Protein–Nanoparticle Binding

DOI: 10.1002/anie.200700465

Detailed Identification of Plasma Proteins Adsorbed on Copolymer Nanoparticles**

Tommy Cedervall, Iseult Lynch, Martina Foy, Tord Berggård, Seamas C. Donnelly, Gerard Cagney, Sara Linse, and Kenneth A. Dawson*

Nanoparticles entering the bloodstream may initially bind highly abundant serum/plasma proteins, such as human serum albumin (HSA). We show here that, as a result of its low affinity and fast exchange, HSA is soon replaced by the higher-affinity and slower-exchanging apolipoproteins AI, AII, AIV, and E, and that these proteins remain associated with the particles under the expected conditions of in vivo exposure, thus conferring their biological identity onto the particles.

The need to understand nanoparticles in a biological environment is now shared by nanobiology, nanomedicine, and nanotoxicology. There is currently considerable debate as to the nanoparticle characteristics that are important in determining biological response, including size, shape, and surface area.[1–3] New and interesting approaches to understanding the impact of interaction with nanoparticles on protein behavior are emerging.[4,5] We have recently argued[6] that the effective unit of interest in the cell–nanomaterial interaction is not the nanoparticle in itself, but the particle and its "corona" of more or less strongly associated proteins from plasma or other bodily fluids. Ultimately, this corona of native-like or unfolded proteins "expressed" at the surface of the particle is "read" by living cells, and is the key phenomenon that scientists need to understand.

Given this, it is surprising that the particle–protein complex is so poorly understood. We believe that the present study is the first reliable analysis of the proteins that associate to a nanoparticle in a complex biological fluid, and we present it as a guide for future studies in this area.

[*] Dr. T. Cedervall, Dr. I. Lynch, Prof. K. A. Dawson
School of Chemistry and Chemical Biology
University College Dublin
Belfield, Dublin 4 (Ireland)
Fax: (+353) 1-716-1178
E-mail: iseult@fiachra.ucd.ie
Dr. M. Foy, Dr. S. C. Donnelly, Dr. G. Cagney
UCD Conway Institute, University College Dublin
Belfield, Dublin 4 (Ireland)
Dr. T. Berggård
Department of Protein Technology, Lund University
22184 Lund (Sweden)
Prof. S. Linse
Biophysical Chemistry, Lund University Chemical Centre
PO Box 124, 22100 Lund (Sweden)

[**] This work results from the EU FP6 project NanoInteract (contract number 033231). Additional funding was from EPA, EI, CIPSNAC, SFI, and VR.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

The composition of the protein corona at any given time will be determined by the concentrations of the over 3700 proteins in plasma, and the kinetic and equilibrium binding constants of each protein for the particular nanoparticle. This corona will not immediately reach equilibrium when exposed to a biological fluid. Proteins with high concentrations and/or high association rate constants will initially occupy the nanoparticle surface but may also dissociate quickly to be replaced by proteins of lower concentration, lower exchange rate, and higher affinity. These relaxation processes may also be important when particles redistribute from one organ to another or between cellular compartments. Furthermore, particles may bind different amounts and types of protein depending on the particle size, shape, and surface characteristics. Exposure to nanoparticles may lead to different outcomes for different individuals, as blood composition varies substantially.[7] Here, we study a particular set of particle systems in plasma, but the issues outlined above must be kept in mind in future studies that concern the interaction of a wide range of nanoparticles with living tissues.

The (model) nanoparticles used here for illustration are polymeric in nature, with controlled sizes and compositions, and consist of essentially random, cross-linked copolymers of *N*-isopropylacrylamide (NIPAM) and *N*-*tert*-butylacrylamide (BAM). A range of sizes (70–700 nm) and two comonomer ratios (50:50 and 85:15) were used to probe the effects of nanoparticle curvature and hydrophobicity on the nature and identity of adsorbed plasma proteins.

There are a number of reports in the literature on plasma proteins interacting with nanoparticles (Supporting Information). The preferred method to separate the nanoparticles from plasma has been centrifugation, but the outcome is affected by the duration of washing and the solution volumes used in these steps. A protein with high abundance in plasma may be identified as being associated with the particles because of insufficient washing. Sedimentation of large proteins, protein aggregates, and co-precipitation may further complicate the picture. However, centrifugation assays are still an efficient way to retrieve enough protein for safe identification. We show here that these assays are reliable if conducted with care and accompanied by proper control experiments. Optimally, other methods should be carried out in tandem to exclude false positives. One such method is to separate plasma proteins from nanoparticles by size-exclusion chromatography.[8]

Nanoparticles that have entered the body may be expected to be rather dilute (unless injected at high concentration in situ), with a large excess of protein over the available nanoparticle surface area. Proteins were thus

retrieved by centrifugation after incubating particles at a range of plasma/particle ratios (Figure 1). Saturation seems to occur at around 100 and 200 μL plasma (40 and 80% plasma)

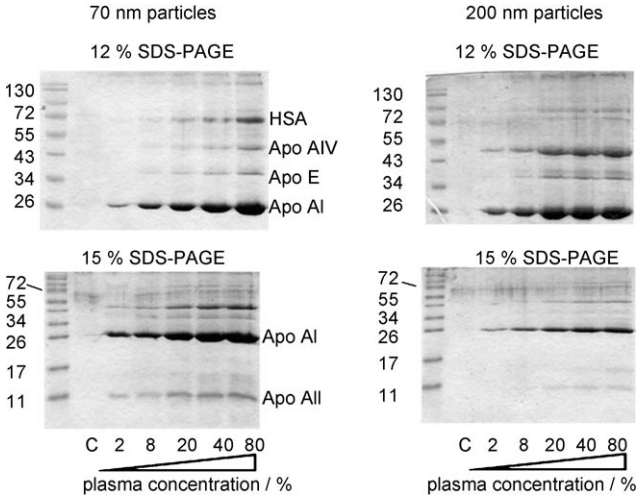

Figure 1. SDS-PAGE (12 and 15% gels) of plasma proteins retrieved from 0.5 mg of 70- or 200-nm 50:50 NIPAM–BAM copolymer particles after centrifugation and triple washing (total washing time 20 min). Particles were incubated with the plasma concentrations indicated; total volume 250 mL. Lane C is a control experiment with 80% plasma but without particles.

bind to the nanoparticles with much higher affinity than HSA. No apolipoprotein was seen in control experiments without particles. In one experiment many other proteins were identified (Table 1 and Supporting Information), of which

Table 1: Identified plasma proteins bound to 50:50 NIPAM–BAM copolymer particles after centrifugation.

Protein	MW [kDa]	Peptides (#)	P[a]	Lipoprotein[b]
apolipoprotein AI[c,d]	28	29/8	1	yes
apolipoprotein AII[c]	9	3/1	0.9	yes
apolipoprotein AIV[c]	43	18/15	1	yes
apolipoprotein E[c]	34	12/15	1	yes
HSA[c]	69	10/25	1	
fibrinogen, alpha	66	10	1	
orosomucoid 1	22	9	1	
paraoxonase 1	40	8	1	yes
C4BP α-chain	67	6	1	
apolipoprotein D	19	4	1	yes
IgM heavy chain	50	3	1	
CETP[e]	53	2	1	yes
galectin-3-binding protein	63	2	1	yes
Ig kappa chain	12	1	1	
LCAT[f]	47	1	1	yes

[a] Protein prophet score. [b] Protein known to associate with lipoproteins. [c] Identified in two independent experiments. [d] Previously identified.[11] [e] Cholesteryl ester transfer protein. [f] Lecithin–cholesterol acyltransferase.

per milligram of particles 200 and 70 nm in diameter, respectively. The protein pattern is dependent on the number of washes (Supporting Information). After one wash, the amount of albumin is still significant but not after three washes. A total washing time of 20 minutes was implemented to retain proteins with relatively high affinity and slow exchange. Five proteins were consistently associated with the particles. Bands were cut out from SDS-PAGE gels, digested by trypsin, and studied by mass spectrometry.[9] In two independent experiments this led to identification of HSA (69 kDa; $p < 10^{-6}$), apolipoprotein AIV (43 kDa; $p < 10^{-6}$), apolipoprotein E (34 kDa; $p < 10^{-6}$), apolipoprotein AI (28 kDa; $p < 10^{-6}$), and apolipoprotein AII (8.7 kDa; $p < 5 \times 10^{-5}$). Apolipoprotein AI (and four other proteins) was identified as associated with the particles in a previous study, in which plasma proteins and particles were separated by gel filtration. The molecular weights (MWs) of these proteins match those estimated from SDS-PAGE in the present experiments. Judging from the band identities, the most abundant protein on the particles is apolipoprotein AI. This protein (1–2 mg mL^{-1} in plasma) is the major component of high-density lipoproteins (HDLs) and is found on chylomicrons, large lipoprotein particles created by the absorptive cells of the small intestine.[8,9] Apolipoproteins AIV, E, and AII (0.13–0.25, 0.03–0.07, and 0.3–0.55 mg mL^{-1}, respectively) are found on different lipoprotein particles in blood.[8,10] The plasma concentrations of the apolipoproteins correlate with their relative abundance on the nanoparticles. In contrast, there is very little HSA on the particles compared to its abundance (35 mg mL^{-1}) in plasma. Thus, the apolipoproteins six are associated with lipoproteins. Their relative abundance on the particles is probably low, but they could still play an important role in determining the biological response to the nanoparticles.

The hydrophobicity of the particle surface influences both the amount and identity of the proteins bound to the particles.[11–14] The less hydrophobic 85:15 NIPAM–BAM copolymer particles (Supporting Information) behave as the negative control, as virtually no protein was retrieved from them (except some HSA), in strong contrast to the more hydrophobic 50:50 particles (Figure 1 and Supporting Information).

To investigate the role of surface curvature, 50:50 NIPAM–BAM particles with diameters from 70 to 700 nm were incubated with plasma (Figure 1 and Supporting Information). The amount of bound protein varied with size, and scaled with the amount of available surface area. However, the protein pattern is the same for all sizes and apolipoprotein AI is always the most abundant protein recovered. This finding indicates that, for these cases and this size range, the surface curvature is not a major determining factor for the relative affinities of proteins for the particles.

Variation in the protein-binding pattern between individuals may occur as a result of serum protein variability.[7] However, copolymer particles incubated with serum from five donors show very similar protein adsorption profiles (Supporting Information), which indicates that, at least among these donors, there are no major differences.

The preferential coverage of 50:50 NIPAN–BAM copolymer particles by apolipoproteins AI, AII, AIV, and E and

other associated proteins, the slow dissociation rate of apolipoprotein AI,[11] and its specificity for the more hydrophobic particles are intriguing. Apolipoproteins in blood associate with lipoprotein particles with similar diameters to those of the particles used here, for example, chylomicrons (> 100 nm) and HDLs (8–10 nm).

The nature of the adsorbed proteins is suggestive of various biological responses that could be elicited by the particles. Apolipoproteins AI, AII, and AIV are key components that modulate lipid metabolism and cardiovascular disease risks.[15] In addition, they are involved in amyloidosis diseases.[16,17] Apolipoprotein E is a cholesterol-transport protein and a risk factor in neurodegenerative diseases, for example, Alzheimer's disease.[18]

The association of apolipoproten AI with lipoprotein particles is thought to depend on eight to nine amphiphatic α-helices of 22-mer repeats.[9,19,20] Two helices make up a hinge region, which gives the protein the flexibility to bind particles of different size.[19,20] Previous results from structural studies of this random copolymer in flat surfaces suggest an exaggerated expression of *tert*-butyl groups at the surface.[21] This finding, combined with the fact that the nanoparticles are of similar size to naturally occurring chylomicrons, may lead to a hydrophobic surface ideally suited to apolipoprotein binding. Another possible explanation is that the hydrophobic copolymer particles are covered with fats from the plasma and are in fact lipoproteins. To distinguish between these explanations further experiments are needed with purified apolipoproteins, fats, and nanoparticles.

Experimental Section

Copolymer nanoparticles were prepared by radical polymerization in SDS micelles (see Supporting Information). Protein binding to nanoparticles was studied by incubating particles (2 mg) in Tris-HCl (0.2 mL, 10 mM, pH 7.5), NaCl (0.15 M), and ethylenediaminetetraacetic acid (EDTA; 1 mM) with increasing amounts of plasma on ice. After 1 h, the temperature of the samples was increased to 23 °C to promote aggregation. The particles were pelleted by centrifugation (13000 rpm, 2 min) and washed three times with Tris-HCl (0.5 mL, 10 mM, pH 7.5), NaCl (0.15 M), and EDTA (1 mL); 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 or 15% SDS-PAGE. Bound proteins were identified in two independent mass spectrometry (MS) experiments: nanoscale liquid chromatography–quadrupole time-of-flight MS/MS and nanoelectrospray liquid chromatography–tandem mass spectrometry (nano-LC MS/MS). Full details of these procedures are given in the Supporting Information.

Received: February 2, 2007
Revised: May 22, 2007
Published online: June 25, 2007

Keywords: adsorption · lipoproteins · nanoparticles · proteomics · toxicology

[1] A. Nel, T. Xia, L. Mädler, N. Li, *Science* **2006**, *311*, 622–627.
[2] D. B. Warheit, T. R. Webb, C. M. Sayes, V. L. Colvin, K. L. Reed, *Toxicol. Sci.* **2006**, *91*, 227–236.
[3] V. L. Colvin, *Nat. Biotechnol.* **2003**, *21*, 1166–1170.
[4] M. Lundqvist, P. Nygren, B.-H. Jonsson, K. Broo, *Angew. Chem.* **2006**, *118*, 8349–8353; *Angew. Chem. Int. Ed.* **2006**, *45*, 8169–8173.
[5] S. Linse, C. Cabaleiro-Lago, W.-F. Xue, I. Lynch, S. Lindman, E. Thulin, S. Radford, K. A. Dawson, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 8691–8696.
[6] I. Lynch, K. A. Dawson, S. Linse, *Science* **2006**, *327*, 14.
[7] B. Muthusamy et al., *Proteomics* **2005**, *5*, 3531–3536.
[8] R. W. Mahley, T. L. Innerarity, S. C. Rall, Jr., K. H. Weisgraber, *J. Lipid Res.* **1984**, *25*, 1277–1294.
[9] T. Berggard, G. Arrigoni, O. Olsson, M. Fex, S. Linse, P. James, *J. Proteome Res.* **2006**, *5*, 669–687.
[10] G. L. Hortin, *Clin. Chem.* **2006**, *52*, 1223–1237.
[11] T. Cedervall, I. Lynch, S. Lindman, H. Nilsson, E. Thulin, S. Linse, K. A. Dawson, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 2050–2055.
[12] S. Lindman, I. Lynch, E. Thulin, H. Nilsson, K. A. Dawson, S. Linse, *Nano Lett.* **2007**, *7*, 914–920.
[13] A. Gessner, R. Waicz, A. Lieske, B.-R. Paulke, K. Mäder, R. H. Müller, *Int. J. Pharm.* **2000**, *196*, 245–249.
[14] M. Lück, B.-R. Paulke, W. Schröder, T. Blunk, R. H. Müller, *J. Biomed. Mater. Res.* **1998**, *39*, 478–485.
[15] C. Q. Lai, L. D. Parnell, J. M. Ordovas, *Curr. Opin. Lipidol.* **2005**, *16*, 153–166.
[16] A. Solomon, C. L. Murphy, D. Kestler, D. Coriu, D. T. Weiss, J. Markovitsky, P. Westermark, *Arthritis Rheum.* **2006**, *54*, 3545–3550.
[17] P. Westermark, *FEBS J.* **2005**, *272*, 5942–5949.
[18] J. Herz, Y. Chen, *Nat. Rev. Neurosci.* **2006**, *7*, 850–859.
[19] P. G. Frank, Y. L. Marcel, *J. Lipid Res.* **2000**, *41*, 853–857.
[20] R. J. Cushley, M. Okon, *Annu. Rev. Biophys. Biomol. Struct.* **2002**, *31*, 177–206.
[21] I. Lynch, I. A. Blute, B. Zhmud, P. MacArtain, M. Tosetto, L. T. Allen, H. J. Byrne, G. F. Farrell, A. K. Keenan, W. M. Gallagher, K. A. Dawson, *Chem. Mater.* **2005**, *17*, 3889–3898.

