein interactions and avoid aggregation in such media over time, implies a suitable design also for preventing the uptake of nanoparticles by phagocytic cells. This supports the hypothesis that it is the protein corona on nanoparticles that determines their fate.

Recent research has simultaneously added support for the suggestion that core-shell particles that were considered stealth and had long term stability in biological media still can have quite abundant protein interactions[108]. However, this discovery doesn´t necessarily undercut the progress made so far,regarding the design of core-shell nanoparticles that prevent cell uptake based on minimizing their nonspecific interactions with proteins. Instead, it emphasizes the importance of understanding the type of interactions that nanoparticles have with blood proteins, and what kind of coronas can result from these interactions, and how they might affect nanoparticle biodistribution. It could be that weakly adsorbed soft coronas with minimal effects on nanoparticle structure and function are no cause for concern, while hard coronas have a larger tendency to change in structure, function, and substantially increase in particle size leading to loss of control. Thus, understanding the effect of the shell on protein interactions when a corona is formed will also help to better predict their fate in vivo[31]leading to better working strategies to functionalize their surfaces without loss of function *in vivo*[109]. The protein corona is a double-edged sword also in these circumstances. A thick protein layer can mask the biological function of a nanoparticle when this is expressed on the surface of the nanoparticle[45]while at the same time as it may trigger immune system recognition and rapid clearance [47]. However, it can also provide higher stability, lower toxicity and lower unspecific uptake in some cases, much like a polymer brush shell[46–48]. Both results are usually consequences of the change in particle colloidal interactions due to the layer.

An interesting recent example of the role of protein adsorption also in the fate of polymer-grafted core–

shell nanoparticles was provided by Walkey et al. They showed that the uptake of Au nanoparticles by macrophages depended on the presence of adsorbed proteins when the particles were grafted with only a low density (<0.16 chains nm^{-2}) of PEG, while at a grafting density higher than 0.64 chains nm^{-2} it was independent[110]. In another seminal study, Paraket et al. showed that none of their designs for PEG-grafted Au nanoparticles could completely suppress the adsorption of proteins[111]. In other actively debated recent studies, it was suggested that controlled protein adsorption on PEG shells could lower the uptake of nanoparticles by several different cell lines[111,112]. These studies were performed on particles with a PEG grafting density that was below what is required for a polymer brush. Thus, in analogy with the results by Walkey *et al*. [110], proteins were able to adsorb on the nanoparticles and change their interaction with cells in a specific way. Recently, we showed that blood serum proteins do indeed interact with nanoparticles modified with polymer brushes [113]. The interaction was quantified using isothermal titration calorimetry and yielded that in the order of one albumin was adsorbed per particle with an average bond strength of approximately one hydrogen bond. Within the uncertainty of the measurements, the type of polymer grafted as a shell on the nanoparticles did not seem to matter in the size regime of 10 nm in diameter, and a grafting density of the polymer brushes in the order of 1 chain/nm^{-2}. As expected, the adsorption of protein was stronger when a lower grafting density of polymer was investigated.

These are the most sensitive and quantitative measurements that have been performed on protein interactions with core-shell nanoparticles, and they were performed on some of the most densely grafted linear polymer brushes that have been characterized for small nanoparticles. It thus appears as if polymer brushes cannot completely suppress protein adsorption, but that the observed level of protein adsorption with very low binding energy does not compromise nanoparticle stability and stealth properties when tested by *in vitro* cell culture. However, it could provide clues as to why nanoparticles that show promising results *in vitro*, still fail to show long circulation times and active targeting *in vivo*. The question is if further refinement of the polymer shell structure, for example,by providing even more densely grafted linear brush shells[97], using dendritic polymers tailored to the curvature of the nanoparticle[114], or by grafting cyclic polymer shells[99] could provide another qualitative and quantitative jump in core-shell nanoparticle performance in terms of protein interaction and circulation in blood.

Conclusions

The most well-controlled architecture for nanoparticles designed for use in complex biofluids such as blood makes use of grafted polymer shells on functional nanoparticle cores. The shell primarily fulfills the function of controlling the nonspecific colloidal interactions with blood proteins. If there is no shell, a complex and evolving adsorbed protein corona will form on the nanoparticle surface that determines the fate of the nanoparticle in vitro and *in vivo*. However, it has been conclusively demonstrated that a dense polymer brush shell can almost wholly suppress protein adsorption. This ability correlates strongly with the so-called stealth behaviour of nanoparticles that allow them to avoid phagocytosis and clearance. While most recent results in the field demonstrate that abundant albumins in particular, still associate weakly with such core-shell nanoparticles, there is also an ongoing development of new polymer architectures, tailored to the nanoscale size of different types of nanoparticles, which may further improve our ability to control protein and cell interactions when blood is the environment.

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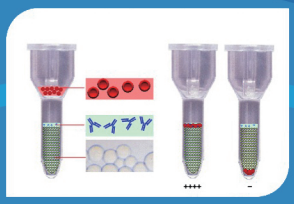

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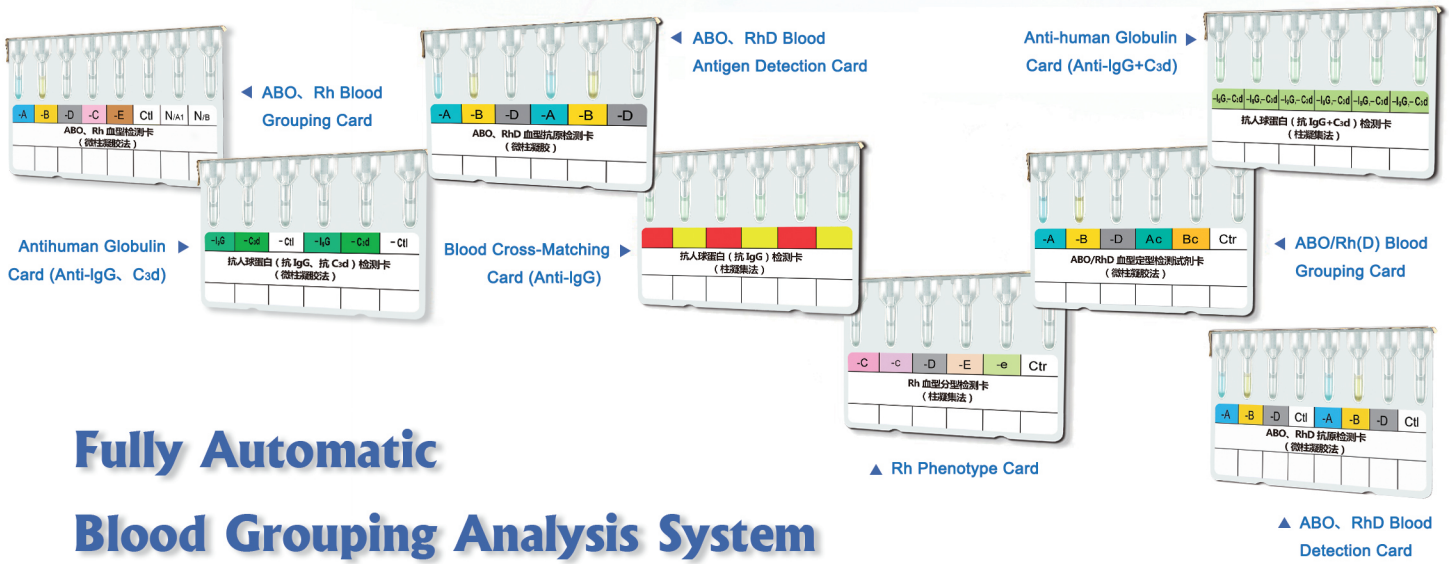

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