The Evolution of the Protein Corona around Nanoparticles: A Test Study

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ABSTRACT The importance of the protein corona formed around nanoparticles upon entering a biological fluid has recently been highlighted. This corona is, when sufficiently long-lived, thought to govern the particles' biological fate. However, even this long-lived "hard" corona evolves and re-equilibrates as particles pass from one biological fluid to another, and may be an important feature for long-term fate. Here we show the evolution of the protein corona as a result of transfer of nanoparticles from one biological fluid (plasma) into another (cytosolic fluid), a simple illustrative model for the uptake of nanoparticles into cells. While no direct comparison can be made to what would happen in, for example, the uptake pathway, 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. This could be evolved to map the transport pathways utilized by nanoparticles, and eventually to predict nanoparticle fate and behavior.

KEYWORDS: protein corona · biological fluid · cytosolic fluid · nanoparticles · proteomics

Nanoparticles may potentially enter our body *via* several different routes, for example, by inhalation, ingestion, or uptake through the skin. However, regardless of the method of entry, biological fluid will surround nanoparticles once they have entered a biological environment. When nanoparticles come into contact with a biological fluid their surface will be covered with a "corona" of biological macromolecules. The composition of the corona depends on the nanoparticle size and surface characteristics,[1,2] which determine protein binding specificities and affinities. Thus, some particles will have a stable hard core of biological macromolecules (that we name the hard corona) that interact strongly with the surface as well as a more loosely bound outer layer of biological macromolecules that associate less strongly both to the particle surface and to the strongly associated biological macromolecules. Some particles will only have a "weak" corona, meaning that most of the biological macromolecules will have a weak association to the surface, for example, pegylated particles. Assuming a sufficiently long residence time, the biological macromolecules that surround a nanoparticle will determine its biological fate, as it is this corona of biomolecules that cells "see" and interact with. The majority of the identified biological macromolecules surrounding nanoparticles are proteins, though recently we have also reported the presence of some lipids.[3] We have reported detailed pictures for the "hard" corona formed around nanoparticles of different materials, including copolymer and polystyrene nanoparticles, of different sizes and with different surface properties.[1,4,5] The importance of the protein corona for determining any possible toxicity from different nanoparticles has been reported.[6,7] Xia *et al.* have a recent publication in which they mapped the adsorption of a set of small-molecule probes to different nanoparticles and transformed the results into a biological surface-adsorption index.[8]

The hard corona around NIPAM:BAM copolymer particles is quite specific, with a small number of proteins contributing to the main part of the corona.[4,5] The interactions between the proteins that build up the corona and the copolymer particles have been characterized.[5,9] These data were used to generate a theoretical model for the formation of the corona around the copolymer particles over time.[9] The model shows that immediately after being introduced into the blood the particle will be surrounded by serum albumin, but with time the serum albumin will be replaced with less abundant proteins that have a higher association rate constant and lower dissociation rate constant.[9] Casals *et al.* have also shown the transition from a loosely attached protein corona from media containing 10% of fetal bovine serum around gold nanoparticles that over time, evolves toward an irreversible attached protein corona.[10]

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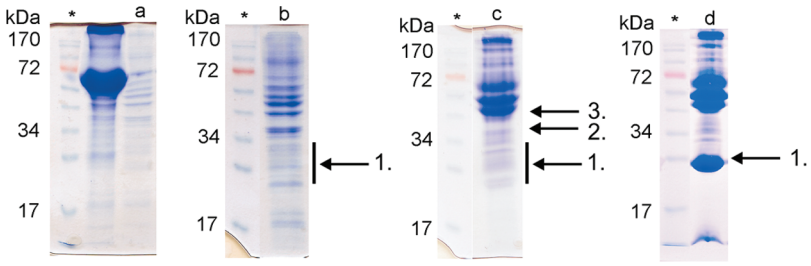

Figure 1. Coomassie stained gel lanes for different samples (lanes cut out from the gels shown in Figure S1 in the Supporting Information). Each sample is represented by a molecular weight standard lane, marked with an asterisk (*), and a lane for the corresponding sample (the middle lane in panel a is not relevant in this study). Panel a shows cytosolic fluid from HeLa-cells. Panel b shows the protein corona formed around silica particles incubated in cytosolic fluid. Panel c shows the protein corona around silica particles that have been transferred from plasma into cytosolic fluid. Panel d shows the protein corona around silica particles incubated in plasma. The arrows in panels b and c indicate from which regions samples were taken for proteomic determination of proteins. The arrow in panel d indicates the region in which apolipoprotein A-I is the dominant protein.

We have previously suggested that the nanoparticle biomolecule corona is not static, but rather evolves as the particles are trafficked with cells.[11] Here we report a case study of how the protein corona may evolve when the particle−protein complex is transferred from one biological fluid into another. This is different from what would occur when a nanoparticle is endocytosed as in that case particles are managed along a fixed pathway, but it is analogous and therefore instructive for that situation. Interestingly, it would correspond to the case where particles escape from the pathway by endosomal or lysosomal disruption, and is therefore likely to be important in understanding associated forms of toxic impacts.

In this study, nanoparticles are incubated first with plasma and are then transferred, 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).

RESULTS AND DISCUSSION

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 the "hard" protein coronas were determined using a similar protocol to that described in previous studies[1,4,5] (for details see Methods section). Briefly, particles were separated from unbound proteins by centrifugation to form a pellet which was washed three times to remove the unbound proteins. Bound proteins (hard corona) were separated by SDS PAGE. Figure 1 shows lanes from coomassie stained gels for the 9 nm silica nanoparticles incubated in the different biological fluids (cytosolic fluid, plasma, and plasma followed by cytosolic fluid). To simplify comparison, the lanes have been cut out from gels (intact gels are shown in Supporting Information, Figure S1). Each panel includes a molecular weight standard lane and a sample lane.

Figure 1, panel a, shows the coomassie stained proteins for the cytosolic fluid fraction from HeLa cells obtained with a ProteoExtract Subcellular Proteome Extraction Kit. The fraction was diluted 1:1 with 2× SDS-loading buffer before it was loaded onto the gel. The faint bands show that the cytosolic fluid obtained is quite dilute, that is, the protein concentration is low. Figure 1b shows the proteins that constitute the protein corona around silica nanoparticles after they have been incubated in cytosolic fluid. A comparison with panel a (in Figure 1) shows that the nanoparticles have significantly concentrated the proteins from the cytosolic fluid. This can be seen even more clearly in Figure 2 which shows the intensity profiles, obtained with the program ImageJ,[12] of the gel lanes from Figure 1. In SDS-PAGE the distance (run length) a protein travels in the gel will depend on its molecular weight, which means that a standard (containing several proteins with known molecular weights) can be used to normalize the run length between different gels. The *x*-axis in Figure 2, which corresponds to the run length, is normalized according to how far the different proteins in the molecular weight standards lane had moved in each respective gel (Figure S2 in Supporting Information shows the overlaid traces for the different molecular weight standards for each panel in Figure 2). The traces for cytosolic fluid, Figure 1a, and the protein corona formed around silica nanoparticles incubated in cytosolic fluid, Figure 1b, are compared in Figure 2a. The figure shows that the intensity patterns differ slightly for the two samples, that is, the relative protein concentrations observed in the nanoparticle corona differ from those in the bulk cytosol. The increased concentration of some proteins relative to the levels in cytosolic fluid indicates a preferential interaction of these proteins with the silica nanoparticles, resulting in a locally increased concentration around the nanoparticles, that is, the formation of a specific protein corona.

Figure 1c shows the proteins detected in the protein corona around silica nanoparticles, which were incubated

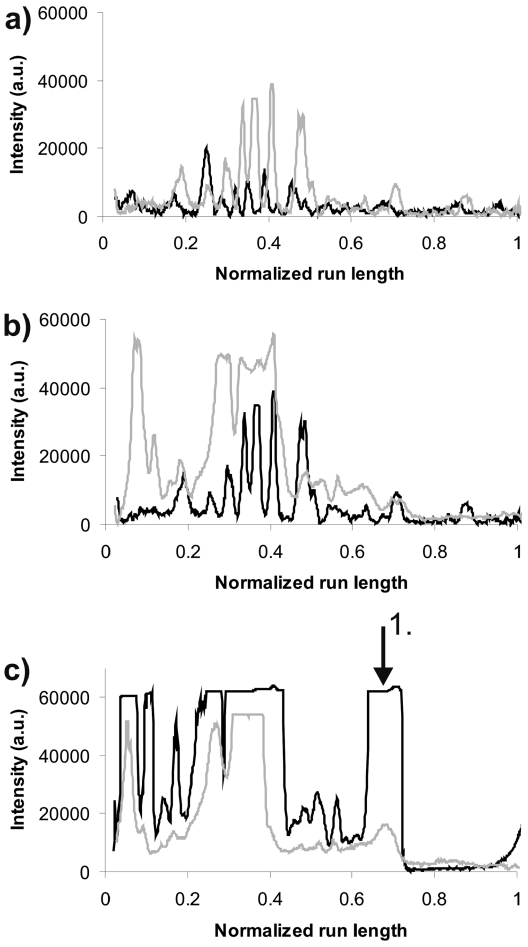

Figure 2. Comparison of the optical intensity across gel lanes between cytosol and nanoparticle corona obtained by incubation in cytosol; graphs were generated using the program ImageJ.[12] Panel a shows a comparison between cytosolic fluid alone (black) and the protein corona around silica particles incubated in cytosolic fluid (gray). Panel b shows comparison between the protein coronas around silica particles incubated in cytosolic fluid (black) and around particles first incubated in plasma and then transferred into cytosolic fluid (gray). Panel c shows a comparison between the protein corona around silica particles incubated in human plasma (black) and the corona from particles first incubated in plasma and then transferred into cytosolic fluid (gray). The run lengths, shown on the *x*-axis, have been normalized to the respective molecular weight standards (shown in Figure S2 in Supporting Information) to compensate for small differences between gels.

first in human plasma and then in cytosolic fluid. After the incubation in plasma the particles were washed extensively with sample buffer to remove any unbound and weakly associated plasma proteins. The proteins that make up the hard corona (the plasma protein corona) can be seen in Figure 1d. A comparison between Figure 1d and Figure 3, lane b3, a sample of diluted plasma, shows that silica nanoparticles preferentially interact with plasma proteins other than serum albumin. Although serum albumin is still found in the corona, its abundance here is much lower compared to its abundance in plasma, indicating only a weak interaction with the particle surface. After the washing procedure (see Methods) the particles and their "hard" plasma protein corona were transferred into and incubated with cytosolic fluid. Thereafter the complexes were washed again extensively with buffer to remove unbound and weakly associated cytosolic proteins. Finally, the protein corona was eluted from the particles and visualized by SDS-PAGE. As can be seen in Figure 1c, the appearance of the SDS-PAGE lane for the double incubated sample resembles the plasma-only incubated corona, Figure 1d, more than the cytosolic fluid corona, Figure 1b. However, a close investigation of the patterns in Figure 1c and the traces in Figure 2 panels b and c reveals clear differences between the double incubated sample and the plasma-incubated sample. Some of the very intense bands in the plasma incubated sample display a remarkable decrease in the double incubated sample, and one band is exceptionally reduced (see arrows marked 1 in Figure 1 and Figure 2c) indicating that the transfer from plasma into cytosolic fluid destabilizes the plasma corona and that some of the plasma proteins consequently dissociate from the particle, likely to be replaced by proteins unique to the cytosolic fluid which have a high affinity for the particle surface and/or the particle−protein corona complex.

Figure 1 clearly shows that the SDS-PAGE gel pattern of nanoparticles coated with a protein corona of plasma proteins is significantly altered from its original composition when the particles are subsequently incubated in cytosolic fluid. A proteomic analysis was conducted to investigate if the cytosolic proteins actually replace some of the plasma proteins or if some plasma proteins in the corona simply dissociates from the complex in the new environment of cytosolic fluid. Selected bands were excised from the SDS-PAGE lanes (see arrows in Figure 1 panels b and c) trypsin digested, and analyzed with MS. The first region that was investigated was the area indicated with an arrow marked 1 in Figure 1c. This region normally contains a high concentration of apolipoprotein A-I for nanoparticles that have been incubated in human plasma.[1,4,5,13−27] Apolipoprotein A-I is still detected in the region marked by arrow 1 in Figure 1c even following incubation in cytosolic fluid. However, the intensity of the band, that is, the protein concentration, has clearly decreased compared to the pure plasma protein corona shown in Figure 1d, and the protein is detected with relatively low sequence coverage, ~26% (see Table S1 in the Supporting Information) and with only six different peptides at best. This should be compared with the results for the determination of the hard plasma protein corona around 6 nm silica particles in which apolipoprotein A-I was identified with 50 different peptides and with almost the full sequence covered (see Figure S4 and Table S2 in the Supporting Information. Taken together these data provide a strong indication that much of the apolipoprotein A-I has dissociated from the protein corona

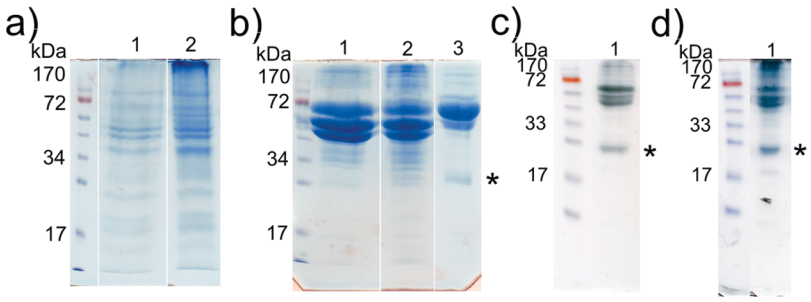

Figure 3. Coomassie stained gels, first lane shows the molecular weight standards (lanes cut from gels which are shown intact in the Supporting Information): (a) lanes 1 and 2 = proteins from cytosolic fluid (from HeLa cells) forming the corona around 50 nm plain and carboxyl-modified polystyrene particles respectively; (b) lanes 1 and 2 = protein corona around plain and carboxyl-modified polystyrene particles respectively, which have transferred from plasma into cytosolic fluid (from HeLa cells); lane 3 = 20 times diluted human plasma; (c) lane 1 = protein corona around plain polystyrene particles incubated in plasma; (d) lane 1 = protein corona around carboxyl-modified polystyrene particles incubated in plasma. The stars indicate the gel region in which apolipoprotein A-I is detected. Panels a and b are 10% gels, whereas panels c and d are 12% gels.

after the protein–particle complex was transferred from plasma into cytosolic fluid.

Apolipoprotein A-I is the major protein in high density lipoprotein (HDL). HDL is an assembly of proteins, lipids, and cholesterol that transports lipids and cholesterol in the bloodstream. Apolipoprotein A-I has been shown to be one of the major proteins in the corona formed around nanoparticles of different materials upon contact with plasma.[4,5,13–27] The suggestion that apolipoprotein A-I is replaced upon transfer from plasma into the cellular environment may have implications for the exocytosis potential of the nanoparticles, and for bioaccumulation of nanoparticles in cells.

Table 1 lists the cytosolic (and nuclei) proteins identified in the band indicated by arrow 1 in Figure 1c, where apolipoprotein A-I is the dominating protein in the plasma corona. Although the region is still dominated by plasma proteins, cytosolic and even nuclei proteins are also detected in the corona— over 35% of the detected proteins stem from inside the cell, that is, from cytosol or nucleus (for complete list of proteins detected see Table S1 in the Supporting Information). The detected cytosolic proteins have reasonable molecular weights for the region in which they are detected. This indicates an exchange of proteins in the nanoparticle corona when the surrounding environment changes. This hypothesis is further supported by proteomics data from two other excised bands, marked by arrows 2 and 3 in Figure 1c. Table 1 lists the cytosolic proteins detected in each of those bands. As for the previously described region, ~35% of the detected proteins in the two bands are defined as cytosolic or nuclear proteins, that is, not plasma proteins (for complete list of proteins detected see Table S1 in Supporting Information). All of these results support the claim that the "hard" protein corona formed around nanoparticles evolves and changes its identity by integrating new proteins when the protein–particle complex is introduced into a new biological environment.

TABLE 1. Detected Proteins in the Different Coronas

name[a]		detected in the cytosolic corona[b]
	Proteins Detected in Area Indicated by Arrow 1 in Figure 1c	
P50914	60S ribosomal protein L14	X
Q06830	Peroxiredoxin-1	X
Q8WW12	PEST proteolytic signal-containing nuclear protein	
P28074	Proteasome subunit beta type 5	X
P28072	Proteasome subunit beta type 6	
P06702	Protein S100-A9	X
Q9H0U4	Ras-related protein Rab-1B	X
P60174	Triosephosphate isomerase	X
	Proteins Detected in Area Indicated by Arrow 2 in Figure 1c	
P27695	DNA-(apurinic or apyrimidinic site) lyase	
P06748	Nucleophosmin	
P04406	Glyceraldehyde-3-phosphate dehydrogenase	
P05198	Eukaryotic translation initiation factor 2 subunit 1	
P04083	Annexin A1	
P29692	Elongation factor 1-delta	
	Proteins Detected in Area Indicated by Arrow 3 in Figure 1c	
P07437	Tubulin beta chain	
P06733	Alpha-enolase	
P68104	Elongation factor 1-alpha 1	
P68371	Tubulin beta-2C chain	
Q16576	Histone-binding protein RBBP7	
P26641	Elongation factor 1-gamma	
P43686	26S protease regulatory subunit 6B	
	Tubulin alpha-1	
Q16401	26S proteasome non-ATPase regulatory subunit 5	

[a] UniProt accession name. [b] Proteins that also was detected in the cytosolic corona.

The region in which apolipoprotein A-I is found was also excised from the gel representing the hard corona formed around silica particles in cytosolic fluid, Figure 1b arrow 1. In the selected region 40 proteins were detected (for full proteomic data see Table S1 in SI). Table 1 shows which of the detected cytosolic proteins (six out of eight) for the double-incubated sample also were detected in the cytosolic protein hard corona. The two other cytosolic proteins contained in the

doubleincubated protein corona may be bound to the particle-protein complex as a result of protein–protein interactions with the plasma proteins remaining in the nanoparticle corona from the first incubation in plasma.

The proteomic investigation of the protein corona formed around the silica particles in cytosolic fluid reveals that the corona is composed of many different proteins (data not shown), some with very low coverage. Additionally, the small size of the nanoparticles (9 nm) which (either in the form of monomer particle, or radius of curvature in the aggregate) is in the range of the size of proteins suggests that there will be significant fluctuations in the corona. However, despite this variability, the predominant proteins will always be found in the corona. For a more detailed investigation of this aspect, a true quantitative proteomics study is needed, and we are developing approaches to address this.

Figure 3 shows another example of how the hard protein corona formed around nanoparticles in a biological fluid evolves if the nanoparticle is transferred into a new biological environment. The trends observed for the silica particles can be reproduced for polystyrene particles. The two different polystyrene particles, 50 nm plain and 50 nm carboxyl-modified, pull down a corona of cytosolic proteins when incubated in cytosolic fluid, as shown in Figure 3a. Figure 3b, lanes 1 and 2 show the hard corona for the two polystyrene particles after they were incubated first in plasma and then in cytosolic fluid, while Figure 3 panels c and d show the hard corona in plasma alone for the plain and carboxyl-modified particles, respectively. Comparing the pattern shown in Figure 3b (double incubation) with that in Figure 3a (hard cytosolic corona) and in Figure 3c,d (hard plasma corona) it is obvious that, as was the case for the silica nanoparticles, the pattern for the hard corona after incubation in first plasma and then cytosolic fluid resembles the hard corona formed in plasma more than that formed in cytosolic fluid, but contains elements of both. In Figure 3c,d, the apolipoprotein A-I band, identified as apolipoprotein A-I with proteomics in Lundqvist *et al.*,[1] is marked with a star (observe that these gels were run with a different percentage of acrylamide than the other gels in Figure 3). The apolipoprotein A-I band is quite intense in the gels shown in Figure 3c,d (plasma), while the pattern for the hard coronas shown in Figure 3b lanes 1 and 2 (double incubation) lacks this intense band (while the strong bands, just under 72 kDa, can be seen in both the plasma and double incubated gels). This means that the two different polystyrene particles show the same trend as silica: apolipoprotein A-I is replaced by cytosolic proteins when the plasma protein covered particles are transferred into cytosolic fluid. It is also possible that apolipoprotein A-I is preferentially binding to phospholipid vesicles left in the cytosolic preparation or is targeted by proteases. Close inspection of the gels in Figure 3a–d also reveals that in the double incubated samples faint bands have appeared under the three dominating bands from the plasma protein corona (for full proteomic investigation of the protein corona around polystyrene particles see Lundqvist *et al.*[1]) in Figure 3b lanes 1 and 2. These faint bands in Figure 3b lanes 1 and 2 correlate with bands in lanes 1 and 2 of Figure 3a (cytosol protein corona) indicating that the bands seen in the double incubated samples have main contributing proteins from the cytosol.

CONCLUSIONS

We have illustrated the scenario (destined we believe to be at the root of modern understanding of trafficking of nanoscale objects) in which the protein corona evolves when nanoparticle-adsorbed-protein complexes are moved from one biological environment into another, with some proteins from the original corona being replaced by proteins from the new biological fluid. It is intriguing that while the corona evolves, it retains a fingerprint of its prior history. This may turn out to be an important paradigm, for understanding *in vitro* and *in vivo* transport. Besides this, however, the protein–particle complex may contain a history-dependent set of protein markers or signals that can be elucidated *via* the protein corona of particles recovered from their final subcellular location.

A future (though challenging if done correctly) next step in this work is to characterize the corona of particles that have been actively taken up by a cell; as to what degree the evolution of the protein corona will in the future assist in the prediction of the nanoparticle uptake and fate remains to be seen. Still, the work presented here points toward the need for a new conceptual framework for QSAR-type approaches.

METHODS

Plasma. Blood was taken from 10 different seemingly healthy donors. Each donor donated blood for 10 × 3 mL tubes containing EDTA to prevent blood clotting. The blood donation was arranged such that the blood samples were labeled anonymously. They could not be traced back to a specific donor; however, it was possible to use plasma from just one of the donors for a specific experiment. The tubes were centrifuged, for 5 min at 800 RCF to pellet the red and white blood cells. The supernatant (the plasma) was transferred to labeled tubes and stored at −80 °C until used. Upon thawing the plasma was centrifuged again for 2 min at 16.1 kRCF to further reduce the presence of red and white blood cells. Plasma was used immediately upon thawing and was never refrozen.

Cytosolic Fluid. Cytosolic fluid was extracted from HeLa cells using the ProteoExtract Subcellular Proteome Extraction Kit purchased from Calbiochem.

Silica Nanoparticles. Silica nanoparticles of 6 and 9 nm were kindly provided by AKZO NOBEL (www.colloidalsilica.com/eka.asp). The particles were supplied at 30 wt % in a basic colloidal solution. Before use, the colloidal nanoparticle solution was diluted 10 times and extensively dialyzed against sample buffer.

Polystyrene Nanoparticles. Polystyrene latex beads were purchased from Polysciences (50 nm unmodified (plain) and carboxyl-modified 50 nm, both labeled with yellow–green fluorophore). The nanoparticles were used as received.

Nanoparticle Characterization. The polystyrene nanoparticles have been characterized in Lundqvist *et al.*[1] The silica particles were used right after the dialysis since silica particles are not stable at the pH or NaCl-concentration used in these experiments.

Detection of the Hard Protein Corona (Formed from Either Plasma or Cytosolic Fluid) around Nanoparticles Used in the Article. Nanoparticles (3.6 μg of silica or 1.3 μg of polystyrene), in 10 mM phosphate, pH 7.5, 0.15 M NaCl and 1 mM EDTA, were incubated with either plasma (200 μL) or cytosolic fluid (500 μL) for 1 h. The particles were pelleted by centrifugation (16.1 kRCF, 3 min), washed three times with 1 mL of 10 mM phosphate, pH 7.5, 0.15 M NaCl, 1 mM EDTA, and the vials were changed after each washing step. Bound proteins were removed from the particles by adding SDS-PAGE loading buffer and were separated by 12% SDS-PAGE. Each gel run included one lane of a molecular weight ladder standard, PageRuler Prestained Protein Ladder (Fermentas).

Detection of the Hard Protein Corona after the Nanoparticle–Protein Complexes Have Been Moved from Plasma to Cytosolic Fluid. The pellet, after the third wash (see above) from plasma incubated nanoparticles was dissolved in 50 μL of 10 mM phosphate, pH 7.5, 0.15 M NaCl, and 1 mM EDTA. A 500 μL portion of cytosolic fluid was added and the sample was incubated overnight at 4 °C. The particles were pelleted by centrifugation (16.1 kRCF, 3 min), washed three times with 1 mL of 10 mM phosphate, pH 7.5, 0.15 M NaCl, 1 mM EDTA, and the vials were changed after each washing step. Bound proteins were removed from the particles by adding SDS-PAGE loading buffer and separated by 12% SDS-PAGE. Each gel run included one lane of a molecular weight ladder standard, PageRuler Prestained Protein Ladder (Fermentas).

Protein Identification by Mass Spectrometry (9 nm Silica Particles). Bands of interest from SDS-PAGE gels (12%) were excised and digested in-gel with trypsin according to the method of Shevchenko *et al.*[28] The resulting peptide mixtures were resuspended in 0.1% formic acid and analyzed by electrospray liquid chromatography mass spectrometry (LC MS/MS). An HPLC (Surveyor, ThermoFinnigan, CA) was interfaced with an LTQ ion trap mass spectrometer (ThermoFinnigan, CA). Chromatography buffer solutions (buffer A, 0.1% formic acid; buffer B, 100% acetonitrile and 0.1% formic acid) were used to deliver a 72 min gradient (5 min sample loading, 32 min to 40% buffer B, 2 min to 80%, hold 11 min, 1 min to 0%, hold for 20 min, 1 min flow adjusting). A flow rate of 150 μL/min was used at the electrospray source. Spectra were searched using the SEQUEST algorithm[29] against the indexed uniprot/swiss prot database (http://www.expasy.org; release 3 July 2007). The probability-based evaluation program, Bioworks Browser was used for filtering identifications; proteins with Xcorr (1,2,3) = (1.90, 2.00, 2.50) and a peptide probability of 1×10^{-5} or better were accepted.

Protein Identification by Mass Spectrometry (6 nm Silica Particles). After the separation of proteins by SDS/PAGE (12%), bands were excised from the gel and identified as described.[30] Briefly, the gel-bands were reduced and alkylated, digested with trypsin and the resulting peptide mixtures were separated and analyzed by nanoscale liquid chromatography quadrupole time-of-flight MS/MS. Spectra were analyzed by MASCOT software to identify tryptic peptide sequences matched to the International Protein Index (IPI) database (www.ebi.ac.uk/IPI/IPIhelp.html).

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Supporting Information Available: Pictures of original gels, normalized run length of the molecular weight standards for the samples shown in Figure 2, gel-lane showing the proteins from the protein corona around 6 nm silica particles, and mass spectroscopy data. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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