Quantitative analysis of the protein corona on FePt nanoparticles formed by transferrin binding

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Nanoparticles are finding a rapidly expanding range of applications in research and technology, finally entering our daily life in medical, cosmetic or food products. Their ability to invade all regions of an organism including cells and cellular organelles offers new strategies for medical diagnosis and therapy (nanomedicine), but their safe use requires a deep knowledge about their interactions with biological systems at the molecular level. Upon incorporation, nanoparticles are exposed to biological fluids from which they adsorb proteins and other biomolecules to form a ‘protein corona’. These nanoparticle–protein interactions are still poorly understood and quantitative studies to characterize them remain scarce. Here we have quantitatively analysed the adsorption of human transferrin onto small (radius approx. 5 nm) polymer-coated FePt nanoparticles by using fluorescence correlation spectroscopy. Transferrin binds to the negatively charged nanoparticles with an affinity of approximately 26 μM in a cooperative fashion and forms a monolayer with a thickness of 7 nm. By using confocal fluorescence microscopy, we have observed that the uptake of FePt nanoparticles by HeLa cells is suppressed by the protein corona compared with the bare nanoparticles.

Keywords: protein corona; fluorescence correlation spectroscopy; cellular nanoparticle uptake; confocal microscopy; nanoparticle–protein interactions; nano-biointerface

1. INTRODUCTION

Colloidal inorganic nanoparticles can be synthesized from many different materials in structurally precisely defined ways (Scher *et al.* 2003; Kudera *et al.* 2006), and highly ordered organic ligand shells can be prepared around the inorganic nanoparticle cores to render them colloiddally stable in aqueous solvents (Jadzinsky *et al.* 2007). Such water-soluble nanoparticles hold great promise as powerful tools for many technological and biomedical applications. Because of their small size, nanoparticles may potentially invade all parts of the body, tissues, cells and even subcellular compartments. While this property offers new possibilities for medicine (nanomedicine), for example in targeted drug delivery, it may also pose biological

hazards if nanoparticle incorporation and migration within the body cannot be tightly controlled.

Despite enormous advances in the nanosciences, little is yet known about the detailed mechanisms by which objects on the nanoscale interact with living organisms. Upon incorporation via the lung, gut or skin, nanoparticles become exposed to biological fluids containing dissolved biomolecules, especially proteins. Adsorption of proteins to nanoparticle surfaces is likely to occur and has been reported in several studies (Lees *et al.* 2008).

However, quantitative studies of such ‘protein coronas’ around nanoparticles are still scarce (Cedervall *et al.* 2007a,b; Röcker *et al.* 2009). It seems obvious that interactions of nanoparticles with biological matter crucially depend on the physico-chemical properties of their surfaces. Detailed studies of how these properties govern the biological coating of nanoparticles with biomolecules are required to understand how these interactions can be controlled, so that the potential of

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Figure 1. Structural depictions of transferrin (TF) and the protein corona. (a) Cartoon representation of the (chicken) TF polypeptide chain (protein database entry 1N04). (b) Sketch showing a polymer-coated FePt nanoparticle covered by a monolayer of TF molecules.

nanoparticles can be fully exploited while avoiding possible safety risks (Colvin 2003).

Here, we have employed fluorescence correlation spectroscopy (FCS), a spectroscopic technique with single-molecule sensitivity, to quantitatively monitor protein adsorption onto fluorescent nanoparticles. This technique is based on the analysis of the duration of short bursts of fluorescence light that are emitted by individual particles diffusing through a small observation volume (about 10^{-15} l) formed by tight focusing of a laser spot (Thompson 1991; Maiti *et al.* 1997; Lamb *et al.* 2000; Rigler & Elson 2001; Zemanova *et al.* 2003). Autocorrelation analysis of the fluorescence emission time traces yields a characteristic time scale of diffusion, τ_D , from which the hydrodynamic radius, R_H , can be calculated. Consequently, the method allows the increasing size of the nanoparticles due to protein deposition on their surfaces to be monitored. A noteworthy advantage of FCS is that only minute amounts of sample are needed, i.e. nanomolar concentrations of the nanoparticles in microlitre-sized volumes, whereas other methods for size determination such as dynamic light scattering or gel-permeation chromatography require much larger amounts of sample.

We recently studied the adsorption of human serum albumin (HSA), a major soluble constituent of human blood plasma, on polymer-coated nanoparticles with a radius of approximately 5 nm by using FCS to explore the structure of the protein corona and the binding affinity of the proteins on the surface (Röcker *et al.* 2009). Here, we have extended these studies to another important and abundant human plasma (glyco-)protein, (apo-)transferrin (TF). It has a molecular mass of 80 kDa, and its 679 amino acid polypeptide chain is folded into two globular lobes connected by a three-turn helix (figure 1a). Each lobe can be described as a prolate ellipsoid of $4.2 \times 5 \times 7$ nm³ (Bailey *et al.* 1988).

TF is an iron carrier crucially involved in maintaining iron homeostasis in the body. It is internalized by specific interaction with its cognate receptor on the cell membrane (Ciechanover *et al.* 1983) and has been extensively investigated as a potential ligand to enable specific targeting of therapeutic agents (Qian *et al.* 2002; Widera *et al.* 2003; Yang *et al.* 2005).

In addition, we have addressed the question as to how the protein corona affects nanoparticle uptake by HeLa cells. By using spinning disc confocal microscopy with fluorescence detection in two separate spectral wavelength bands, we have analysed the uptake of FePt nanoparticles in the absence and presence of proteins in the solution.

2. MATERIAL AND METHODS

2.1. Preparation and properties of FePt nanoparticles

FePt nanoparticle cores were synthesized following a previously published protocol (Sun *et al.* 2000). For water solubilization, they were coated with an amphiphilic polymer synthesized from dodecylamine and poly(isobutylene-alt-maleic anhydride) that was labelled with the amino-modified fluorescent dye DY-636 (Dyomics, Jena, Germany; Lin *et al.* 2008). These nanoparticles carry carboxylic acid groups on their surfaces so as to endow them with colloidal stability. A detailed description of the synthesis of these bifunctional, optical and magnetic probes for biomedical imaging has been published (Röcker *et al.* 2009).

2.2. Fluorescence correlation spectroscopy

FCS experiments were performed on a home-built confocal microscope similar in design to those published

earlier by our group (Wiedenmann *et al.* 2002; Schenk *et al.* 2004). The 635 nm excitation light from a diode laser (Chromalase 635, Blue Sky Research, Milpitas, CA, USA) was delivered to the back port of an inverted epifluorescence microscope (Axiovert 200, Carl Zeiss, Göttingen, Germany) by a single-mode optical fibre (QSMJ, OZ Optics, Ottawa, Canada). The emitted light was collected by a water-immersion objective (UPLAPO 60 $\times$ /1.2w, Olympus, Hamburg, Germany), passed through a dichroic mirror (z532/633xr, AHF, Tübingen, Germany) and a band pass filter (HQ 690/80, AHF) and focused onto a 62.5 μ m diameter gradient index fibre (Thorlabs, Newton, NJ, USA), which acted as the confocal pinhole. The fluorescence signal was detected by an avalanche photodiode (SPCM-CD3017, Perkin Elmer, Fremont, CA, USA) and processed by a digital correlator (ALV-5000/E, ALV, Langen, Germany). All FCS measurements were performed with an excitation power of 6–10 μ W at the sample; the laboratory temperature was set to 22°C.

FCS samples were dissolved in phosphate-buffered saline (PBS) buffer (Dulbecco's PBS without Ca^{2+} and Mg^{2+} , PAA Labs, Linz, Austria). Gel-filtration cartridges (Edge BioSystems, Gaithersburg, MD, USA) and centrifugation filters (Pall Nanosep, Ann Arbor, MI, USA) were employed for buffer exchange and removal of aggregates, respectively. For FCS measurements of protein association, samples containing 1–4 nM nanoparticles and protein concentrations ranging from approximately 0.1 to 1000 μ M were prepared by mixing equal volumes of the corresponding stock solutions. Sample solutions were held between two standard cover slips separated by 200 μ m mylar foils, leaving a 3 mm wide channel for the sample solution in the middle.

The stability of the set-up was ensured by control measurements at the beginning and end of each series with a calibration sample consisting of an aqueous solution of 3 nM ATTO 655 (ATTO-TEC, Siegen, Germany) at 22°C. Its diffusion coefficient was measured by two-focus FCS at 25°C (Dertinger *et al.* 2007). Rescaling to 22°C by the known temperature dependence of the viscosity of water yielded $3.93 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$.

In FCS, the fluorescence emission emanating from a small confocal volume within the sample solution is recorded as a function of time, and autocorrelation curves (figure 2) are calculated as

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}, \quad (2.1)$$

where F denotes the fluorescence intensity, δF its fluctuations around the mean, t time, τ correlation time and the angular brackets represent the time average (Rieger *et al.* 2005). Such data are severely compromised by the presence of even minute quantities of large fluorescent aggregates. Because of their brightness and slow diffusion, even a single transit through the observation volume may significantly distort the autocorrelation function. Therefore, 5–15 independent autocorrelation functions were measured (15–60 s each) and averaged. Datasets that were affected by aggregates, as judged by inspection of the autocorrelation

Figure 2. Effect of TF binding on the FCS autocorrelation functions of polymer-coated FePt nanoparticles. Correlation curves measured with 10 μ M TF and 180 μ M TF in the solution are shifted towards longer times when compared with bare nanoparticles. The best-fit model curves are shown as solid lines.

functions, were excluded from further analysis. The experimental data were fitted with autocorrelation functions, $G(\tau)$, using a one-species, three-dimensional diffusion model with an additional exponential reaction term

$$G(\tau) = \frac{1}{\langle N \rangle} \left(1 + \frac{\tau}{\tau_D} \right)^{-1} \left(1 + \left(\frac{r_0}{z_0} \right)^2 \frac{\tau}{\tau_D} \right)^{-1/2} \times (1 + A e^{-k_R \tau}). \quad (2.2)$$

Here, $\langle N \rangle$ denotes the average number of particles in the confocal observation volume assumed to have the shape of a three-dimensional Gaussian. Its radial and axial extensions, r_0 and z_0 , respectively, are determined by measurements with the calibration sample. The experimentally determined diffusion time, τ_D , is converted to the translational diffusion coefficient, D , of the particles by the equation $D = r_0^2 / 4\tau_D$. The exponential term accounts for fast photodynamics of the dye; its parameters, A and k_R , improve the fit of the autocorrelation function but are otherwise irrelevant for the analysis. Hydrodynamic radii were calculated from D by using the Stokes–Einstein relation, $R_H = kT / 6\pi\eta D$, with Boltzmann constant, k , absolute temperature, T , and solvent viscosity, η . Statistical errors are specified as standard deviations in the text; systematic errors introduced by FCS modelling and analysis are estimated to contribute with less than 5 per cent of the absolute R_H values. Changes in viscosity owing to the increasing protein concentration were taken into account by using a linear approximation for the contribution of the solute to the solution viscosity based on the intrinsic viscosities of the proteins studied, i.e. $4.4 \text{ cm}^3 \text{ g}^{-1}$ for transferrin (Armstrong *et al.* 2004) and $4.2 \text{ cm}^3 \text{ g}^{-1}$ for HSA (as specified by the supplier Sigma-Aldrich).

2.3. Confocal imaging

HeLa cells were allowed to attach in eight-well chamber slides (Nunc, Langenselbold, Germany) overnight

(37°C, 96% humidity and 5% CO₂). After serum depletion by 3× washing with PBS, the cell membranes were stained with 0.25 µg ml⁻¹ CellMask Orange (Invitrogen, Carlsbad, CA, USA) in PBS buffer for 5 min. Nanoparticle uptake experiments were performed in PBS and not in cell culture medium containing serum proteins to avoid binding of these proteins to the nanoparticle surface. Nanoparticle solutions of approximately 1 nM in PBS were applied to the cells immediately prior to the measurement. Particle uptake was visualized using a spinning disc confocal laser microscope assembled from individual components including an inverted microscope (Axio Observer, Zeiss, Göttingen, Germany) with environmental control (PECON, Erbach, Germany), four solid-state lasers for excitation (405, 473, 532 and 637 nm), a CSU10 scan head (Yokogawa, Tokyo, Japan), an image splitter unit (OptoSplit II, Cairn Research, Faversham, UK) and an EMCCD camera (DV-887, Andor, Belfast, UK).

Nanoparticle uptake was measured by collecting dual-colour images of confocal cross sections through the cells. The data were analysed using our own software written with Matlab (The MathWorks, Natick, MA, USA). The image in the orange emission channel (membrane stain) was used to generate binary masks for membrane and intracellular space. These masks were subsequently applied to the image in the deep red region from the nanoparticle emission to calculate the average intensity per cell area of the nanoparticles within the cell and at the membrane. The membrane mask was generated by edge detection using a Sobel filter (Duda & Hart 1973) after exclusion of artefacts based on intensity histogram analysis and size filtering. To enforce a closed shape of the intracellular mask even if the membrane contour is discontinuous owing to small regions with insufficient staining (dark pixels), a convex shell was generated and refined using gradient vector flow fields (Xu & Prince 1998). Finally, the binary masks were applied to the particle image and an average background value was subtracted before normalization by the cross-sectional area of each cell as given by the size of the whole-cell mask.

3. RESULTS AND DISCUSSION

3.1. Fluorescence correlation spectroscopy studies of protein binding to FePt nanoparticles

Autocorrelation functions were measured on nanoparticle solutions containing TF concentrations ranging from 400 nM to 200 µM. Analysis with equation (2.2) yields a characteristic time scale of diffusion, τ_D , from which the diffusion coefficient, D , and by means of the Stokes–Einstein relation, the hydrodynamic radius, R_H , is obtained (see §2.2). The resultant (average) R_H values increase with the protein concentration in a step-wise fashion (figure 3a), which clearly shows that the nanoparticles have a limited capacity to bind TF. Saturation was reached slightly above physiological concentrations of TF in human blood plasma (approx. 30 µM). We describe the dependence of the hydrodynamic radius on the number, N , of

Figure 3. Protein adsorption onto polymer-coated FePt nanoparticles measured by FCS. Hydrodynamic radii of the particles are plotted as a function of (a) transferrin and (b) human serum albumin concentration. The data points are averages from three independent series of measurements. The solid lines represent best-fit curves of a cooperative binding model (equation (3.3)) to the data. The dashed lines are Langmuir-binding isotherms fitted to the data points corresponding to the first and last 20 per cent of the transitions.

bound proteins, $R_H(N)$, by the expression (Röcker *et al.* 2009)

$$R_H(N) = R_H(0) \sqrt[3]{1 + cN}, \quad (3.1)$$

where c is a scaling factor and N represents the average number of protein molecules bound to the nanoparticles at a specific protein concentration in the solution. Therefore, at saturation,

$$R_H(N_{\max}) = R_H(0) + \Delta R = R_H(0) \sqrt[3]{1 + cN_{\max}}. \quad (3.2)$$

The number of protein molecules adsorbed to the nanoparticle increases with protein concentration [protein]. This dependence can be modelled by the Hill equation,

$$N = N_{\max} \frac{1}{1 + (K'_D/[\text{protein}])^n}, \quad (3.3)$$

with the maximum number of proteins binding to the nanoparticle, $N_{\max}$, Hill coefficient, n , and dissociation coefficient, K'_D , which gives the concentration of free protein that produces half occupation and quantifies the strength of the protein–nanoparticle interaction. The solid line through the data points (figure 3a) indicates that this binding model affords an excellent fit of the TF data, with $K'_D = 26 \pm 6$ µM, and an overall thickness of the protein shell, $\Delta R = 7.0 \pm 0.4$ nm. The Hill coefficient ($n = 1.7 \pm 0.2$) is markedly above one, which indicates cooperative binding. Langmuir isotherms (non-cooperative binding, $n = 1$) fitted to the first and last 20 per cent of the transition, are also plotted in figure 3a. They are less steep, and their

displacement indicates an effective affinity increase by about a factor of six across the transition. On general grounds, one could expect anti-cooperative behaviour ($n < 1$) owing to crowding and concomitant steric hindrance at higher protein coverage. However, stabilizing interactions between adjacent protein molecules on the nanoparticle surface can overcompensate this effect and result in cooperative binding with $n = 1.7$.

The two lobes of TF occupy an overall volume of approximately $4.2 \times 10 \times 7 \text{ nm}^3$. The observed increase of the nanoparticle radius by 7 nm at saturation as measured by FCS is compatible with a monolayer of protein molecules formed around the nanoparticles (figure 1*b*), so that the $4.2 \times 10 \text{ nm}^2$ face attaches to the nanoparticle. The size increase is also in accordance with the hydrodynamic diameter of 7.4 nm (Armstrong *et al.* 2004). For complete surface coverage, approximately 22 TF molecules adsorb onto each FePt nanoparticle, as estimated from the surface area of a sphere with radius $R_0 + \Delta R/2$. An alternative approach to determine the binding stoichiometry involves the scaling factor c in equations (3.1) and (3.2), which represents the ratio of the molecular volume of protein and bare nanoparticle, $c = V_P/V_0$ (Röcker *et al.* 2009), which allows one to confirm the structural model-based estimation of the number of protein molecules bound to the particle. With $R_H(0) = 5.1 \text{ nm}$, the nanoparticle volume $V_0 = 556 \text{ nm}^3$, and the overall volume occupied by TF, $V_P \approx 4.2 \times 10 \times 7 \text{ nm}^3 = 294 \text{ nm}^3$. With $\Delta R = 7 \text{ nm}$, we arrive at a maximum number of approximately 24 bound TF molecules with equation (3.2).

Our earlier measurements of HSA binding to similar FePt nanoparticles with $R_H = 5.6 \text{ nm}$ (figure 3*b*) yielded $K_D = 5.1 \pm 1.3 \mu\text{M}$, and $\Delta R = 3.3 \pm 0.3 \text{ nm}$ at saturation (Röcker *et al.* 2009). HSA has roughly the form of an equilateral triangular prism (He & Carter 1992; Ferrer *et al.* 2001), with a thickness of 3.15 nm and triangular sides of 8.4 nm. Based on the dimensions of the protein, the ΔR value of 3.3 nm gives strong evidence that approximately 20 HSA molecules form a monolayer around the 5.6 nm FePt nanoparticles covering the surface with their large triangular faces. In contrast to the experiments on TF, the HSA data showed a Hill coefficient ($n = 0.74 \pm 0.1$) below one, which indicates anti-cooperative binding (Röcker *et al.* 2009). Langmuir isotherms (non-cooperative binding, $n = 1$) are also plotted in figure 3*b*; they are steeper and suggest an effective affinity decrease by about a factor of three across the transition. The anti-cooperativity may reflect steric hindrance at high protein coverage, imposing an energetic penalty on further proteins upon binding to the nanoparticle.

Our FCS measurements revealed that both serum proteins adsorb onto FePt nanoparticles with significant affinities in the micromolar range and create single protein monolayers on the nanoparticle surface. Given that the concentrations of TF and HSA in blood serum are approximately 30 and 800 μM , respectively, a nanoparticle with a comparable surface structure in the blood flow is thus expected to be completely coated by these proteins. The measured thicknesses of the protein layers are very different but correlate with the molecular dimensions of the

two proteins, which may suggest that the proteins preserve at least their overall shapes upon binding. The observation that both proteins differ markedly in their binding cooperativity appears noteworthy. The cooperativity results from protein–protein interactions that affect the energetics of protein binding to the nanoparticles. HSA binds more strongly to the FePt nanoparticles than TF and shows an anti-cooperative behaviour. HSA is present in the bloodstream in very high concentrations, where it stores and transports small compounds, e.g. fatty acids. To be colloiddally stable at high concentrations, it should only minimally interact with other protein molecules. Therefore, we believe that HSA binding (figure 3*b*) to the nanoparticles is driven by fairly strong protein–nanoparticle interactions; protein–protein interactions are most likely repulsive, and protein crowding on the surface will naturally lead to anti-cooperative behaviour. By contrast, TF interacts with its specific receptor on the cell surface and may also exhibit significant interactions with other protein molecules. As a matter of fact, TF’s N-terminal lobe was observed to associate with the TF receptor in non-specific ways, possibly owing to the presence of a hydrophobic patch that may make it non-specifically ‘sticky’ (Cheng *et al.* 2004). TF’s affinity to the FePt nanoparticles (26 μM) is five-fold lower than that of HSA (5.1 μM), and therefore it seems reasonable that its cooperative binding results from stabilizing protein–protein interactions that may significantly contribute to forming the protein corona.

3.2. Cellular uptake of FePt nanoparticles

The formation of a protein corona on the nanoparticle surface immediately raises the question as to whether its presence has consequences for nanoparticle uptake by living cells. Endocytosis, the process by which cells take up nanoparticles, actually comprises several distinct active mechanisms that provide cells with the ability to internalize nutrients including macromolecules and larger particles. Whereas phagocytosis is carried out by specialized cell types that internalize larger particles on the micrometre scale, smaller particles up to several hundred nanometres are internalized by pinocytosis, which occurs in almost all cells. Four basic pinocytic mechanisms are being distinguished: macropinocytosis, clathrin- or caveolae-mediated endocytosis, and mechanisms that involve neither clathrin nor caveolae (Conner & Schmid 2003). Particles may also penetrate cells by still other mechanisms (Rothen-Rutishauser *et al.* 2006). The efficiency of nanoparticle incorporation is known to depend on size (Rejman *et al.* 2004; Chithrani *et al.* 2006), shape (Chithrani *et al.* 2006), surface charge (Labhasetwar *et al.* 1998; Arbab *et al.* 2003; Oyewumi *et al.* 2004; Sun *et al.* 2005) and surface chemistry (Labhasetwar *et al.* 1998; Holzapfel *et al.* 2006; Lorenz *et al.* 2006; Nativo *et al.* 2008) of the particles.

Before studying nanoparticle uptake, we assessed the ability of human cancer (HeLa) cells to internalize the two proteins, TF and HSA, without the nanoparticles. HeLa cells were incubated for 1 h with 1 nM fluorescently labelled protein in PBS buffer. The cells were

Figure 4. Uptake of labelled proteins by HeLa cells. Confocal images of HeLa cells after 1 h incubation with 1 nM labelled TF (a) and HSA (b); scale bar: 10 μm . The protein molecules were labelled with AlexaFluor 647; CellMask Orange was used as a membrane stain.

kept at 37°C in the live-cell imaging chamber on the microscope. Confocal images were acquired in two separate colour channels to simultaneously observe both the stained membrane and the red-labelled proteins (figure 4). For TF, which is well known to be internalized by its specific receptor, we have observed strong intracellular fluorescence all over the cell except for the nuclear region (figure 4a). By contrast, fluorescently labelled HSA is barely endocytosed by HeLa cells under otherwise identical conditions (figure 4b).

Upon incubation of HeLa cells with bare 1 nM FePt nanoparticles in PBS, internalization was noticeable as early as 1 min after incubation, and the nanoparticles gradually accumulated in the cells thereafter with a characteristic time scale of approximately 10 min. After 1 h, a large amount of particles is enriched on the cell membrane or incorporated by the cells (figure 5a). Large particle aggregates are visible as bright spots inside the cell and, as in the experiments shown in figure 4, much lower fluorescence intensity is visible in the region of the nucleus, which indicates that the nuclear membrane poses an efficient barrier to penetration by nanoparticles or aggregates. While individual nanoparticles escape detection in these experiments, a detailed analysis reveals that they contribute to an enhanced overall red fluorescence within the cell. We note that, in figure 5, no nanoparticle aggregates are visible outside the cell. This observation is consistent with the FCS data which revealed that our nanoparticle preparations in PBS contain only single nanoparticles. Consequently, the bright aggregates inside the cell are assembled during the uptake process, probably in endosomal compartments.

For studying the effects of the protein corona, we added the respective proteins at a concentration of 100 μM to the 1 nM nanoparticle solutions to achieve essentially complete protein coverage of the particle

surface, as judged from the saturation curves (figure 3). Compared with the bare nanoparticles, their amount on the cell surface and in the intracellular space is substantially reduced in the presence of 100 μM TF (figure 5b). For HSA, the same effect was observed (figure 5c). We analysed the images quantitatively to distinguish between membrane-associated and internalized particles. The results in figure 6 are based on an ensemble of typically 15 cells from two to three independent preparations and show that the total cellular fluorescence is reduced by a factor of approximately 3 due to the presence of 100 μM protein. Here, the intracellular fluorescence from the endocytosed particles is more strongly reduced (approx. factor 4) than the fluorescence from membrane-associated particles (approx. factor 2) for both proteins. The similar suppression of nanoparticle uptake by the protein corona despite the very different uptake behaviour for the proteins themselves (figure 4) is remarkable. For TF, we may argue that the cellular endocytosis machinery is occupied with internalization of the free protein, which is present in 10^5 -fold excess over the 1 nM nanoparticles. Indeed, in uptake experiments with 20 nM labelled TF in the presence of 100 μM unlabelled TF (data not shown), intracellular fluorescence was essentially absent after washing (which was required due to the strongly fluorescent solution). A similar competitive effect has been reported for the uptake of TF-conjugated gold nanoparticles by NPC cells in the presence of free TF (Yang *et al.* 2005). However, we may also argue that TF adsorption to the nanoparticle conceals the receptor-binding interface so that the specific uptake mechanism is blocked. For HSA, we suggest that the protein corona acts as a protective layer, shielding the nanoparticle surface from interactions with the cell membrane. In fact, nanoparticle uptake is known to correlate with the binding affinity to the outer cell membrane (Munoz Javier *et al.* 2006).

Figure 5. Effect of the protein corona on the uptake of FePt nanoparticles by HeLa cells. Confocal images of HeLa cells after 1 h incubation with PBS solutions containing 1 nM FePt nanoparticles only (a) and, in addition, 100 μ M TF (b) and 100 μ M HSA (c); scale bar: 10 μ m. The cell membrane was stained with CellMask Orange.

Figure 6. Quantitative analysis of nanoparticle uptake by HeLa cells within 1 h. Integrated intensity of nanoparticles per cell area after incubation with 1 nM FePt nanoparticles (black bar) and, in addition, 100 μ M TF (dark grey) and 100 μ M HSA (light grey). Typically, 15 cells from two to three independent preparations were analysed.

4. CONCLUSIONS

Both TF and HSA form protein monolayers on the surface of small, carboxy-functionalized FePt nanoparticles. TF shows cooperative binding with 26 μ M affinity, whereas

HSA binds about fivefold more strongly with an anti-cooperative binding isotherm. Protein adsorption to the nanoparticle surface is governed by both protein–nanoparticle and protein–protein interactions. The protein–nanoparticle interaction governs the interaction at low protein occupation and thus the onset of the saturation curve and, by and large, the dissociation coefficient. The surface-mediated protein–protein interactions are reflected in the cooperativity of the binding isotherm, as characterized by the Hill coefficient. The quantitative FCS technique employed here appears powerful for the precise determination of the equilibrium and structural parameters of protein binding to nanoparticles, using only micromolar volumes of nanomolar nanoparticle solutions. We will extend these *in vitro* studies of the protein corona to other relevant proteins (Cedervall *et al.* 2007a,b) and nanoparticles with specifically tailored surface properties, including competition assays. The fluorescence microscopy work presented here has given a clear example that protein adsorption may markedly change the uptake behaviour. These results underscore the importance of advancing our understanding of protein–nanoparticle interactions at the molecular level.

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