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Nanoparticles in the Biological Context: Surface Morphology and Protein Corona Formation

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A recent paper demonstrated that the formation of a protein corona is not a general property of all types of nanosized objects. In fact, it varies between a massive aggregation of plasma proteins onto the nanoparticle down to traces (e.g., a few proteins per 10 nanoparticles), which can only be determined by mass spectrometry in comparison to appropriate negative controls and background subtraction. Here, differences between various types of nanosized objects are discussed in order to determine general structure–property-relations from a physico-chemical viewpoint. It is highlighted that "not all nanoparticles are alike" and shown that their internal morphology, especially the difference between a strongly hydrated/swollen shell versus a sharp "hard" surface and its accessibility, is most relevant for biomedical applications.

During the last decades, the interest in nanosized objects increased world-wide. And, they might come into contact with the living world. Thus, their toxicology and—from a scientific point—the basis for their interaction with living matter became of great interest.[1–3] Parallel to this development, but with a completely different perspective, nanosized drug delivery systems attracted the attention as nanomedicines, entered into clinical trials and became approved drugs.[4–6]

Obviously, the behavior of a nanoparticle in biological environments is extremely relevant, however, the term "nanoparticles" is often used for nanosized objects of different types and thus often does not reflect fundamentally different properties. This makes the comparison between different systems complicated. One motivation of this contribution is to highlight these differences and to look for general concepts, which can explain the different behavior from a physico-chemical perspective.

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In the biological context, the potential formation of a protein corona around nanoparticles is of primary interest. It is the first indication of an unspecific interaction of the nanoparticle with living matter. Additionally, since the process changes the nanoparticle surface properties, it can thus be expected to modify the biological profile in the body.[7–9] Protein corona formation of various nanoparticles has been intensively studied.[10–14] Thereby, the tested nanoparticles were mainly inorganic or organic colloidal nanoparticles, because they represent the mostly prepared systems.[15–18] For these kind of nanoparticles a pronounced corona formation was always observed upon contact with plasma proteins. Due to their high density, it is easy to separate the nanoparticles by centrifugation from the protein solution used for incubation.[19] The formed protein corona also modifies the interaction with cells[12] and can shield recognition units, leading to the loss of targeting functionalities.[20]

However, other types of nanosized drug delivery systems, such as polymeric micelles[21] had been hardly investigated with respect to protein corona formation.[22–25] This is astonishing especially since polymeric micelles are in advanced stages of clinical testing (e.g., CPC634, (phase II)[26] and NC-6004 NanoplatinTM (phase III)[27]) or in the case of Genexol-PM are even approved nanomedicines (approved in Korea and marketed in Europe).[28] Thus, they are particularly relevant concerning the "biological context". Additionally, the few studies aimed to investigate the interactions between proteins and polymeric micelles seem to have mostly positive conclusions indicating that micellar structures tend to be stable in plasma and hold great potential as stealth-like nanocarriers.[22–25]

Recently, a study investigated the protein corona formation in such nanocarriers (core crosslinked micelles and molecular polymer brushes).[29] For this purpose, the nanocarriers were separated by asymmetrical flow field-flow fractionation (AF4) from human blood plasma.[30–33] (for a discussion of different separation methods (AF4 or centrifugation), see the Supporting Information). It is claimed that the sensitivity of this method is high enough to allow the separation of the soft corona proteins with the nanoparticles. Afterward, only a negligible corona formation was found, which means that a large number of all nanoparticles (at least 80%) is not associated with any protein.[29] Thus, also these particles are not absolutely free of some plasma protein interactions, but this low amount is far too small to change the identity of the nanoparticles. This demonstrates that the immediate formation of a significant protein

corona is not a general property of all nanosized objects, which are in contact with plasma proteins.

Now, during publication of these results, many raised questions were just of the type "corona formation has been studied so intensively, why is it not found here?" This is the case because not all nanosized systems are alike. And, this is the starting point for this concept paper. During writing, we were looking for general concepts, which can explain differences in the interaction of various artificial nanoparticulate systems with plasma proteins from a physico-chemical perspective (colloid science). We decided to start from different, relatively simple (straight forward) structures, although some of the nanoparticulate structures used for biomedical applications are quite complex.[14,34,35] For these more simple structures, we discuss different possibilities to reduce the amount of plasma interaction and preconditions to prepare such structures.

It is thereby not the intention to claim that plasma adsorption is generally negative and especially not that nanosized systems, which are different from core crosslinked micelles, will not be useful in the biomedical context. In addition, in vivo incubation of these nanoparticles in the body would probably lead to a larger amount of enriched proteins as reported by Hadjidemetriou, Dawson, Kostarelos, and coworkers for liposomal nanoparticles recollected from the blood of animals and patients.[36,37] That is not astonishing, as things, which happen in the complex living body with all enzymatic activities are beyond such general concepts.

At first, the formation of a protein layer on macroscopic surfaces in contact with an aqueous protein solution (like, e.g., plasma) is known for a long time.[38–40] It happens on implants, but also needles, stents, and on lab equipment used to analyze biological samples. This has led to many attempts to reduce the formation of a plasma film on the surface. Considering the surface material, it is important that it is selected under the terms of the so-called "Whitesides rules"[41,42] to reduce interactions with plasma proteins, which are mostly driven by electrostatic and hydrophobic interactions. According to the Whitesides rules, a surface-material is protein resistant if it is hydrophilic, not charged and, at best, has only hydrogen bond acceptor (no donor) properties. An efficient way to prevent protein adsorption is to coat a surface with a dense layer of strongly hydrated, water-swollen polymer brushes of this type of material. This brush system can then prevent the direct contact of the proteins to the (hydrophobic) surface by entropic repulsion, but without any attractive interaction of the brush with the water-soluble protein. This protection holds as far as the surface coverage is defect-free (see **Figure 1**).

This concept is also valid for nanoparticles. However, in this case, it is more difficult to assure (and to prove) that there are no defects in the hydrophilic shell. This is a limiting step for the use of amphiphiles, which can diffuse away and change places in equilibrium (see Figure 1).

Then, what is the difference between the core crosslinked micelles[21] for which only a negligible corona formation is found[29] and the colloidal nanoparticles studied earlier (and much more intensively)[15–17] for which corona formation is prominent? Thus, it is of prime interest to understand the basic interaction of different polymers and different general types of nanosized objects (see **Figure 2**) with the "biological surrounding".

In short, the core crosslinked polymer micelles discussed and studied recently[29] consist of a hydrophobic core, which is densely grafted with a hydrophilic shell. Any protein will, at first, get in contact with the highly swollen hydrophilic polymer brush, which gets denser as the protein tries to reach the hydrophobic part of the nanoparticle. In this situation, the hydrophilic polymer brushes act as an entropic cushion, keeping the proteins rather effectively away from the interface to the hydrophobic core if they are selected according to the "Whitesides rules". Thus, they resemble–from the side of nanomaterials–most closely surfaces, treated to prevent protein adsorption. From a structural point of view these nanosized objects can be located in between colloidal nanoparticles and micro- and nanogels (see Figure 2). In addition, since a core crosslinked polymeric micelle is, in fact, only one large molecule (every part is connected by covalent bonds), it cannot dissociate partly into unimers and change its surface topology, as it usually happens with non-crosslinked micelles (and also with detergents stabilizing a colloid) upon contact with the concentrated protein solution.

Figure 2 illustrates the fundamental differences between nano-objects of different kinds. Rigid, hard nanoparticles (left) display a sharp surface (surface roughness is typically negligible compared to the particle diameter). Such rigid nanoparticles need to be stabilized in aqueous media, independent of their chemical nature (e.g inorganic particles as silica, gold or iron oxide or organic nanoparticles as polystyrene latex particles). The stabilization needs to overcome the van-der-Waals attraction and can be achieved by i) electrostatic stabilization (surface charges can be generated either by dissociation of ionizable moieties at the surface or via adsorption of ions from the solution), ii) steric stabilization by macromolecules that are soluble in the aqueous medium and somehow fixed to the surface of the particle or iii) by "electrosteric" stabilization, that is a combination of (i) and (ii).[43]

Steric stabilization is also most prominent in nanohydrogels and core crosslinked polymer micelles, so it can be a unifying property. In colloids, it is, however, often achieved by reversible adsorption of amphiphilic polymers. Thus, the hard surface of colloids is—over time—usually directly accessible to small proteins as discussed above for planar surfaces in Figure 1.

Steric stabilization depends on the thickness of the hydrophilic polymer shell, as well as on the density of polymer segments within the shell. The latter typically decreases from the surface of the nanoparticle towards the periphery and can be controlled via i) the grafting density of the polymers at the surface, ii) the length, and iii) the architecture (especially possible branching) of the macromolecules.[43]

The fact that the density of polymer segments decays smoothly towards the periphery has the consequence that the surface is "fuzzy" as compared to hard nanoparticles and thus the "size" is less well-defined. Even for simple isotropic, spherical objects, different experimental techniques probe different properties and thus can lead to different "diameters". Dynamic light scattering, for example, determines the diffusion of objects in solution and is sensitive to the dangling chains in the periphery.[44,45] As a consequence, the hydrodynamic diameter is often bigger than the diameter obtained from, for example, electron microscopy.

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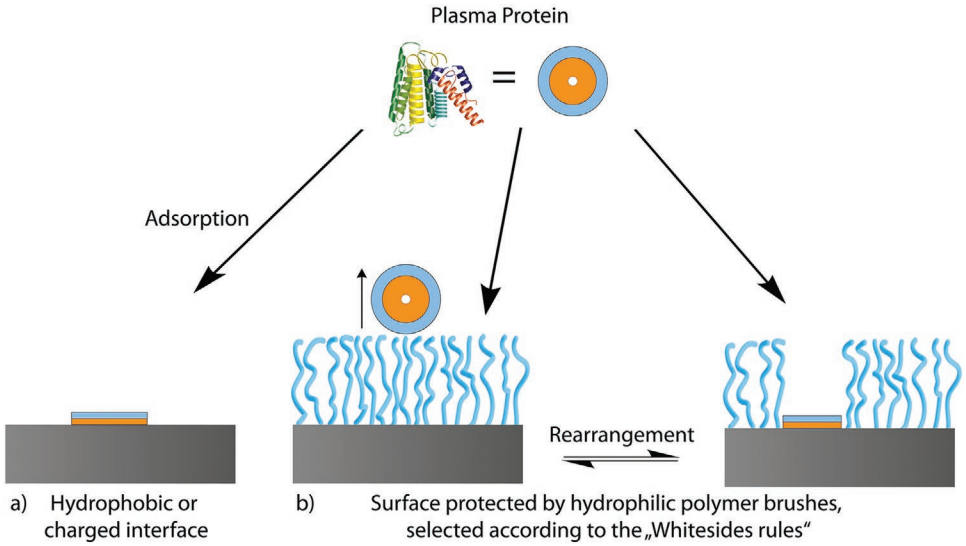

Figure 1. Schematic presentation of the interactions of water-soluble proteins (blue: hydrophilic, orange: hydrophobic) and surfaces: a) On a plain hard surface the protein can adsorb and change its conformation to maximize hydrophobic and electrostatic interactions. b) If the surface is coated with strongly hydrated swollen polymer brushes, the direct interaction between protein and polymer may be very close to zero (as both the polymer and the surface of the protein prefer the interaction with water). This non-interacting brush may, nevertheless, keep the protein away from the hard surface by entropic interactions. This requires, however, that the brush structure is and stays intact.

Nanohydrogels, on the other hand (Figure 2, right), consist of crosslinked water-soluble polymers, and thus are inherently stable as long as the aqueous medium is a good solvent.[46,47] The degree of swelling of nanogels depends on the crosslinking density which might not be homogeneous inside the microgel. The surface of nanogels is characterized by dangling chains. Often, the solubility of polymers in water depends on parameters such as temperature, pH, and ionic strength. Nanogels based on temperature-sensitive macromolecules, for example, can collapse above the so-called volume phase transition temperature (VPTT) and thus become a nanoparticle that needs to be stabilized as discussed above in order to avoid flocculation.

As mentioned before, polymeric micelles (both uncrosslinked and core crosslinked) are a further class of nano-objects that have similarities and differences as compared to colloidal structures and nanogels, respectively. After a self-assembly step, the core is not swollen by the solvent (water) (see **Figure 3**). Thus, the hydrophobic core (crosslinked or not) resembles a rigid nanoparticle (a colloid) but not a nanogel. Crosslinking of the core can provide—in addition—structural integrity of the micelles as compared to non-crosslinked micelles, where individual chains can be exchanged or even an entire micelle can disassemble. Generally, such polymeric micelles are under a lot of investigation and the significance

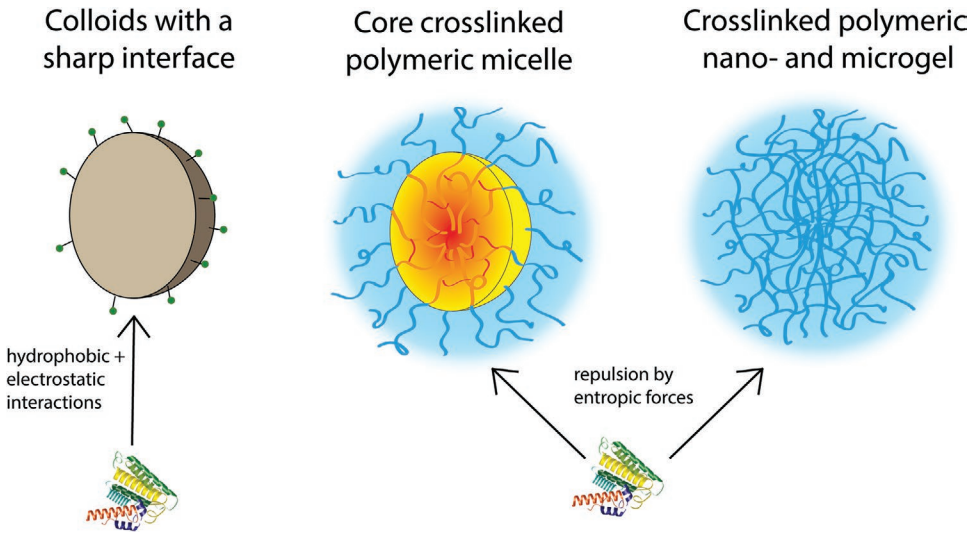

Figure 2. Various nano-objects, which are studied in the biological context (e.g., interaction with proteins). From left to right: colloids, polymeric micelles and nano- and microgels; while colloids possess a sharp interface, polymeric micelles and hydrogels extend solvated (hydrated) and highly swollen polymer chains into the environment.

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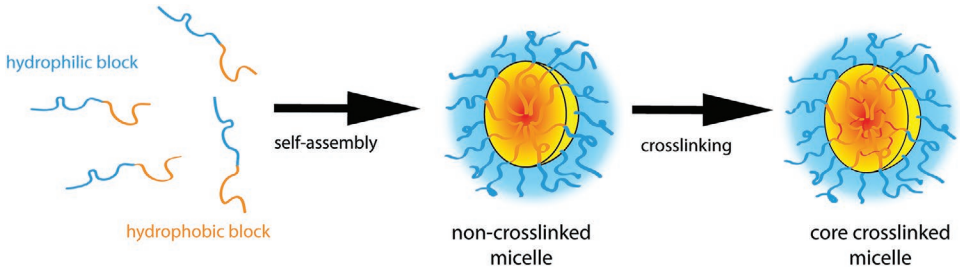

Figure 3. Preparation and crosslinking of micelles composed of amphiphilic block copolymers. After self-assembly of the polymers into micelles, they are crosslinked to freeze the dynamic system.

of stable (core crosslinked) structures for nanomedicines has been pointed out quite recently.[21]

The hydrophilic shell of the polymer micelles leads to a steric stabilization similar to sterically stabilized colloids or hydrogels; however, the details of the shell are different. Since the micelles are formed by block copolymers, the "grafting density" of the hydrophilic chains is very high (the Supporting Information in ref. [29] gives a surface density of 5–6 nm^2 per chain as an estimate of the grafting density). Similar to nanogels, the segment density in the hydrophilic shell will decay towards the periphery.

Furthermore there are nanohydrogels or polyplexes with a cationic core, which can be used to transport short interfering RNA (siRNA), mRNA, or pDNA.[48–51] Their inner core changes its properties from cationic, hydrophilic, and mobile to neutral, more hydrophobic and relatively solid due to polyplex formation after loading. They add the complexity of a charged and partly shielded core and shall thus not be discussed further in this context, as well as core multi-shell nanocarriers,[14,34,35,52] which can have a rather complex internal structure.

The different internal structure of colloidal nanoparticles, core crosslinked micelles, and nanogels, respectively, are illustrated in **Figure 4**, where the volume fraction of the (polymer) material is plotted versus the radial distance from the particle center. Hard colloids reveal a box profile: the particle consists of the dense material and has a sharp surface. Micelles have the same type of core, but a smoothly decaying segment density in the shell, whereas microgels are entirely swollen by the solvent and thus have a low polymer concentration in the center and the density decreases smoothly to the periphery.

This different internal structure leads to distinctly different physico-chemical properties. For nanoparticles with a sharp surface, the determination of size and electrophoretic mobility (zeta-potential) is rather simple, at least in aqueous media. On the other hand, the "fuzzy" surface of micelles and nanogels renders the concept of size and "surface" charge much more complex. First, different experimental techniques for the determination of the size probe different properties and thus can lead to a different result. Furthermore, the size can change upon interaction with other species in the solution and at interfaces because of the deformability of the soft object. It is, for example, well known that nanogels can penetrate through pores that are much smaller than the nanogels diameter and even very large microgels can be taken up by cells if they have a low cross linker content.[53–56]

The concept of zeta-potential is not well-defined for soft objects as charged groups as well as their counter-ions can be distributed inside the object. Despite the fact that models for soft, penetrable charged objects have been developed,[57] one should rather report electrophoretic mobilities instead of zeta-potential, which are calculated using models for rigid nanoparticles with a sharp surface.

The interaction of the different types of nanosized objects with plasma proteins will also be different. It happens in water as a solvent and is dominated by hydrophobic and ionic interactions.[58] For colloidal systems, it is relatively easy for the protein

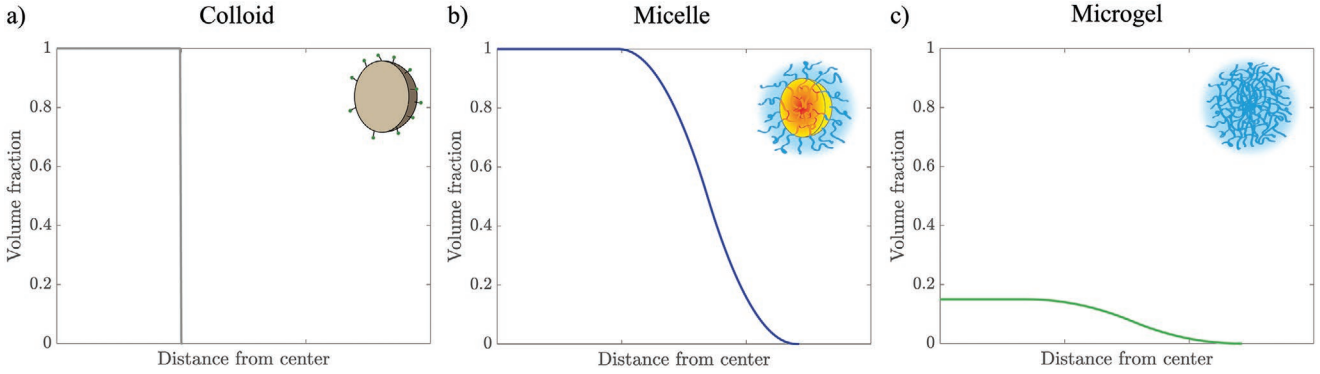

Figure 4. Radial density profile for different types of nanosized objects. a) Colloid with a hard sphere and sharp surface (left); b) Micelle with a hard core and a swollen shell (middle); c) Swollen microgel (right). The volume fraction of the (polymer) material is plotted versus the distance from the center of the object.

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to reach the sharp interface (see Figure 2) if it is only stabilized by charges or detergents with a low CMC (such structures will rearrange with time and leave hydrophobic patches on the surface, Figure 1). Such systems are known to form a protein corona. In addition, the proteins can change their conformation at the sharp interface (denature) and this can induce the adsorption of more proteins.[59] Landfester and coworkers characterized the protein adsorption on polystyrene nanoparticles. Thereby, they determined the amount of adsorbed proteins to be 350 to 1330 proteins per nanoparticle (depending on the functionalization and the pH). [60] Also for nanocapsules from hydroxyethyl starch (HES), which are prepared by dense crosslinking in an inverse miniemulsion (note: due to this process they are more like a hollow colloid, contain detergents and are not sterically stabilized towards the outside!) similarly high amounts of adsorbed protein were observed.[61] This presents a huge contrast to the mentioned findings for core crosslinked micelles, where much less than one protein per micelle was found to be associated.[29] In another recent study the amount of adsorbed proteins on pNIPAM and pNIPMAM nanogels were found to be more than 1000-fold lower as compared to colloidal structures.[62]

We think that the observed difference can be explained in the way that in polymeric micelles and hydrogels, the protein solution will get in contact with a dense array of hydrophilic, water-soluble and thus strongly hydrated polymer chains. This dense polymer brush layer expels proteins for entropic reasons.

In addition, the polymeric shell material can be selected to be protein resistant (i.e., without any attractive interaction with proteins), a classification, which comes from the work to reduce the protein adsorption onto solid surfaces in contact with body fluids.[41,63–65] For this case, such highly swollen polymer brushes have been shown to be very effective if they follow the "Whitesides rules" (see above).[41,42] Typical examples for such polymers are PEG, poly(2-oxazoline) and polysarcosine.[63,64,66]

The radially decaying density profile of the strongly hydrated shell of micelles as well as the strongly swollen state of nanogels also affects the interaction with proteins. Although strongly hydrated polymer chains interact less with the proteins, both these nano-objects can in principle interact with proteins such that they are adsorbed near the fuzzy surface or "absorbed", that is, within the shell or nanogel, respectively. Whether adsorption and/or absorption occurs will depend on the type of interaction (hydrophobic, electrostatic) but also on the size of the protein as well as on the segment density and mesh-size of the micelle and nanogel. The segment density can be increased by a higher cross linker content or via branching. Hereby, the use of dendritic moieties was shown to increase colloidal stability and to reduce interaction with proteins.[14,67,62]

Binding of guest species to soft nano-objects can lead to a decrease in the size of the soft host, which can deswell a bit upon complexation with the guest. This was also described for liposomal structures, which consist of a hollow, aqueous core. The adsorbed layer of the guest species (proteins) leads to osmotic pressure resulting in shrinkage of the host structure.[68] Whether the binding of oppositely charged guest species leads to charge reversal, depends on how far the guest penetrates the host.[69,70]

Concerning nanogels, there seem to be only very few reports in the literature that specifically address the protein corona. Temperature-sensitive nanogels are of particular interest as the temperature might be employable to control/switch a protein corona. O'Brien et al. reported that chemical functionalization of pNIPAM nanogels provides the possibility to tune the protein corona by temperature as well as by pH and the type of buffer.[71]

While pNIPAM microgels show only little adsorption of proteins,[71,14] Cedervall et al. reported that pNIPAM microgels hydrophobically modified by copolymerisation with *N-tert*-butylacrylamide show higher protein adsorption.[72] Miceli et al. recently investigated the protein corona in detail for pNIPAM and pNIPMAM based microgels, the latter have a VPTT >40 °C and therefore are swollen and colloidally stable at body temperature.[62] Copolymerization with dendritic polyglycerol renders the microgels more hydrophilic, which enhances colloidal stability and further reduces the amount of adsorbed proteins by one order of magnitude compared to microgels with the more hydrophobic *N,N'*-methylenebisacrylamide as crosslinker.

So, a view at the molecular structure of the nanoparticle helps to understand their interaction with plasma proteins. But, are nanosized objects without a significant protein corona relevant? Generally, nanosized drug delivery systems offer the potential to substantially change the body distribution of small molecular active components attached to it. Especially relevant is thereby the possibility to modulate pharmacokinetics, for example, to increase the circulation time in order to enhance the accumulation at the target location in the body due to the EPR effect (passive targeting)[73] or by functionalization of the nanoparticle with specific target ligands (active targeting).[74] Now, the blood clearance of nanoparticulate structures is not yet understood on a molecular level. So, it might be that one nanosized system circulates very long because it does not have a protein corona, while another one circulates for a long time because it adsorbs "perfect" proteins such as dysopsonins (for example, lipoproteins and human serum albumin). Dysopsonin proteins were found to have a positive effect on the circulation time and biodistribution of nanocarriers, on which they adsorb. In contrary, opsonins such as immunoglobulins, complement proteins, or coagulation proteins can mark nanocarriers by adsorbing on the surface and present them to the immune system resulting in decreased circulation times and higher accumulation in liver and spleen.[68,75]

The problem with a protein corona that might support particle circulation is, however, that according to present knowledge, it depends strongly on the individual person, their disease, current therapy, and possibly their diet (heavy meal or not).[76,77] But this means that the pharmacokinetics of any nanoparticulate system, which relies on a spontaneous protein corona formation, will hardly be predictable and differ between patients. It may thus even require a personalized optimization of drugs. A proper precoating of nanoparticles[78] may solve this problem if the artificially prepared corona is stable enough. On the other side, nanoparticles like, for example, CPC634, which do not have a detectable protein corona, show enhanced circulation times—per se—and very little variability between the patients.[26,29,79]

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In this context, it is also advisable to look for the macrophage uptake of the nanoparticles, because a low macrophage uptake is linked to prolonged circulation times.[80] Here, polymer micelles with a hydrophilic polysarcosine shell show a low unspecific uptake and a long circulation time.[81]

Now, given that it has some advantages to prepare nanoparticles with a neglectable protein corona, what are reasonable methods to make them? At first, the proteins should be kept away from the sharp interface and here steric repulsion seems most reasonable. First, this requires the use of protein resistant materials, like PEG, poly(2-oxazoline), or polysarcosine. Second, core-shell structures are required to avoid access to such interfaces. Generally, this can be done either by chemical grafting of polymers to the interface or by a self-assembly process of amphiphilic molecules. Here, self-organization seems to be most robust, because during this process the structures assemble naturally in such a way that the surface tension is minimized. Thus, the hydrophilic polymer chains will get distributed homogeneously on the surface of the hydrophobic inner core, whilst all hydrophobic structures try to "hide" in the inner part of the micellar structure. On the other hand, during a chemical surface modification (to stabilize a colloidal system and prevent it from aggregation), parts of the surface might not react and will thus not be covered with the "protein-repellent" material. Another important point is the grafting density of the hydrophilic stealth material. While in a self-organized system the grafting density is determined by the ratio of hydrophilic and hydrophobic blocks, the density of chemical grafting to a sharp surface will always depend on all the details of the reaction process. By self-assembly, it is easy to reach densities of 5–6 nm^2 per hydrophilic chain (see ref. [10] and estimate in the Supporting Information of ref. [29]). Interestingly, this is just the packing density for which Farokhzad and coworkers found prolonged circulation times in mice.[10]

At last, the steric stabilization should be stable for hours or better a day to effectively modulate pharmacokinetics. For this purpose, the diffusion of the shielding polymers away from the surface has to be prevented. Linking it to the surface by hydrophobic interactions, as in the case of detergents, is probably not enough, except when the CMC gets extremely low. Thus, uncrosslinked polymer micelles tend to disintegrate with time.[19,82,83] Chemical crosslinking is successful, as shown here.[21] However, kinetic concepts like the use of a glassy hydrophobic polymer, a crystalline hydrophobic polymer like polylactid[25] or a strongly interacting polymer as hydrophobic block might work also, to keep the shielding polymers at the desired location on the surface of the inner core. In this context, Shi et al. compared physical and chemical stabilization strategies (e.g., π–π stacking, stereocomplexation, free radical polymerization, click chemistry, etc.) for polymeric micelles, to prevent the dissociation of unimers in the body.[84] In conclusion, we would like to state that as long as the crosslinking is efficient, self-assembled systems can be very stable and recommendable.

The presence or absence of plasma proteins on the nanoparticles will also have some influence on cellular uptake, circulation time, active and passive targeting, and loss of cargo.[13] For some thoughts in this direction, see the Supporting Information.

As outlined above, the internal structure of nanosized objects—especially the strongly hydrated/swollen shell or network versus a sharp "hard" surface—is relevant for biomedical applications. Rigid colloids, core crosslinked micelles, nanogels and many other more complex nanoparticles are used in the biomedical context.[14] They all are nanoparticles, although the joint term "nanoparticle" hides their difference. The term "nanoparticle" has two disadvantages: i) it is used differently in different scientific communities and ii) it does not provide information on the presence/absence of a strongly hydrated/swollen shell, on the internal structure and especially on the accessibility of the interface. Determining and specifying the internal structure of nanosized objects is therefore indispensable in order to develop a rational design of such systems for applications in the biological context.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

colloids, microgels, nano-sized drug delivery particles, polymeric micelles, protein corona, surface interactions

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