



Nanoparticle size matters in the formation of plasma protein coronas on Fe₃O₄ nanoparticles



Zhengyan Hu^{a,b}, Hongyan Zhang^{a,b}, Yi Zhang^{a,b}, Ren'an Wu^{a,*}, Hanfa Zou^{a,**}

^a CAS Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic R & A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences (CAS), Dalian 116023, China

^b University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:

Received 19 February 2014

Received in revised form 4 June 2014

Accepted 5 June 2014

Available online 12 June 2014

Keywords:

Protein corona

Fe₃O₄ NPs

Particle size

Quantitative proteomics

ABSTRACT

When nanoparticles (NPs) enter into biological systems, proteins would interact with NPs to form the protein corona that can critically impact the biological identity of the nanomaterial. Owing to their fundamental scientific interest and potential applications, Fe₃O₄ NPs of different sizes have been developed for applications in cell separation and protein separation and as contrast agents in magnetic resonance imaging (MRI), etc. Here, we investigated whether nanoparticle size affects the formation of protein coronas around Fe₃O₄ NPs. Both the identification and quantification results demonstrated that particle size does play an important role in the formation of plasma protein coronas on Fe₃O₄ NPs; it not only influenced the protein composition of the formed plasma protein corona but also affected the abundances of the plasma proteins within the coronas. Understanding the different binding profiles of human plasma proteins on Fe₃O₄ NPs of different sizes would facilitate the exploration of the bio-distributions and biological fates of Fe₃O₄ NPs in biological systems.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Nanoparticles (NPs) have been widely applied in drug/gene delivery, disease diagnosis and biosensing due to their unique properties compared with their corresponding bulk counterparts [1–5]. When NPs enter biological systems, proteins can bind onto their surfaces, leading to the formation of so-called “protein coronas”, which represents the first interfacial physicochemical properties of the NPs [6,7]. It has been observed that protein coronas bound on the surfaces of NPs critically affect the biological identity of the nanomaterial [6–9] and/or result in physiological and pathological changes, including macrophage uptake, blood coagulation, complement activation and cellular toxicity [10–17].

Studies of protein coronas for different types of NPs, such as copolymers [18,19], silica [15,20,21], polystyrene [21–23] and metallic NPs [24,25], have been performed extensively. It was found that protein coronas on these NPs could be affected by several factors, such as the chemical composition [26], cell culture medium [27], serum inactivation [28], plasma concentration [21,29], surface

properties [24,30] and particle size [20,23] of the NPs. For instance, differences in plasma proteins bound on the surfaces of SiO₂, TiO₂ and ZnO NPs have been observed due to differences in the chemical compositions of the materials [26]. Additionally, it has been reported that the formation of a protein corona on cationic liposomes is strongly related to the membrane charge density [30]. Moreover, it has been illustrated that particle size could critically influence human plasma proteins bound on the surfaces of silica NPs [20].

Fe₃O₄ NPs have been extensively developed for applications in cell separation, protein separation, targeted drug delivery and as contrast agents in magnetic resonance imaging (MRI) [31–36]. Although many types of Fe₃O₄ NPs have been synthesized with different sizes and functionalities to meet the needs of various applications, studies of the interactions between Fe₃O₄ NPs and human plasma proteins are relatively rare. Recently, Mahmoudi et al. found that superparamagnetic iron oxide NPs induced irreversible changes in the conformation of human transferrin protein [37]. Additionally, it was found that the plasma proteins adsorbed onto iron oxide NPs vary depending on the initial NP surface coating and the concentrations of plasma, while the formation of protein corona would further influence the internalization of the iron oxide NPs into macrophages [38]. However, the above-mentioned studies only focused on protein coronas formed on superparamagnetic

* Corresponding author. Tel.: +86 411 84379828; fax: +86 411 84379617.

** Corresponding author. Tel.: +86 411 84379610; fax: +86 411 84379620.

E-mail addresses: wurenan@dicp.ac.cn, wurnan@gmail.com (R. Wu),

hanfazou@dicp.ac.cn (H. Zou).

iron oxide NPs less than 10 nm in size and with coatings of polymers, the protein coronas on the pristine Fe₃O₄ NPs in larger sizes were not investigated. Furthermore, whether the particle size of Fe₃O₄ NPs influences the formation of the plasma protein corona is unknown. Although pristine Fe₃O₄ NPs are not directly applied in biomedical fields, they are precursors of the magnetic NPs used in biological and biomedical applications. During the fabrication process, pristine Fe₃O₄ NPs can be inhaled by humans, pass through membranes and barriers, and finally enter the blood [39]. Here, we quantified the plasma protein coronas bound on three different sizes of pristine Fe₃O₄ NPs (~30 nm, 200 nm and 400 nm) by means of label-free quantitative proteomic strategy. Our results demonstrated that the particle size was an important factor that not only influenced the protein composition of the human plasma protein coronas on Fe₃O₄ NPs but also affected the abundance of plasma proteins in the protein coronas.

2. Materials and methods

2.1. Chemicals and materials

Fe₃O₄-30 (~30 nm), dithiothreitol (DTT), iodoacetamide (IAA), TPCK-treated trypsin, bovine serum albumin (BSA), formic acid (FA), tetraethyl orthosilicate (TEOS) and (3-aminopropyl) triethoxysilane (APTES) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade) and ammonium solution (25%) were from Merck (Darmstadt, Germany). Iron (III) chloride hexahydrate (FeCl₃·6H₂O), anhydrous sodium acetate (NaAc), ethylene diamine, isopropanol, ethylene glycol (EG) and ethanol were bought from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). SDS-PAGE marker and loading buffer were obtained from Thermo Scientific (San Jose, CA). The water used in the experiments was doubly distilled and purified by a Mill-Q system (Millipore, Bedford, MA, USA).

2.2. Human plasma

Human blood was taken from 20 different healthy donors and collected in tubes containing EDTA to prevent blood clotting. The tubes were centrifuged for 5 min at 1000 RCF to pellet the blood cells. The supernatant (the plasma) was transferred to new tubes and mixed together in order to eliminate individual differences. Then, the plasma was divided into portions and stored at –80 °C until use. Upon thawing, the plasma was centrifuged again for 2 min at 16,000 RCF to further reduce the presence of red and white blood cells. The plasma was used immediately upon thawing and was never refrozen.

2.3. Synthesis of Fe₃O₄ NPs of different sizes

Fe₃O₄ NPs with sizes of ~200 and ~400 nm were synthesized according to previous literature [40]. Briefly, FeCl₃·6H₂O (1.35 g, 5 mmol) was dissolved in EG (40 mL) to form a clear solution, followed by the addition of NaAc (3.6 g) and polyethylene glycol (1.0 g). The mixture was stirred vigorously for 30 min and then sealed in a Teflon-lined, stainless-steel autoclave. The autoclave was heated and maintained at 200 °C for 8 h and 48 h for the preparation of the ~200 nm and ~400 nm Fe₃O₄ NPs, respectively. Finally, the mixture was allowed to cool to room temperature. The black products were washed several times with ethanol and dried under vacuum at 60 °C overnight.

2.4. The formation of human plasma protein corona on Fe₃O₄ NPs

To ensure comparability between the identification results for the protein coronas on the Fe₃O₄ NPs of different sizes, the ratio

of the total particle surface area to the plasma concentration was kept the same for the three different particle sizes. For this purpose, the mass of Fe₃O₄ NPs of different sizes (suspended in PBS with a concentration of 0.2%, w/w) possessing a total surface area ($A = 4\pi R^2$) of 0.01 m² was calculated and incubated in a constant amount of human plasma at 37 °C with shaking at 1000 rpm for 1 h. The ratio of the plasma volume to the particle surface area was adjusted to a constant value of 5.56 mL/m², as reported previously (PBS was added to obtain a total volume of 1 mL) [23,24]. After that, the nanoparticle–protein complexes were collected by a magnet and washed at least 3 times to remove the loosely bound and unbound proteins. The amount of plasma proteins absorbed onto the Fe₃O₄ NPs was roughly calculated by subtraction of the amount of plasma proteins before and after incubation. In addition, the microcentrifuge tubes were changed after each washing step in order to eliminate the potential influences of proteins adsorbed on the surfaces of the tubes. Then, the nanoparticle–protein complexes were resuspended in 500 µL of 100 mM NH₄HCO₃ solution (pH ~8.1) and divided into two parts. One portion was pelleted, resuspended in SDS-PAGE loading buffer, then boiled at 95 °C for 5 min and finally separated with 1-D SDS-PAGE to illustrate the binding patterns of the plasma proteins on the Fe₃O₄ NPs. The other portion was analyzed with the on nanoparticle digestion shotgun proteomics method [41]. Briefly, the nanoparticle–protein complexes were resuspended in a solution containing 8 M urea and 100 mM NH₄HCO₃ (pH ~8.1) to denature the proteins, then reduced and alkylated with DTT (10 mM, 2 h at 37 °C) and IAA (20 mM, 30 min in the dark at room temperature), respectively [42]. Next, the solution was diluted with 100 mM NH₄HCO₃ to 1 M urea, and the proteins were digested with trypsin at 37 °C overnight with shaking (the amount of trypsin applied to digest the plasma proteins was set to an enzyme:protein ratio of 1:40 according to the amount of adsorbed proteins). Finally, the digestion solution was adjusted to pH 2–3 with a 10% TFA solution and desalted with homemade C18 SPE columns. Briefly, after the C18 SPE columns were sequentially activated and equilibrated with methanol and 0.1% TFA solution, respectively, the digestion solutions were loaded onto the SPE columns, washed extensively with 0.1% TFA solution and then eluted with 0.1% TFA in 80% ACN. Finally, the elutions were lyophilized in a vacuum freeze dryer. The samples were stored at –40 °C until RPLC-MS/MS analysis.

Furthermore, to investigate the plasma protein adsorption dynamics of the Fe₃O₄ NPs, Fe₃O₄ NPs of different sizes and with a total surface area of 0.005 m² were also incubated with the same amount of human plasma at 37 °C with shaking at 1000 rpm for 1 min and 30 min. After that, the nanoparticle–protein complexes were collected and washed at least 3 times to obtain the hard protein coronas. Finally, the nanoparticle–protein complexes were resuspended in 1× SDS-PAGE loading buffer, boiled at 95 °C for 5 min and separated with 1-D SDS-PAGE to illustrate the binding dynamics of the Fe₃O₄ NPs of different sizes.

2.5. RPLC-MS/MS analysis

The digested peptides from the human plasma protein corona were dissolved in 100 µL of 0.1% FA solution. The analysis was carried out on a 1-D nano LC-MS/MS system with 5 µL of the resuspended peptides injected each time. The C18 trap column (7 cm × 200 µm i.d.) was connected to a reverse phase (RP) C18 column (12 cm × 75 µm i.d.) packed with C18 AQ beads (5 µm, 120 Å pore size) from Microm BioResources in tandem by a union. For the RPLC separation, a Waters Nano-ACQUITY UPLC system was used to deliver the mobile phases of 0.1% FA in water (mobile phase A) and ACN (mobile phase B) with a total flow rate of ~350 nL/min; the binary separation gradient of mobile phase B was increased from 2% to 30% in 60 min. Tandem mass spectrometry was performed on

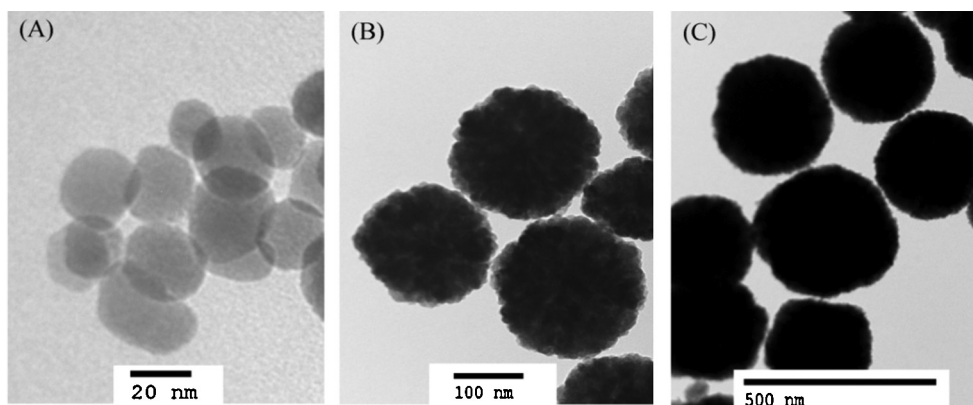


Fig. 1. TEM images of the Fe_3O_4 NPs of different sizes (Fe_3O_4 -30 (A), Fe_3O_4 -200 (B) and Fe_3O_4 -400 (C)).

a triple TOF 5600 mass spectrometer (AB SCIEX, USA) operated in positive ion data dependent (IDA) mode with one MS survey scan followed by 30 MS/MS scans using a 120 s exclusion window. The scan range of the full MS was set from m/z 350 to m/z 1250, while the MS/MS was from m/z 100 to m/z 1500.

2.6. Database searching

The MS/MS spectral data acquired by the triple TOF 5600 mass spectrometer were searched by the “Paragon Algorithm” of the Protein Pilot software (AB SCIEX, USA) with trypsin specificity in “Thorough ID” mode [43]. The peptide false discovery rate (FDR) was controlled <1% by setting the criterion for the corresponding confidence of global FDR of hit. The results from the human plasma protein coronas were searched against the International Protein Index (ipi.human.3.80.fasta). To confirm the identification results for the plasma proteins bound on Fe_3O_4 NPs, each sample was analyzed in at least three replicates, and only proteins that were both detected at least 2 times in triplicate analysis and identified by at least 2 unique peptides in each run were considered as positively identified.

3. Results and discussion

3.1. Characterization of Fe_3O_4 NPs

The Fe_3O_4 NPs of different sizes were characterized by transmission electron microscopy (TEM, Fig. 1). The size distributions and zeta potentials of the Fe_3O_4 NPs with different particle sizes of 30, 200 and 400 nm (denoted as Fe_3O_4 -30, Fe_3O_4 -200 and Fe_3O_4 -400) are listed in Table 1. The Fe_3O_4 NPs exhibited approximate zeta potential values of -28.9 mV, -23.4 mV and -24.3 mV for Fe_3O_4 -30, Fe_3O_4 -200 and Fe_3O_4 -400, respectively. The particle size of NPs influences their surface curvature and free energy, which may result in different aggregation states of the NPs in suspensions. This could explain why the Fe_3O_4 NPs of different particle sizes were found to be aggregated in PBS buffer to different degrees (shown in Table 1).

Table 1
The sizes and zeta potentials of the Fe_3O_4 NPs.

Fe_3O_4 nanoparticle	Size (nm)	Zeta potential (mV)	Z-Ave diameter (nm)	PDI
Fe_3O_4 -30	30 ± 10.3	-28.9 ± 0.87	822.4 ± 73.9	0.41 ± 0.02
Fe_3O_4 -200	225 ± 11.5	-23.4 ± 0.35	643.1 ± 34.6	0.38 ± 0.03
Fe_3O_4 -400	375 ± 16.3	-24.3 ± 1.34	571.1 ± 10.8	0.44 ± 0.06

The error bars represent the standard deviation from the mean ($n=3$).

3.2. Plasma protein binding patterns for Fe_3O_4 NPs with different sizes

Once introduced into biological systems, proteins would compete for binding sites on the surfaces of NPs. After incubation in human plasma for 1 min, 30 min and 1 h, the obtained nanoparticle–protein complexes were washed at least 3 times with PBS to obtain the hard protein corona of Fe_3O_4 NPs. The nanoparticle–protein complexes were resuspended in SDS loading buffer and separated using 1-D SDS-PAGE. The adsorption dynamics of the human plasma protein coronas on the Fe_3O_4 NPs are shown in Fig. 2. It was observed that the adsorption of plasma proteins on the three different sizes of Fe_3O_4 NPs evolved as the incubation time went along and reached a relative equilibrium state after 1 h of incubation in human plasma. On the other hand, the plasma proteins displayed different binding patterns depending on the size of the Fe_3O_4 NPs, illustrating that nanoparticle size impacted the formation of the protein corona. Additionally, the total band intensities for the plasma protein coronas exhibited obvious differences depending on NP size (shown in Fig. 2D); i.e., there were much more plasma proteins bound on the Fe_3O_4 -200 and Fe_3O_4 -400 NPs than on the Fe_3O_4 -30 NPs, which may be due to the obvious aggregation of the Fe_3O_4 -30 NPs, indicating that NP aggregation also affects the formation of protein coronas. Moreover, the optical intensity distributions of the plasma protein coronas formed on Fe_3O_4 NPs of different particle sizes were further analyzed with the Image Lab program (shown in Fig. 3), which also confirmed the different adsorption patterns on the Fe_3O_4 NPs and the equilibrium state achieved after 1 h of incubation in human plasma.

3.3. Identification and quantification of plasma proteins bound on Fe_3O_4 NPs with different sizes

In our study, to guarantee identification accuracy, each sample was analyzed in at least three replicates, and only proteins detected in at least two runs and identified by at least two unique peptides in each analysis were considered as constituents of the protein coronas formed on the Fe_3O_4 NPs. The protein coronas bound on the Fe_3O_4 NPs after 1 h of incubation with human plasma were

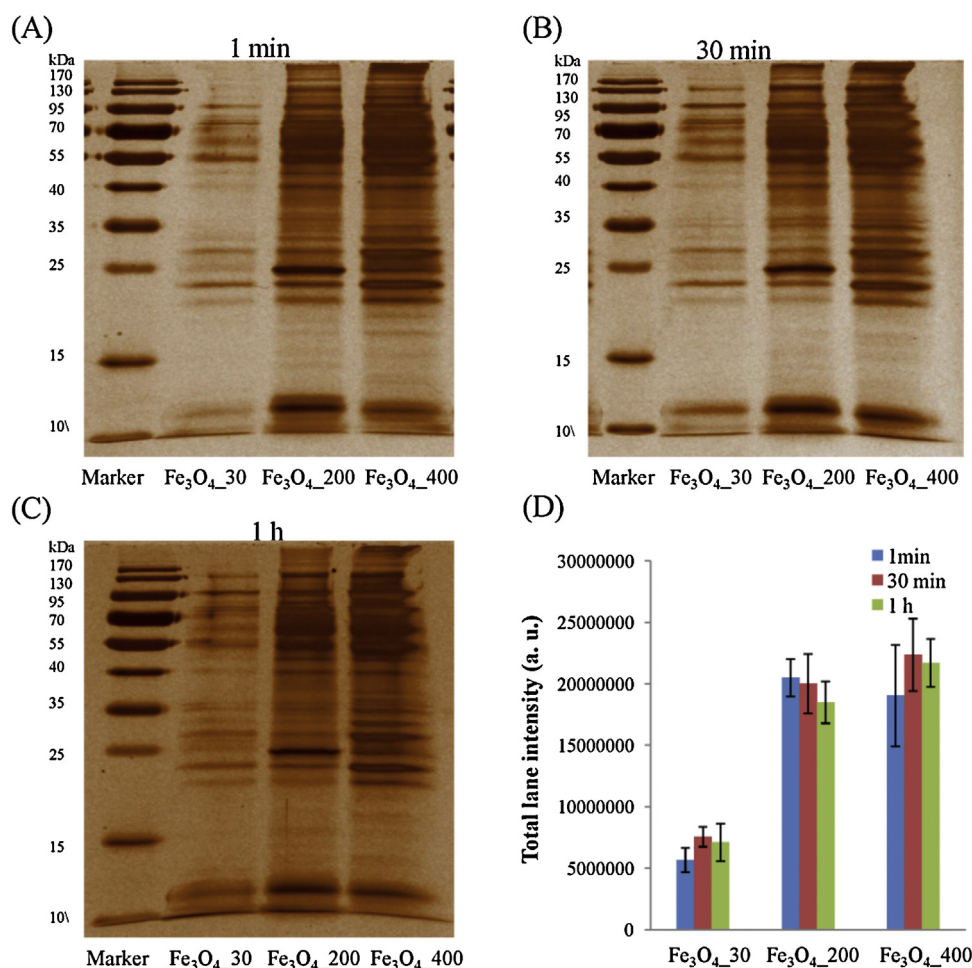


Fig. 2. Silver stained SDS-PAGE gel lanes showing the plasma protein binding profiles on different sizes of Fe₃O₄ NPs after incubation in human plasma for 1 min (A), 30 min (B) and 1 h (C) and the corresponding optical intensities of each gel lanes for the plasma proteins adsorbed on the Fe₃O₄ NPs. The error bars represent the standard deviation from the mean ($n = 3$).

used to investigate the size effects on the formation of protein coronas on the Fe₃O₄ NPs. In total, there were 117, 133 and 100 plasma proteins identified in the protein coronas of the Fe₃O₄_30, Fe₃O₄_200 and Fe₃O₄_400 NPs, respectively (the detailed identification results were listed in Table S1). A total of 71 human plasma proteins were detected in the protein coronas of Fe₃O₄ NPs of the three different sizes, with 22, 31 and 11 distinct proteins for the Fe₃O₄_30, Fe₃O₄_200 and Fe₃O₄_400 NPs, respectively (shown in Fig. 4). For example, serum albumin, apolipoprotein A-I and Platelet factor, among others, were observed to bind on the surfaces of all three types of Fe₃O₄ NPs, while ceruloplasmin and serum amyloid P-component, among others, were found to be uniquely bound on the surface of only the Fe₃O₄_30 NPs. Fibronectin, serotransferrin and serum paraoxonase/arylesterase 1 were observed to only be adsorbed on the Fe₃O₄_200 NPs, and coagulation factor XIII A chain and inter-alpha (Globulin) inhibitor H2 were only bound on the Fe₃O₄_400 NPs (Table S1). In addition, with the analysis of GoMiner and Panther databases [44,45], it was found that most of the 71 common proteins bound on the Fe₃O₄ NPs of all sizes were related to cellular process, metabolic process, response to stimulus, immune system process and other processes (shown in Fig. 5A). By contrast, the plasma proteins uniquely bound on the surfaces of the Fe₃O₄ NPs of only one specific size (Fe₃O₄_30/Fe₃O₄_200/Fe₃O₄_400) were observed to participate in different biological processes (shown in Fig. 5B). For instance, plasma proteins that related to metabolic processes were

found to comprise 33.3% of the total distinct proteins bound on the Fe₃O₄_30 NPs, which were observed to take up 9.5% and 16.7% of the total distinct proteins bound on the Fe₃O₄_200 and Fe₃O₄_400 NPs, respectively. Additionally, the plasma proteins uniquely bound on the Fe₃O₄_30 NPs were observed to be related to the cell cycle (6.7%), while it was not found for plasma proteins that uniquely bound on the Fe₃O₄_200 and Fe₃O₄_400 NPs. Thus, the size of the Fe₃O₄ NPs seemed to be an important factor regulating the composition of the plasma protein corona on the Fe₃O₄ NPs, which may pose great impacts on the biological fates of NPs.

On the other hand, based on the spectral counting (SpC) method [46], we applied the label-free quantitative proteomic strategy to quantitatively analyze the plasma proteins bound on the surfaces of the Fe₃O₄ NPs of different sizes. Here, the SpC of each protein identity was normalized to the protein mass and described as the relative protein quantity by applying the following equation:

$$MWSpC_k = \left(\frac{(\text{SpC}/\text{Mw})_k}{\sum_{i=1}^n (\text{SpC}/\text{Mw})_i} \right) \times 100$$

where MWSpC is the percentage normalized spectral count for protein k , SpC is the spectral count identified and Mw is the molecular weight in kDa for protein k . This correction takes into account the protein size and evaluates the real contribution of each protein to the hard corona composition [20,21]. By using this equation, the relative protein abundances for the plasma proteins bound on the

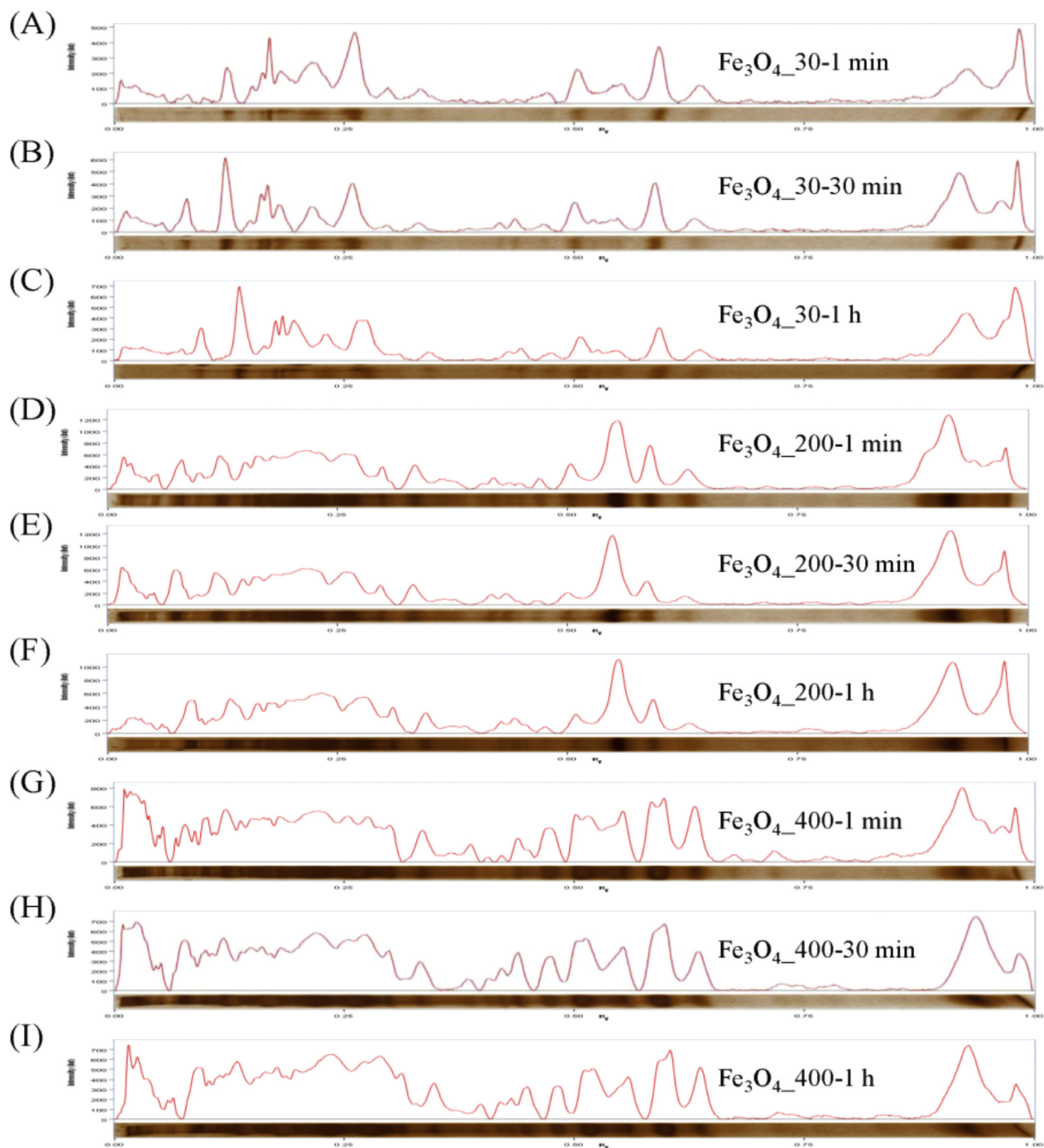


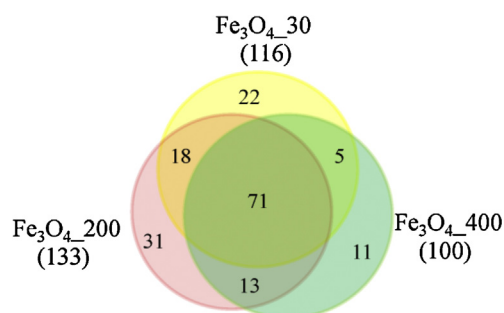
Fig. 3. The optical intensities across the SDS-PAGE gel lanes for the plasma protein binding profiles on different sizes of Fe_3O_4 NPs (Fe_3O_4 _30 (A–C)/ Fe_3O_4 _200 (D–F)/ Fe_3O_4 _400 (G–I)) after incubation in human plasma for 1 min (A, D and G), 30 min (B, E and H) and 1 h (C, F and I).

Fe_3O_4 NPs were calculated and listed in Table S1. It was found that although there were 71 common plasma proteins bound on the different sizes of Fe_3O_4 NPs, the relative protein abundances for these proteins differed. For instance, serum albumin was observed in the protein corona of the Fe_3O_4 _200 NPs with an average MWSpC of 9.01, while the average MWSpC for serum albumin in the

protein coronas of the Fe_3O_4 _30 and Fe_3O_4 _200 NPs were 1.27 and 2.50, respectively, indicating the selective binding of plasma proteins on different sizes of Fe_3O_4 NPs. Additionally, platelet factor 4 was observed in the corona of Fe_3O_4 _30 with an average MWSpC of 8.57, which accounted for 3.51% and 4.01% in the protein coronas of the Fe_3O_4 _200 and Fe_3O_4 _400 NPs, respectively. Furthermore, the

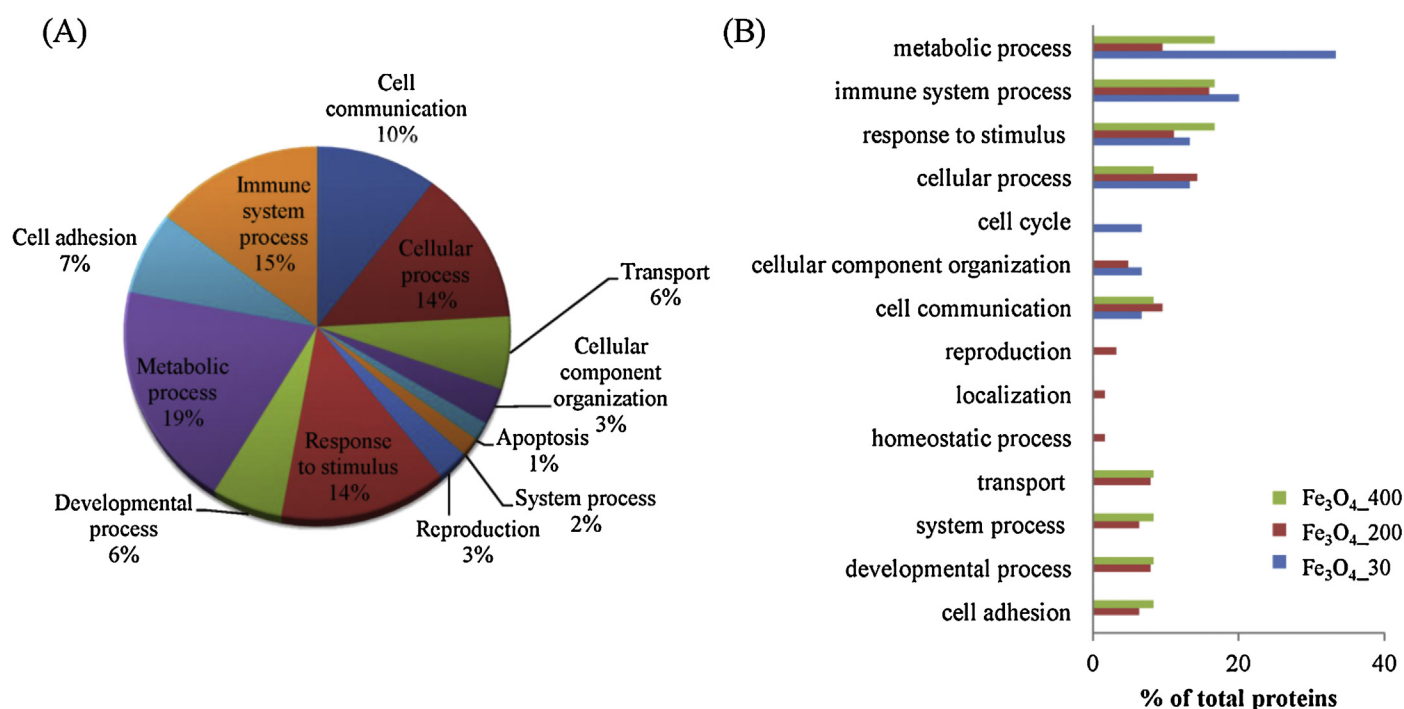
Table 2The top 20 most abundant plasma proteins bound on the Fe₃O₄ NPs of different sizes.

Rank	Fe ₃ O ₄ _30	Fe ₃ O ₄ _200	Fe ₃ O ₄ _400
1	Platelet factor 4	Apolipoprotein A-I	Apolipoprotein E
2	Apolipoprotein E	Serum albumin	Vitronectin
3	Vitronectin	Apolipoprotein C-III variant 1	Histidine-rich glycoprotein
4	Platelet basic protein	Apolipoprotein C-I	Platelet basic protein
5	IGK@ IGK@ protein	Apolipoprotein E	Platelet factor 4
6	IGKC Anti-RhD monoclonal T125 kappa light chain	Isoform LMW of Kininogen-1	Complement C1q subcomponent subunit C
7	Putative uncharacterized protein	Vitronectin	Complement C1q subcomponent subunit A
8	Complement C1q subcomponent subunit C	Platelet factor 4	Isoform 1 of Gelsolin
9	IGHG1	Histidine-rich glycoprotein	APOC1 Apolipoprotein C-I
10	Apolipoprotein C-III variant 1	Platelet basic protein	Complement C3 (Fragment)
11	IGHG1	Isoform HMW of Kininogen-1	Prothrombin (Fragment)
12	IGHG1	Isoform 1 of Gelsolin	Serum albumin
13	Prothrombin (Fragment)	Apolipoprotein A-II	C1QB Uncharacterized protein
14	Complement C1q subcomponent subunit A	Complement C3 (Fragment)	IGHG1
15	CLEC3B Putative uncharacterized protein DKFZp686H17246	Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H4	IGHG1
16	Apolipoprotein A-I	clusterin isoform 3	IGHG1
17	Isoform LMW of Kininogen-1	ITIH4 protein	APOH Beta-2-glycoprotein 1
18	Complement C3 (Fragment)	inter-alpha (globulin) inhibitor H4 isoform 2 precursor	IGKC Putative uncharacterized protein
19	Beta-2-glycoprotein 1	Prothrombin (Fragment)	Complement component 4B
20	IGHM Putative uncharacterized protein DKFZp686I15212	Apolipoprotein C-II	IGKC Anti-RhD monoclonal T125 kappa light chain

**Fig. 4.** Venn diagram displaying the number of proteins identified in the protein coronas of the different sizes of Fe₃O₄ NPs.

plasma proteins with an average MWSpC ≥ 1 in the protein corona on the Fe₃O₄ NPs are illustrated in Fig. 6, which also reveals the distinctive properties of the plasma protein coronas formed on the different sizes of Fe₃O₄ NPs.

Moreover, it has been reported that adsorbed protein layer on NPs would affect the cellular uptake and subsequent trafficking in cells, while in vivo, the specific binding of proteins would influence bio-distribution and blood clearance [47]. For example, the adsorption of opsonins such as IgG and complement factors is supposed to promote rapid clearance from the bloodstream, while the binding of dysopsonins, such as serum albumin and apolipoproteins, would promote prolonged circulation time in blood [21,47,48]. Although proteins with higher relative

**Fig. 5.** Biological processes related to the common proteins identified on all three sizes of Fe₃O₄ NPs (A) and those related to the plasma proteins uniquely bound on only one specific NP size (Fe₃O₄_30/Fe₃O₄_200/Fe₃O₄_400).

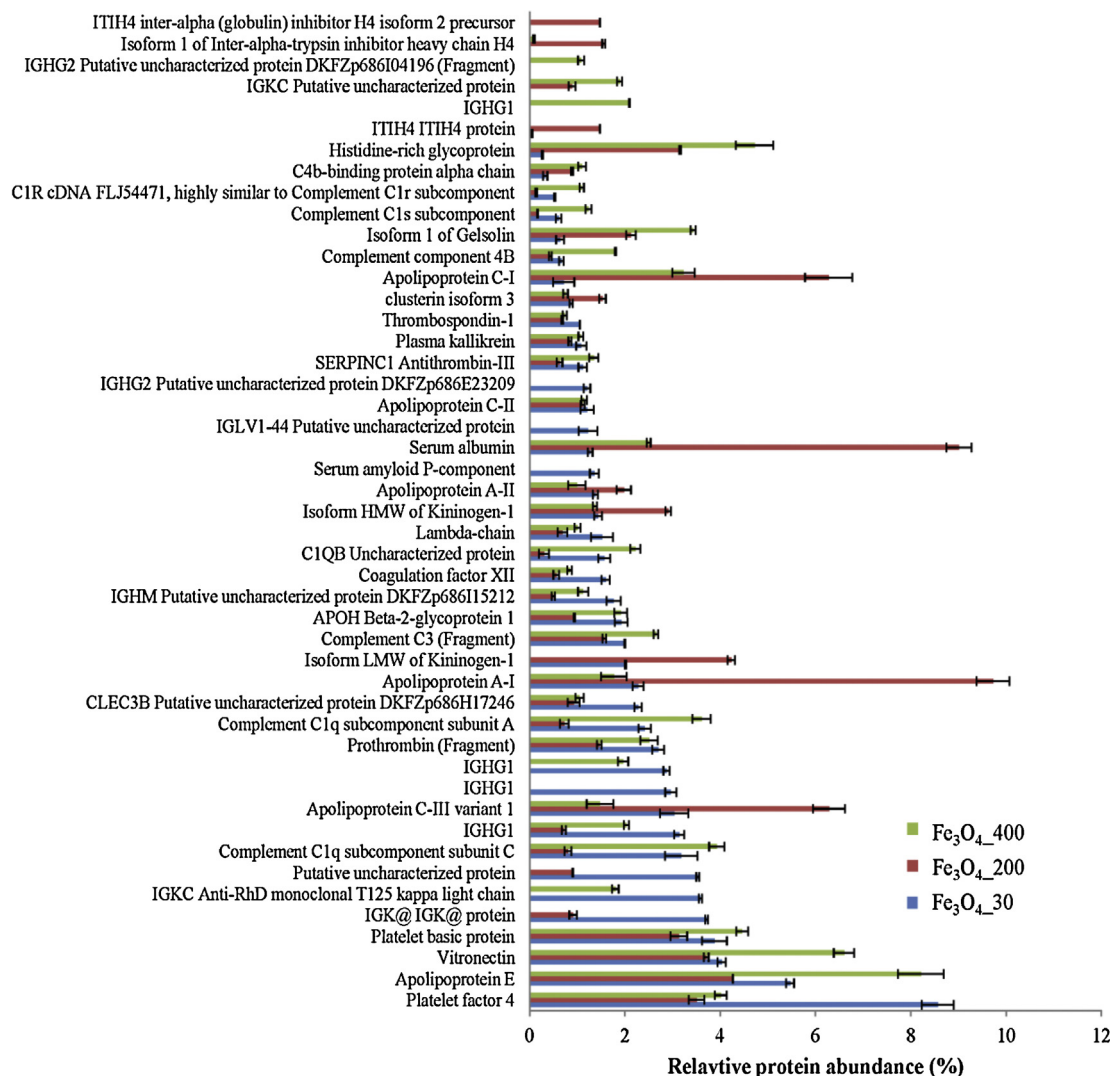


Fig. 6. Relative protein abundance of proteins (normalized to molecular weight, MWSpC) satisfying the criterion that MWSpC ≥ 1 in the coronas of the different sizes of Fe_3O_4 NPs. As the proteins were detected in more than 2 replicates, the error bars represent the standard deviation from the mean ($n = 2$ or $n = 3$).

abundances do not directly reflect biological impacts, proteins with extremely low relative abundances are not expected to be critical factors that determine the bio-distributions and biological fates of NPs [48,49]. Here, we list the top 20 most abundant plasma proteins comprising the protein coronas formed on the Fe_3O_4 NPs in Table 2 to further illustrate the different binding patterns of the plasma proteins and understand the following potential biological impacts. It was found that as the most abundant protein in human plasma, serum albumin was the 2nd most abundant protein in the protein corona around the Fe_3O_4 -200 NPs, while it was the 12th most abundant protein in the protein corona on the Fe_3O_4 -400 NPs and was not among the top 20 most abundant proteins for the Fe_3O_4 -30 NPs (27th). Thus, the quantitative results confirmed the selective binding of plasma proteins to different sizes of Fe_3O_4 NPs. Additionally, platelet factor 4, which is supposed to promote rapid clearance from the bloodstream, was found to bind on the aggregated Fe_3O_4 -30 NPs with the highest protein abundance. Apolipoprotein A-I and apolipoprotein E, which are supposed to promote prolonged circulation times, were the most abundant proteins bound on the Fe_3O_4 -200 and Fe_3O_4 -400 NPs, respectively. Therefore, the different binding profiles of plasma proteins on the different sizes of Fe_3O_4 NPs may have different impacts in human organisms.

4. Conclusions

In this study, the plasma protein coronas formed on pristine Fe_3O_4 NPs of three different particle sizes were investigated for the first time. According to the results, it could be concluded that the particle size induced different surface curvature and aggregation state of the Fe_3O_4 NPs, both of which may have played important roles in the formation of plasma protein coronas on these Fe_3O_4 NPs; the protein compositions as well as the abundances of the plasma proteins in the protein coronas of Fe_3O_4 NPs were found to be critically affected by particle size. Moreover, the proteins bound on the Fe_3O_4 NPs of different particle sizes may pose great influences on the biological distribution and fates of the Fe_3O_4 NPs.

Acknowledgements

Financial supports from the National Natural Science Foundation of China (Nos. 21175134, 21375125), the Creative Research Group Project of National Natural Science Foundation of China (21321064) and the Analytical Method Innovation Program of MOST (2012IM030900) are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2014.06.016>.

References

- [1] S. Laurent, D. Forge, M. Port, A. Roch, C. Robic, L.V. Elst, R.N. Muller, Magnetic iron oxide nanoparticles: synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications, *Chem. Rev.* 108 (2008) 2064–2110.
- [2] M.G. Lines, Nanomaterials for practical functional uses, *J. Alloy. Compd.* 449 (2008) 242–245.
- [3] J.A. Barreto, W. O'Malley, M. Kubeil, B. Graham, H. Stephan, L. Spiccia, Nanomaterials applications in cancer imaging and therapy, *Adv. Mater.* 23 (2011) H18–H40.
- [4] N.A. Saunders, Application of nanomaterials to arrays for infectious disease diagnosis, *Nanomedicine* 6 (2011) 271–280.
- [5] L. Zhang, T.J. Webster, Nanotechnology and nanomaterials promises for improved tissue regeneration, *Nano Today* 4 (2009) 66–80.
- [6] I. Lynch, K.A. Dawson, Protein–nanoparticle interactions, *Nano Today* 3 (2008) 40–47.
- [7] C.D. Walkey, W.C.W. Chan, Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment, *Chem. Soc. Rev.* 41 (2012) 2780–2799.
- [8] M.P. Monopoli, F.B. Bombelli, K.A. Dawson, Nanobiotechnology nanoparticle coronas take shape, *Nat. Nanotechnol.* 6 (2011) 11–12.
- [9] I. Lynch, A. Salvati, K.A. Dawson, Protein–nanoparticle interactions what does the cell see? *Nat. Nanotechnol.* 4 (2009) 546–547.
- [10] M.S. Ehrenberg, A.E. Friedman, J.N. Finkelstein, G. Oberdorster, J.L. McGrath, The influence of protein adsorption on nanoparticle association with cultured endothelial cells, *Biomaterials* 30 (2009) 603–610.
- [11] Z.J. Deng, Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation, *Nat. Nanotechnol.* 6 (2011) 39–44.
- [12] W. Hu, C. Peng, M. Lv, X. Li, Y. Zhang, N. Chen, C. Fan, Q. Huang, Protein corona-mediated mitigation of cytotoxicity of graphene oxide, *ACS Nano* 5 (2011) 3693–3700.
- [13] D. Dutta, S.K. Sundaram, J.G. Teeguarden, B.J. Riley, L.S. Fifield, J.M. Jacobs, S.R. Addleman, G.A. Kaysen, B.M. Moudgil, T.J. Weber, Adsorbed proteins influence the biological activity and molecular targeting of nanomaterials, *Toxicol. Sci.* 100 (2007) 303–315.
- [14] M.A. Dobrovolskaia, P. Aggarwal, J.B. Hall, S.E. McNeil, Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution, *Mol. Pharm.* 5 (2008) 487–495.
- [15] A. Lesniak, F. Fenaroli, M.R. Monopoli, C. Aberg, K.A. Dawson, A. Salvati, Effects of the presence or absence of a protein corona on silica nanoparticle uptake and impact on cells, *ACS Nano* 6 (2012) 5845–5857.
- [16] C. Vauthier, B. Persson, P. Lindner, B. Cabane, Protein adsorption and complement activation for di-block copolymer nanoparticles, *Biomaterials* 32 (2011) 1646–1656.
- [17] H. Mao, W. Chen, S. Laurent, C. Thirifays, C. Burtea, F. Rezaee, M. Mahmoudi, Hard corona composition and cellular toxicities of the graphene sheets, *Colloid. Surf. B* 109 (2013) 212–218.
- [18] T. Cedervall, I. Lynch, M. Foy, T. Berggård, S.C. Donnelly, G. Cagney, S. Linse, K.A. Dawson, Detailed identification of plasma proteins adsorbed on copolymer nanoparticles, *Angew. Chem. Int. Ed.* 46 (2007) 5754–5756.
- [19] T. Cedervall, I. Lynch, S. Lindman, T. Berggård, E. Thulin, H. Nilsson, K.A. Dawson, S. Linse, Understanding the nanoparticle–protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles, *Proc. Natl. Acad. Sci. U S A* 104 (2007) 2050–2055.
- [20] S. Tenzer, D. Docter, S. Rosfa, A. Wlodarski, J. Kuharev, A. Rekić, S.K. Knauer, C. Bantz, T. Nawroth, C. Bier, J. Sirirattanapan, W. Mann, L. Treuel, R. Zellner, M. Maskos, H. Schild, R.H. Stauber, Nanoparticle size is a critical physicochemical determinant of the human blood plasma corona: a comprehensive quantitative proteomic analysis, *ACS Nano* 5 (2011) 7155–7167.
- [21] M.P. Monopoli, D. Walczyk, A. Campbell, G. Elia, I. Lynch, F.B. Bombelli, K.A. Dawson, Physical–chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles, *J. Am. Chem. Soc.* 133 (2011) 2525–2534.
- [22] D. Walczyk, F.B. Bombelli, M.P. Monopoli, I. Lynch, K.A. Dawson, What the cell sees in bionanoscience, *J. Am. Chem. Soc.* 132 (2010) 5761–5768.
- [23] M. Lundqvist, J. Stigler, G. Elia, I. Lynch, T. Cedervall, K.A. Dawson, Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts, *Proc. Natl. Acad. Sci. U S A* 105 (2008) 14265–14270.
- [24] C.D. Walkey, J.B. Olsen, H. Guo, A. Emili, W.C.W. Chan, Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake, *J. Am. Chem. Soc.* 134 (2012) 2139–2147.
- [25] Z.J. Deng, M. Liang, I. Toth, M.J. Monteiro, R.F. Minchin, Molecular interaction of poly(acrylic acid) gold nanoparticles with human fibrinogen, *ACS Nano* 6 (2012) 8962–8969.
- [26] Z.J. Deng, G. Mortimer, T. Schiller, A. Musumeci, D. Martin, R.F. Minchin, Differential plasma protein binding to metal oxide nanoparticles, *Nanotechnology* 20 (2009).
- [27] G. Maiorano, S. Sabella, B. Sorce, V. Brunetti, M.A. Malvindi, R. Cingolani, P.P. Pompa, Effects of cell culture media on the dynamic formation of protein–nanoparticle complexes and influence on the cellular response, *ACS Nano* 4 (2010) 7481–7491.
- [28] A. Lesniak, A. Campbell, M.P. Monopoli, I. Lynch, A. Salvati, K.A. Dawson, Serum heat inactivation affects protein corona composition and nanoparticle uptake, *Biomaterials* 31 (2010) 9511–9518.
- [29] G. Caracciolo, D. Pozzi, A.L. Capriotti, C. Cavaliere, P. Foglia, H. Amenitsch, A. Laganà, Evolution of the protein corona of lipid gene vectors as a function of plasma concentration, *Langmuir* 27 (2011) 15048–15053.
- [30] A.L. Capriotti, G. Caracciolo, C. Cavaliere, P. Foglia, D. Pozzi, R. Samperi, A. Laganà, Do plasma proteins distinguish between liposomes of varying charge density? *J. Proteomics* 75 (2012) 1924–1932.
- [31] H.W. Gu, P.L. Ho, K.W.T. Tsang, L. Wang, B. Xu, Using biofunctional magnetic nanoparticles to capture vancomycin-resistant Enterococci and other gram-positive bacteria at ultralow concentration, *J. Am. Chem. Soc.* 125 (2003) 15702–15703.
- [32] S. Bucak, D.A. Jones, P.E. Laibinis, T.A. Hatton, Protein separations using colloidal magnetic nanoparticles, *Biotechnol. Progr.* 19 (2003) 477–484.
- [33] A.S. Arbab, G.T. Yocum, H. Kalish, E.K. Jordan, S.A. Anderson, A.Y. Khakoo, E.J. Read, J.A. Frank, Efficient magnetic cell labeling with protamine sulfate complexed to ferumoxides for cellular MRI, *Blood* 104 (2004) 1217–1223.
- [34] S.J.H. Soenen, U. Himmelreich, N. Nuytten, M. De Cuyper, Cytotoxic effects of iron oxide nanoparticles and implications for safety in cell labelling, *Biomaterials* 32 (2011) 195–205.
- [35] Y.F. Zhu, T. Ikoma, N. Hanagata, S. Kaskel, Rattle-type Fe₃O₄@SiO₂ hollow mesoporous spheres as carriers for drug delivery, *Small* 6 (2010) 471–478.
- [36] C. Alexiou, R.J. Schmid, R. Jurgons, M. Kremer, G. Wanner, C. Bergemann, E. Huenges, T. Nawroth, W. Arnold, F.G. Parak, Targeting cancer cells: magnetic nanoparticles as drug carriers, *Eur. Biophys. J.* 35 (2006) 446–450.
- [37] M. Mahmoudi, M.A. Shokrgozar, S. Sardari, M.K. Moghadam, H. Vali, S. Laurent, P. Stroeve, Irreversible changes in protein conformation due to interaction with superparamagnetic iron oxide nanoparticles, *Nanoscale* 3 (2011) 1127–1138.
- [38] L. Lartigue, C. Wilhelm, J. Servais, C. Factor, A. Dencausse, J.-C. Bacri, N. Luciani, F. Gazeau, Nanomagnetic sensing of blood plasma protein interactions with iron oxide nanoparticles: impact on macrophage uptake, *ACS Nano* 6 (2012) 2665–2678.
- [39] A.A. Shemetov, I. Nabiev, A. Sukhanova, Molecular interaction of proteins and peptides with nanoparticles, *ACS Nano* 6 (2012) 4585–4602.
- [40] H. Deng, X. Li, Q. Peng, X. Wang, J. Chen, Y. Li, Monodisperse magnetic single-crystal ferrite microspheres, *Angew. Chem. Int. Ed.* 44 (2005) 2782–2785.
- [41] H. Zhang, K.E. Burnum, M.L. Luna, B.O. Petritis, J.-S. Kim, W.-J. Qian, R.J. Moore, A. Heredia-Langner, B.-J.M. Webb-Robertson, B.D. Thrall, D.G. Camp II, R.D. Smith, J.G. Pounds, T. Liu, Quantitative proteomics analysis of adsorbed plasma proteins classifies nanoparticles with different surface properties and size, *Proteomics* 11 (2011) 4569–4577.
- [42] A.A. Klammer, M.J. MacCoss, Effects of modified digestion schemes on the identification of proteins from complex mixtures, *J. Proteome Res.* 5 (2006) 695–700.
- [43] I.V. Shilov, S.L. Seymour, A.A. Patel, A. Loboda, W.H. Tang, S.P. Keating, C.L. Hunter, L.M. Nuwaysir, D.A. Schaeffer, The paragon algorithm, a next-generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra, *Mol. Cell. Proteomics* 6 (2007) 1638–1655.
- [44] B. Zeeberg, W. Feng, G. Wang, M. Wang, A. Fojo, M. Sunshine, S. Narasimhan, D. Kane, W. Reinhold, S. Lababidi, K. Bussey, J. Riss, J. Barrett, J. Weinstein, Gminer: a resource for biological interpretation of genomic and proteomic data, *Genome Biol.* 4 (2003) R28.
- [45] P.D. Thomas, M.J. Campbell, A. Kejariwal, H. Mi, B. Karlak, R. Daverman, K. Diemer, A. Muruganujan, A. Narechania, Panther: a library of protein families and subfamilies indexed by function, *Genome Res.* 13 (2003) 2129–2141.
- [46] D.H. Lundgren, S.-I. Hwang, L. Wu, D.K. Han, Role of spectral counting in quantitative proteomics, *Expert Rev. Proteomics* 7 (2010) 39–53.
- [47] P.P. Karmali, D. Simberg, Interactions of nanoparticles with plasma proteins: implication on clearance and toxicity of drug delivery systems, *Expert Opin. Drug Deliv.* 8 (2011) 343–357.
- [48] Z.J. Deng, M. Liang, M. Monteiro, I. Toth, R.F. Minchin, Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation, *Nat. Nanotechnol.* 6 (2011) 39–44.
- [49] R.Y. Prasad, K. Wallace, K.M. Daniel, A.H. Tennant, R.M. Zucker, J. Strickland, K. Dreher, A.D. Kligerman, C.F. Blackman, D.M. DeMarini, Effect of treatment media on the agglomeration of titanium dioxide nanoparticles: impact on genotoxicity, cellular interaction, and cell cycle, *ACS Nano* 7 (2013) 1929–1942.

Supporting Information

Nanoparticle Size Matters **in** the Formation of Plasma Protein

Coronas on Fe₃O₄ nanoparticles

Zhengyan Hu^{a, b}, Hongyan Zhang^{a, b}, Yi Zhang^{a, b}, Ren'an Wu^{a, *}, Hanfa Zou^{a, *}

^a CAS Key Laboratory of Separation Science for Analytical Chemistry, National Chromatographic R & A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences (CAS), Dalian 116023, China

^b University of Chinese Academy of Sciences, Beijing 100049, China

*To whom correspondence should be addressed:

Prof. Dr. Ren'an Wu (e-mail: wurenan@dicp.ac.cn)

Tel: +86-411-84379828, Fax: +86-411-84379617;

&

Prof. Dr. Hanfa Zou (e-mail: hanfazou@dicp.ac.cn)

Tel: +86-411-84379610, Fax: +86-411-84379620.

Table S1 Total identified plasma proteins in the protein coronas of the Fe₃O₄ NPs of different sizes and their isoelectric points (pI), molecular weights (MW) and relative protein abundance (blank spaces indicate that the corresponding proteins were not identified in the analysis). **Because** the proteins were detected in more than 2 replicates, the standard deviation was from the mean (n=2 or n = 3).

IP1	Names	pI	MW (kD)	FeO ₄ _30					FeO ₄ _400					FeO ₄ _400				
				MWSpC _1	MWSpC _2	MWSpC _3	Average	SD	MWSpC _1	MWSpC _2	MWSpC _3	Average	SD	MWSpC _1	MWSpC _2	MWSpC _3	Average	SD
IP100021842.1	Apolipoprotein E	5.65	36.13	5.52	5.38	5.52	5.47	0.08	4.27	4.26	4.27	4.27	0.01	8.55	7.66	8.43	8.21	0.48
IP100298971.1	Vitronectin	5.55	54.27	4.09	4.07	3.94	4.03	0.08	3.75	3.73	3.66	3.71	0.05	6.37	6.64	6.79	6.60	0.21
IP100022371.1	Histidine-rich glycoprotein	7.09	59.54	0.26	0.26	0.28	0.27	0.01	3.14	3.17	3.18	3.16	0.02	4.42	5.16	4.59	4.72	0.39
IP100022445.1	Platelet basic protein	9.04	13.89	3.71	3.75	4.18	3.88	0.26	3.00	3.33	3.07	3.14	0.17	4.32	4.55	4.51	4.46	0.12
IP10002246.1	Platelet factor 4	8.93	10.84	8.95	8.36	8.39	8.57	0.33	3.55	3.52	3.64	3.51	0.16	4.09	4.08	3.87	4.01	0.12
IP100022394.2	Complement C1q subcomponent subunit C	8.61	25.76	3.47	2.81	3.28	3.19	0.34	0.82	0.86	0.72	0.80	0.07	4.08	3.96	3.76	3.93	0.16
IP100022392.1	Complement C1q subcomponent subunit A	9.26	26.00	2.30	2.39	2.56	2.42	0.13	0.62	0.76	0.80	0.73	0.09	3.44	3.82	3.58	3.61	0.19
IP100026314.1	Isoform 1 of Geloslin	5.90	85.64	0.61	0.57	0.74	0.64	0.09	2.01	2.19	2.18	2.13	0.10	3.40	3.49	3.41	3.44	0.05
IP100021855.1	Apolipoprotein C-I	8.01	9.33	0.95	0.51	0.69	0.72	0.22	5.83	6.81	6.19	6.28	0.50	3.07	3.50	3.13	3.23	0.23
IP100783987.2	Complement C3 (Fragment)	6.02	187.03	1.99	1.99	2.01	2.00	0.01	1.53	1.59	1.59	1.57	0.04	2.65	2.60	2.70	2.65	0.05
IP100019568.1	Prothrombin (Fragment)	5.64	69.99	2.76	2.56	2.78	2.70	0.13	1.41	1.51	1.47	1.46	0.05	2.47	2.34	2.70	2.51	0.18
IP100745872.2	Serum albumin	5.92	69.32	1.33	1.24	1.25	1.27	0.05	8.83	9.32	8.89	9.01	0.27	2.53	2.52	2.45	2.50	0.04
IP100967849.1	C1QB Uncharacterized protein	8.83	26.44	1.48	1.52	1.71	1.57	0.12		0.23	0.38	0.30	0.11	2.24	2.11	2.31	2.22	0.10
IP100785084.2	IGHG1	8.91	51.05												2.10	2.09	2.10	0.01
IP100876888.1	IGHG1	8.44	51.56	3.21		3.07	3.14	0.10	0.67	0.73	0.76	0.72	0.04	2.00		2.07	2.03	0.05
IP100784817.1	IGHG1	8.64	52.33		2.83	2.92	2.88	0.07						1.89	2.04		1.96	0.11
IP100298828.3	APOH Beta-2-glycoprotein 1	8.34	38.27	1.92	1.79	2.06	1.92	0.13	0.96	0.94	0.93	0.94	0.02	2.07	1.83	1.84	1.91	0.13

IP100969456.1	IGKC Putative uncharacterized protein	8.17	25.79							0.93	0.93	0.81	0.89	0.07	1.85	1.95	1.88	1.89	0.05
IP100887154.2	Complement component 4B	6.89	192.63	0.64	0.63	0.73	0.67	0.05	0.46			0.42	0.44	0.03	1.80	1.81		1.81	0.01
IP100853045.1	IGKC Anti-RND monoclonal T125 kappa light chain	8.69	25.68	3.62	3.55		3.58	0.03							1.75	1.88	1.77	1.80	0.07
IP100021841.1	Apolipoprotein A-I	5.56	30.76	2.21	2.21	2.41	2.28	0.11	9.44	10.11	9.63	9.73	0.35	0.35	1.77	2.03	1.50	1.77	0.27
IP100657670.1	Apolipoprotein C-III variant 1	7.90	12.81	3.37	2.91	2.82	3.04	0.30	6.67	6.15	6.05	6.29	0.34	1.22	1.43	1.78	1.48		0.28
IP100032328.2	Isoform HMW of Kinogen-1	6.33	71.91	1.46	1.35	1.51	1.44	0.08	2.96	2.93	2.84	2.91	0.06	1.41	1.37	1.33	1.37		0.04
IP100032179.3	SERPINC1 Antithrombin-III	6.32	52.57	1.01	1.16	1.18	1.12	0.09	0.69	0.56	0.64	0.63	0.06	1.45	1.32	1.27	1.35		0.09
IP100017696.1	Complement C1s subcomponent	4.86	76.63	0.55	0.63	0.66	0.61	0.06	0.16	0.17	0.18	0.17	0.01	1.21	1.30	1.19	1.23		0.06
IP100021856.3	Apolipoprotein C-II	4.72	11.28	1.05	1.31	1.26	1.21	0.14	1.16	1.10	1.08	1.11	0.04	1.15	1.09	1.20	1.14		0.06
IP100418153.1	IGHM Putative uncharacterized protein	8.39	56.98	1.85	1.59	1.86	1.77	0.15	0.46	0.50	0.54	0.50	0.04	1.02	1.23	1.12	1.12		0.11
IP100021727.1	DKFZp686I15212 C4b-binding protein alpha chain	7.15	66.99	0.28	0.34	0.37	0.33	0.04	0.88	0.92	0.88	0.89	0.02	1.01	1.17	1.14	1.10		0.08
IP100296165.6	C1R cDNA FLJ54471, highly similar to Complement C1r subcomponent	5.96	81.84	0.52	0.53	0.54	0.53	0.01	0.14	0.13	0.16	0.14	0.02	1.04	1.13	1.11	1.09		0.05
IP100399007.7	IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	7.63	46.03											1.04		1.13		1.08	0.06
IP100654888.4	KLKB1 Plasma kallikrein	8.60	71.32	1.10	0.97	1.19	1.09	0.11	0.82	0.88	0.84	0.85	0.03	1.02	1.09	1.12	1.08		0.05
IP100792115.1	CLEC3B Putative uncharacterized protein DKFZp686H17246	4.96	17.78	2.36	2.26	2.21	2.28	0.08	0.95	1.03	0.79	0.92	0.13	1.00	1.15	1.00	1.05		0.09
IP100827875.1	Lambda-chain	7.54	24.64	1.27	1.56	1.73	1.52	0.23	0.67	0.80	0.60	0.69	0.10	1.05	0.97		1.01		0.06
IP100021854.1	Apolipoprotein A-II	6.27	11.17	1.32	1.43	1.39	1.38	0.05	1.87	2.15	1.93	1.98	0.15	0.79	1.04	1.15	0.99		0.18
IP100011264.2	Complement factor	7.39	37.63	0.52	0.50	0.48	0.50	0.02	0.85	0.76	0.87	0.83	0.06	0.83	0.87	0.83	0.84		0.02

IP100478809.4	F5 Coagulation factor V	5.68	251.55	0.12	0.13	0.12	0.12	0.01	0.08	0.08	0.08	0.08	0.00	0.08	0.16	0.15	0.13	0.15	0.01
IP100879931.1	SERPINC1 cDNA FLJ58826, highly similar to Plasma protease C1 inhibitor	6.28	59.45										0.00	0.00	0.00	0.10	0.18	0.14	0.05
IP100656092.1	Isoform F of Proteoglycan 4	9.50	146.38	0.08	0.09	0.13	0.10	0.03	0.11	0.11		0.11	0.00	0.11	0.15	0.12	0.12	0.14	0.02
IP100964840.1	IG1 Uncharacterized protein	5.92	17.67							0.00	0.00	0.00	0.00	0.15	0.12	0.12	0.13	0.02	
IP100010295.1	Carboxypeptidase N catalytic chain	6.86	52.25						0.03	0.05	0.07	0.05	0.02	0.09	0.12	0.14	0.11	0.02	
IP100847179.1	apolipoprotein A-IV precursor	5.28	45.34						0.87	0.89	0.86	0.87	0.01	0.12	0.09		0.11	0.02	
IP100553177.1	SERPINA1 Isoform 1 of Alpha-1-antitrypsin	5.37	46.71	0.09	0.10	0.07	0.09	0.02	0.29	0.32	0.32	0.31	0.01	0.10	0.13	0.08	0.10	0.03	
IP100027462.1	Protein S100-A9	5.71	13.23		0.18	0.20	0.19	0.01			0.00	0.00	0.00	0.10	0.10		0.10	0.00	
IP100019359.4	Keratin, type I cytoskeletal 9	5.14	62.03						0.19	0.19	0.19	0.19	0.00	0.10	0.10	0.10	0.10	0.00	
IP100843942.1	Complement factor H-related protein 5	6.81	64.38	0.17		0.15	0.16	0.02	0.16	0.16	0.13	0.15	0.02	0.07	0.08	0.13	0.10	0.03	
IP100292532.6	Cathelicidin antimicrobial peptide precursor	9.48	19.58	0.06		0.07	0.06	0.00	0.05	0.07	0.04	0.05	0.02	0.07	0.10	0.11	0.09	0.02	
IP100896419.3	Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H4	6.51	103.29						1.54	1.59	1.53	1.56	0.03	0.11	0.07	0.09	0.09	0.02	
IP100879573.1	SERPIND1 Heparin cofactor 2	6.41	57.03		0.08	0.08	0.08	0.00	0.09	0.12	0.11	0.11	0.01	0.06		0.07	0.07	0.01	
IP100641737.1	Haploglobin	6.28	46.69						0.18	0.17	0.25	0.20	0.04	0.10	0.04	0.05	0.06	0.03	
IP100019576.1	F10 Coagulation factor X	5.68	54.70	0.45	0.52	0.51	0.49	0.03	0.05	0.03	0.03	0.03	0.01	0.06	0.06	0.07	0.06	0.00	
IP100925635.1	IGFALS insulin-like growth factor-binding protein complex acid labile subunit isoform 1 precursor	6.48	70.19						0.04	0.05	0.05	0.05	0.01	0.05	0.09	0.05	0.06	0.02	
IP100554504.1	KRIT1 krev interaction trapped protein 1 isoform 2	8.97	78.60								0.00	0.00	0.00	0.06		0.06	0.06	0.00	

IP100021364.1	CFP Propertidin	8.32	51.24	0.09	0.08	0.11	0.10	0.02	0.02	0.01	0.01	0.04	0.04	0.07	0.06	0.05	0.01
IP100025204.1	CD5 antigen-like	5.28	38.06	0.03		0.03	0.03	0.00	0.06	0.07	0.08	0.08	0.07	0.01	0.04	0.05	0.03
IP100914948.1	apolipoprotein L1 isoform c precursor	5.58	42.13	0.07	0.07	0.06	0.07	0.01	0.08	0.10		0.09	0.01	0.06	0.03	0.05	0.02
IP100032311.4	Lipopolysaccharide-binding protein	6.23	53.35	0.02	0.08	0.07	0.06	0.03	0.08	0.08	0.08	0.08	0.08	0.00	0.05	0.05	0.02
IP100032291.2	Complement C5	6.11	188.19	0.06	0.08	0.05	0.06	0.01	0.04	0.03	0.03	0.03	0.03	0.00	0.04	0.04	0.00
IP100947307.1	CP gDNA FLJ58075, highly similar to Centropomus	5.49	108.75							0.00	0.00	0.00	0.00	0.04	0.03	0.04	0.01
IP100967086.1	HGFAC Uncharacterized protein	6.69	71.44						0.03	0.03	0.04	0.03	0.03	0.00	0.05	0.02	0.02
IP100645849.1	ECM1 Isoform 4 of Extracellular matrix protein 1	6.43	63.52						0.01	0.01	0.02	0.01	0.00	0.04	0.02	0.03	0.01
IP100029168.1	Apolipoprotein(a)	5.58	501.00	0.03	0.03	0.03	0.03	0.00		0.00	0.00	0.00	0.00	0.03	0.03	0.03	0.00
IP100478003.3	Alpha-2-macroglobulin	6.03	163.19						0.04	0.05	0.05	0.05	0.00	0.03	0.03	0.03	0.01
IP100298497.3	Fibrinogen beta chain	8.54	55.89	0.06	0.10	0.10	0.09	0.02	0.08	0.04	0.07	0.06	0.02		0.02	0.02	0.00
IP100297550.8	F13A1 Coagulation factor XIII A chain	5.75	83.21							0.00	0.00	0.00	0.00	0.02		0.02	0.00
IP100879709.3	Complement component 6 precursor	6.41	105.68	0.03	0.02	0.05	0.04	0.02	0.07	0.09	0.09	0.08	0.01	0.03		0.02	0.01
IP100215894.1	Isoform LMW of Kininogen-1	6.29	47.85	2.00		2.02	2.01	0.01	4.30	4.26	4.15	4.24	0.07				
IP100944960.1	ITIH4 protein	6.43	103.82	0.04		0.06	0.05	0.02	1.48		1.47	1.48	0.01				
IP100896413.1	inter-alpha (globulin) inhibitor H4 isoform 2 precursor	6.06	99.80						1.48		1.47	1.47	0.00				
IP100784985.1	IGK@IGK@protein	6.14	25.50	3.73		3.69	3.71	0.03	0.97		0.86	0.91	0.08				
IP100550731.2	Putative uncharacterized protein	8.24	26.22	3.57	3.50		3.53	0.03	0.90	0.92		0.91	0.01				
IP100304273.2	Apolipoprotein A-IV	5.28	45.37	0.22		0.24	0.23	0.01	0.87	0.92	0.89	0.89	0.02				
IP100552578.2	SAAI	6.28	13.52						0.57	0.55	0.61	0.58	0.03				
IP100953689.1	Alpha-2-HS-glycoprotein	5.43	39.30	0.48	0.44	0.46	0.46	0.02	0.58	0.49	0.55	0.54	0.04				

IP100007047.1	S100A8 Protein S100-A8	6.51	10.83	0.16	0.22		0.19	0.04											
IP100453473.6	HIST1H4D	11.36	11.36		0.10	0.23	0.17	0.09											
IP100926941.2	KRIT1 Uncharacterized protein	7.23	64.77	0.18	0.15		0.16	0.03											
IP100973032.1	V1-17 protein	5.55	12.28	0.14		0.16	0.15	0.01											
IP100873210.1	FNI 263 kDa protein	5.38	262.39	0.05	0.05	0.05	0.05	0.00											
IP100643034.3	PLTP cDNA FLJ39690 fis, clone SMINT2010639, highly similar to PHOSPHOLIPID TRANSFER PROTEIN	6.19	56.60		0.02	0.06	0.04	0.03											
IP100328609.3	SERPINA4 Kalistatin	7.34	48.51		0.02	0.03	0.03	0.00											
IP100236556.1	Isoform H7 of Myeloperoxidase	9.29	87.19		0.03	0.01	0.02	0.01											
IP100925547.1	Lactoferrin	8.51	77.93		0.02	0.02	0.02	0.00											
IP100607777.2	KALRN kalirin isoform 1	5.72	340.05	0.01	0.01		0.01	0.00											