



<b>Title</b>	Mapping protein binding sites on the biomolecular corona of nanoparticles
<b>Authors(s)</b>	Kelly, Philip, Åberg, Christoffer, Polo, Ester, O'Connell, Ann, Cookman, Jennifer, Fallon, Jonathan, Krpetic, Zeljka, Dawson, Kenneth A.
<b>Publication date</b>	2015-03-30
<b>Publication information</b>	Kelly, Philip, Christoffer Åberg, Ester Polo, Ann O'Connell, Jennifer Cookman, Jonathan Fallon, Zeljka Krpetic, and Kenneth A. Dawson. "Mapping Protein Binding Sites on the Biomolecular Corona of Nanoparticles." Springer Nature, March 30, 2015. <a href="https://doi.org/10.1038/nnano.2015.47">https://doi.org/10.1038/nnano.2015.47</a> .
<b>Publisher</b>	Springer Nature
<b>Item record/more information</b>	<a href="http://hdl.handle.net/10197/12170">http://hdl.handle.net/10197/12170</a>
<b>Publisher's statement</b>	This is a post-peer-review, pre-copyedit version of an article published in Nature Nanotechnology. The final authenticated version is available online at: <a href="http://dx.doi.org/10.1038/nnano.2015.47">http://dx.doi.org/10.1038/nnano.2015.47</a> .
<b>Publisher's version (DOI)</b>	<a href="https://doi.org/10.1038/nnano.2015.47">10.1038/nnano.2015.47</a>

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# **Mapping protein binding sites on the biomolecular corona of nanoparticles**

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**Nanoparticles in a biological milieu are known to form a sufficiently long lived and well-organised ‘corona’ of biomolecules which confer a biological identity to the particle. Because this nanoparticle-biomolecule complex interacts with cells and biological barriers, potentially engaging with different biological pathways, it is important to clarify the presentation of functional biomolecular motifs at its interface. Here we demonstrate that using antibody-labelled gold nanoparticles, differential centrifugal sedimentation and various imaging techniques, it is possible to identify the spatial location of proteins, their functional motifs and binding sites. We show that for transferrin-coated polystyrene nanoparticles only a minority of adsorbed proteins exhibit functional motifs and that the spatial organisation appears random, overall consistent with a stochastic and irreversible adsorption process. Our methods are applicable to a wide array of nanoparticles and can offer a microscopic molecular description of the biological identity of nanoparticles.**

The biomolecular coronae of nanoparticles exposed to animal-derived fluids<sup>1,2</sup> are known to include proteins<sup>3,4</sup>, lipids<sup>5</sup> and sugars,<sup>6</sup> of which the proteins are the most studied.<sup>4,7-9</sup> Currently, the most tightly nanoparticle-bound biomolecules (so-called hard corona) are stripped from the nanoparticles, and identified *via* mass spectrometry, yielding a macroscopically averaged composition of the biomolecules on the particles.<sup>4,10</sup> This does not address their detailed organisation on the nanoparticle surface, nor if the molecules are presented appropriately to activate specific cellular processes, but is nevertheless suggestive of such possibilities.<sup>11-17</sup>

Few doubt that our future biological understanding of nanomaterials will require a microscopic link between nanoparticle properties, biological milieu, and biological pathways<sup>14,18</sup> and especially a detailed description of the corona structure. For example, of the biomolecules grafted and adsorbed to nanoparticles, only a small fraction will have biologically relevant peptide sequences facing externally, away from the nanoparticle surface, making it difficult to predict how these nanoparticle-corona complexes might further interact with circulating molecules such as antibodies, scaffolding biopolymers, cell-receptors, or key structures in organs such as the liver. Here, using suitably designed immunogold labels and subsequent image processing, we determined the statistical distribution of exposed protein epitopes presented across the nanoparticle corona surface on a particle-by-particle basis, identifying both the epitopes expressed, as well as their organisation in relation to each other.

## Epitope Labelling, Classification and Counting

We began by labelling and counting epitopes in a model system in which transferrin protein (Tf) was adsorbed onto polystyrene particles of diameter 220 nm (determined by electron microscopy; Supplementary Table S3 gives basic physicochemical characterisation).<sup>19,20</sup> Appropriate antibodies were adsorbed onto 5 nm gold nanoparticles.<sup>21,22</sup> For illustration, we used a monoclonal antibody (mTf) for an epitope proximate to the transferrin receptor binding region (Pro142-Pro145;<sup>23,24</sup> see Scheme 1) as well as a polyclonal antibody (pTf). Mapping the epitopes of proteins adsorbed onto nanoparticles requires specifically designed high quality gold nanoparticle-antibody conjugates and their preparation and characterisation is described in the Supplementary Material (see Supplementary Fig. S1 and Supplementary Table S1-S2). Indeed, careful control of the whole system is required for reproducibility, as described in the Supplementary Material (Sections 2-3, Supplementary Fig. S3 and S8).

The first step is to identify the total number of target epitopes. We therefore titrated the immunogold labels against the transferrin-coated polystyrene particles and observed the change in size of the complex using Differential Centrifugal Sedimentation (DCS).<sup>10,25</sup> DCS measures the sedimentation time of the complex under centrifugation (Scheme 2) which can then be related to its mass and size. We present the results in terms of an “apparent diameter” which is calculated on the assumption that the complex has the same uniform density (1.052 g/ml) as the bare particle (see Supplementary Discussion 1 for a more quantitative interpretation of DCS data). Figure 1a illustrates the shift in this apparent diameter between the bare polystyrene particle, the transferrin-coated polystyrene particle complex and, finally, the transferrin-coated polystyrene particle complex binding progressively more immunogold labels. From such distributions one can extract the peak positions and build titration curves showing the extent of binding of the immunogold labels to the transferrin-coated nanoparticles as a function of immunogold concentration (Fig. 1b).

One can rapidly identify the saturation conditions under which all (accessible) surface epitopes have been bound by the immunogold, and use those conditions in imaging experiments to count and categorise the epitopes. Thus, while the DCS shift is not necessarily directly proportional to the amount of antibody bound (Supplementary Discussion 1), the saturation point can still be identified. For instance Fig. 1b

shows that binding of the polyclonal immunogold label (pTf) saturates at 100-150 nM, while for the monoclonal (mTf) this occurs at 250 nM.

This technique also allows us to categorise epitopes by using multiple sequential titrations, thereby simultaneously identifying different (or different classes of) epitopes. In Fig. 1a this is illustrated by the double titration curve in which first the monoclonal immunogold labels were bound to saturation and then the polyclonal labels titrated in, allowing us to detect the more extended class of epitopes. In all cases, gold particles (coated with BSA but without attached antibodies) and immunogold labels using antibodies to a fluorescent protein found in marine algae (with little homology to humans) were used as controls (see Fig. 1b).

Epitopes, having been labelled, can now be imaged and counted. Figure 1c shows images of immunogold labelled nanoparticle-corona complexes acquired using Transmission Electron Microscopy (TEM), representative of the combinations investigated using DCS just discussed. Polystyrene is semi-transparent to the electron beam and the denser immunogold labels are readily distinguished. The number of immunogold labels identified in such images is qualitatively consistent with the DCS data (Fig. 1b); one may also note the near complete absence of labels under control conditions.

Significant numbers of such immunogold-labelled nanoparticles can be imaged and processed using electron microscopy, but for our systems only modest sample sizes are required for the averages.

Furthermore, multiple methods can be used to identify the positions of the immunogold labels, including TEM (Fig. 1c, 2a-b, Supplementary Fig. S17 and S21), Scanning TEM (STEM; Fig. 2c and Supplementary Fig. S23), helium ion microscopy (Supplementary Fig. S19) and Energy-Dispersive X-ray spectroscopy (EDX; Supplementary Fig. S18 and S20).

Thereby, the number of immunogold labels per particle was counted for several points along the titration curves of the monoclonal and polyclonal antibodies. Exemplar electron microscopy images in which immunogold labels were counted are shown in Fig. 2a, while the average numbers are included in Fig. 2b next to the corresponding DCS data points (Supplementary Fig. S9 shows robustness of the averages). The results suggest far fewer identifiable epitopes ( $259 \pm 42$  for the monoclonal antibody) compared to estimated number of proteins (ca. 2600 from Supplementary Fig. S3) covering the surface. We also

determined monoclonal epitope numbers for 80 nm polystyrene nanoparticles at saturation and found  $41 \pm 6$  (Supplementary Fig. S16), suggesting these available epitope numbers scale with nanoparticle surface area in this size regime.

Limitations of the labelling method could arise from immunogold self-blocking during binding because the size of gold and antibody is comparable to the target epitope. The small numbers of specific epitopes available on the nanoparticle in many cases of interest make such effects less likely, but the gold particle size and nature of the antibody chosen could reflect specific circumstance. The fact that (via imaging) absolute numbers of expressed epitopes are counted along the titration curve (Fig. 2b) allows for the construction of binding curves (see Scatchard plots Supplementary Fig. S33). Though qualitative, these indicate if binding is co-operative, or anti co-operative. In the present example the sparseness of target epitopes leads to linear (independent) binding, suggesting that all of those epitopes have been identified.

We stress that each mapping experiment gives limited information on the organization of the protein corona. Additional monoclonal and polyclonal antibodies can shed light on broader aspects of the interface. This assumes prior identification of the adsorbed proteins<sup>1,4</sup> and then, depending on those antibodies that are available, or can be made, the presentation (orientation and position) of a range of epitopes can be determined. Other complimentary surface studies may also be useful. For instance, in the polystyrene-transferrin case, tryptophan fluorescence suggests that the adsorbed transferrin is not denatured (see Supplementary Fig. S30-S32), and not therefore the cause of the relatively small number of these epitopes detected. Rather, our observations are consistent with corona formation by a stochastic process in which there is negligible directional preference for the adsorption, and many of the relevant epitopes are obscured by the particle surface or adjacent protein packing.

### **Spatial Organization of Epitopes on Nanoparticle Corona**

It is believed that multivalency of biomolecules conjugated to the nanoparticles is key in controlling biological interactions.<sup>26</sup> Even though such biomolecular arrangements must be (using current synthetic techniques) statistically defined, rather than fixed positions, in future it will likely become necessary to determine and characterize these spatial arrangements between epitopes as well as those derived from interaction with the biological milieu. Such determinations will play a similar role in the specification and

characterization of nanoparticles for medical and other applications in the same way as structure currently does for small molecules.

To illustrate the idea we determine the spatial distribution of epitopes derived from adsorbed transferrin. While the spatial resolution of the positions is limited by the size and irregularity of antibody-gold attachment, organizations at longer scale contain important information. Thus, STEM imaging allows an approximate identification of the immunogold label positions in all three dimensions (Supplementary Fig. S22-23), and a full reconstruction can thereby be found (Fig. 2c). It is less tedious to identify the positions projected onto the plane defined by the electron beam, and subsequently calculate statistical observables such as the inter-particle distance distribution.<sup>27,28</sup> These distances may subsequently be averaged over polystyrene nanoparticles as a means to characterize the population. Thus, Fig. 2d shows the measured distribution of distances between the polyclonal (pTf) immunogold labels (Supplementary Fig. S29 shows robustness of the averages) compared to a completely random arrangement (see Supplementary Discussion 2). The large overlap suggests that these transferrin epitopes (labelled by polyclonal immunogolds) are (spatially) approximately pairwise distributed randomly. This observation is again consistent with the concept of the corona being formed by random adsorption. In future, patterns of more organized assemblies of ligands or epitopes on nanoparticles can be studied explicitly by this method.

Again, complimentary information can be helpful in building up a picture of the organization of the corona. To probe the more proximate arrangements of binding epitopes and the capacity of the epitopes to bind the receptor we use a soluble model of the homodimer transferrin receptor (TfR, ecto-domain Cys89-Phe760;<sup>23,29</sup> Supplementary Fig. S25). In Fig. 3a we show results for the titration of TfR against polystyrene particles coated with iron-free (*apo*) and iron-complexed (*holo*) transferrin, respectively. When the transferrin coating the polystyrene nanoparticle binds iron (*holo*) there is a clear shift in DCS apparent diameter, suggesting the presence of intact receptor binding epitopes on the transferrin-coated polystyrene particles. Iron-free transferrin (*apo*) acts as a control; the absence of DCS shift shows that the binding with iron-bound transferrin (*holo*) is specific.

Fig. 3b shows DCS data for the fraction of epitopes lost as a consequence of receptor binding, by first binding the receptor and then binding with the monoclonal immunogold labels at saturating concentrations

(determined from Fig. 1b). Only a minority of the epitopes (maximum reduction 18%) is lost due to the prior binding of the receptor. Corresponding imaging experiments (Fig. 3c-d) show similar results. Thus, Fig. 3c shows representative TEM images where the modest reduction in the number of immunogold labels between transferrin-coated polystyrene, respectively, unblocked and blocked by prior binding of the receptor can be visualized qualitatively. Figure 3d shows a quantitative measurement where the number of immunogold labels has been counted for tens of particles under the same conditions, again showing only a minor reduction in extent of immunogold labelling after prior blocking with the receptor. Overall, the results could suggest that a minority of the transferrin epitopes are appropriately arranged suitably for receptor binding.

Other particles are readily studied using the method, for example, 45 nm gold nanoparticles coated with transferrin (Supplementary Fig. S15 shows corresponding titration curves with both monoclonal and polyclonal immunogold labels). However, in this case we observe a degree of denaturation of the adsorbed proteins (Supplementary Fig. S30-31) and we cannot directly conclude that the reduced number of target epitopes is a result solely of random adsorption, though that is likely a significant factor in the outcome.

### **Application to Complex Coronae; Example of Human Plasma**

The mapping of the hard corona of nanoparticles exposed to biological fluids is potentially of considerable interest. For instance, it is believed that the corona peptides expressed on the surface of the nanoparticles circulating in the bloodstream will determine their interactions at cell, organ and immune system level.<sup>1,2</sup> We briefly illustrate the approach using carboxylated polystyrene nanoparticles (diameter 200 nm by electron microscopy) dispersed in human plasma. Hard corona proteins were identified using mass spectrometry (Supplementary Table S4), and two representative examples of the identified proteins, transferrin and immunoglobulin G (IgG), were epitope mapped with suitable immunogold labels. For transferrin we used the same two antibodies (mTf and pTf) as above. We also used two different antibodies against human IgG, a monoclonal which recognises the Fc domain (mIgG) and a polyclonal raised against the full protein (pIgG). Controls for antibody specificity are shown in Supplementary Fig. S12 (DCS titrations of corona formed from human plasma depleted of transferrin) and Supplementary Fig. S13-S14 (antibody cross reactivity dot blots).

Figure 4a shows DCS titration curves of these immunogold labels for the human plasma corona, while Fig. 4b shows the corresponding DCS distributions. Corresponding electron microscopy images are given in Fig. 4c illustrating the number of immunogold particles bound at saturation (250 nM) for each antibody type. As expected, there are far fewer available epitopes of a given type on such mixed coronas (Fig. 4a and c) than for single protein layers (Fig. 1b and c). IgG appears to be more abundant at the hard corona interface than transferrin, in line with the relative composition of the corona from mass spectrometry (Supplementary Table S4) or antibody blots (Supplementary Fig. S14). Possibly significant for the future is the wider variance found (for example in numbers of immunogold labels on nanoparticles, reflected in the broad peaks of Fig. 4b) suggesting that particles exposed to plasma have a greater variety of biological presentations than simple model systems (Fig. 1a). In future systematic mapping of nanoparticle coronae could be undertaken, using immunogold labels derived from the main proteins in the hard corona as identified by mass spectrometry.

## **Conclusions**

It is of considerable interest to understand, and ultimately predict, the outcome of interactions between engineered nanoscale objects with living organisms. In the presence of biological milieu, the formation of a strongly associated and long lived biomolecular boundary layer (hard corona) at the interface between nanoparticle surface and broader biological environment suggests that it is the biomolecular composition and organization at this interface that could determine many of those outcomes. Numerous key processes are driven by the recognition of specific sequences of peptides and other molecules, ultimately leading to receptor-mediated outcomes such as particle retention in organs, intracellular and cross-biological-barrier trafficking. A substantive basis on which to understand these processes will require knowledge of the numbers and arrangements of functional biomolecule motifs on nanoparticles. The present work has demonstrated several different methods for investigating the microscopic organisation (on a particle-by-particle basis) of the corona of transferrin and human plasma coronae formed on polystyrene particles, demonstrating also applicability for more realistic systems.

It is worth noting that analogous issues exist for the biomolecules themselves, and these have been studied over several decades in an effort to understand underlying signalling and trafficking mechanism. The methods that can be applied to determine the structure and surface of peptides on proteins certainly include epitope mapping. Here, however, since every nanoparticle corona is a different, scattering and other methods requiring structural coherence are more limited, suggesting that epitope mapping tools (such as illustrated here), will be a key path of future development.

## Methods

### *Transferrin-Coated Polystyrene Nanoparticle Titrations*

The kinetics of immunogold binding was investigated by DCS showing that steady-state is reached after 30 minutes (Supplementary Fig. S6). For immunogold titrations with transferrin-coated polystyrene nanoparticles (220 nm by TEM) the polystyrene nanoparticle concentration was fixed at 50 µg/ml while the concentrations of immunogold labels were varied between 1-300 nM; samples were incubated for 1 h at room temperature prior to DCS analysis. The samples were injected onto the disc without any further processing as sedimentation through the disc separates the unbound immunogold labels from the polystyrene nanoparticles (Supplementary Fig. S5). The (apparent) diameter reported by the instrument implicitly assumes a uniform density of the full complex. For definiteness, we used a density of 1.052 g/ml, which is the density of the polystyrene particles used as calibration standard. See Supplementary Discussion 1 for a discussion of how to relate this to physical parameters. The change in the apparent diameter of the particles was calculated using Equation 1, where  $d_i$  is the diameter of the protein-coated particles and  $d_s$  is the apparent diameter of the sample post immunogold-labelling.

$$\text{Relative Shift} = d_s - d_i \quad (1)$$

For the double titration, the transferrin-coated polystyrene particles were saturated with monoclonal (mTf) immunogold labels and washed free of excess immunogold by three cycles of centrifugation at 20000 x g for 10 min. These particles were subsequently titrated with polyclonal (pTf) immunogold labels. Analysis of the washed samples indicated that negligible amounts of immunogold were removed from the surface during the washing procedure.

Two controls were used to check the level of nonspecific binding of the immunogold labels. The first was immunogold with no antibodies in which the surface was blocked with BSA (BSA). The second control used an antibody raised against R-phycoerythrin (cAb), carefully chosen to have minimal cross-reactivity with human plasma proteins. Both controls showed minimal binding at the same concentrations where the monoclonal (mTf) and polyclonal (pTf) immunogold labels were already saturated, confirming that the shifts are due to specific binding of the antibodies to their target epitopes (Fig. 1a-b, Supplementary Fig. S11).

#### *Plasma-Derived Polystyrene Corona Titrations*

Carboxylated polystyrene nanoparticles (diameter 200 nm by TEM) were incubated for 1 h at room temperature in 80% v/v plasma at a nanoparticle concentration of 1 mg/ml. These nanoparticles complete with corona were washed six times by centrifuging 250  $\mu$ l at 20000 x g for 10 min and re-dispersing in PBS (137 mM, pH 7.4, with 1 mM EDTA). The biomolecular corona was analysed by SDS-PAGE (Supplementary Fig. S4). Immunogold labelling was carried out by incubating the plasma-coated polystyrene nanoparticles with different immunogold concentrations (1-300 nM) overnight at 4°C, prior to analysis using DCS. These incubation conditions were chosen to reduce nonspecific binding of immunogold labels. The results shown are the average of three measurements carried out on independently prepared samples (Fig. 4).

The same immunogold controls that were used for the transferrin-coated polystyrene nanoparticles were applied to the corona particles. A small shift (20 nm *versus* 40-70 nm for the other immunogold labels) was observed for both these controls when incubated with plasma-coated polystyrene nanoparticles, demonstrating some level of nonspecific interaction (Fig. 4a and Supplementary Fig. S11).

Human plasma was depleted of transferrin following a previously reported method.<sup>29</sup> This was used to form a corona on carboxylated polystyrene nanoparticles, which were subsequently labelled with mTf, pTf, BSA and cAb immunogolds. The results show a reduction in mTf immunogold binding; however an element of nonspecific binding was observed for pTf immunogold, potentially due to cross-reactivity or detection of homologous epitopes in other proteins (Supplementary Fig. S12). This was later confirmed

using immunoblots, both for proteins in solution (Supplementary Fig. S13) and adsorbed onto nanoparticles (Supplementary Fig. S14).

### *Investigation of Transferrin Receptor Interactions*

Transferrin-coated polystyrene particles (50 µg/ml) were incubated with increasing concentrations (1-300 nM) of soluble TfR for 1 h at room temperature. The apparent diameter of these particles was measured by DCS and compared with particles in the absence of any receptor. The results shown are representative of duplicate measurements (Fig. 3a); a single data point at high receptor concentrations 350 nM was measured to ensure saturation had been reached. The dispersion, structure and iron content of *apo* and *holo* transferrin were characterised (Supplementary Fig. S27-S28 and Supplementary Table S5).

Transferrin-coated polystyrene nanoparticles were incubated with different concentrations of TfR and subsequently labelled using monoclonal (mTf) immunogolds at a concentration corresponding to saturation. The samples were analysed by DCS and the percentage reduction in immunogold binding was calculated using Equation (2), where  $d_{block}$  is the apparent diameter of the sample after blocking of the surface with TfR and labelling with monoclonal (mTf) immunogolds, and  $d_{sat}$  is the diameter for the particles saturated with monoclonal immunogold (Fig. 3b). Samples corresponding to medium (50 nM) and high (200 nM) TfR concentrations were imaged by electron microscopy (Fig. 3c) and the number of immunogold particles attached counted (Fig. 3d).

$$\%reduction = 1 - \left( \frac{d_{block}}{d_{sat}} \right) * 100 \quad (2)$$

### *Scanning Transmission Electron Microscopy (STEM) and 3D Reconstruction*

STEM imaging was used to generate a 3D reconstruction of transferrin-coated polystyrene saturated with monoclonal (mTf) immunogold labels (Fig. 2c). A schematic designed to show how particles distributed around a sphere can be viewed in STEM mode is shown in Supplementary Fig. S24. In particular, the difference in focus between particles on the top and the bottom can be achieved through careful analysis of each particle on the surface by comparing bright field, and the two STEM modes. It should be noted that identification around the circumference of the sphere is the most problematic.

### *Protein Structures*

Protein structures were obtained from the protein databank [www.rcsb.org](http://www.rcsb.org)<sup>30</sup> (PDB ID: 1SUV) (Scheme 1).<sup>23</sup> These structures were visualised and labelled using UCSF Chimera.<sup>31</sup>

### **Acknowledgements:**

This work was supported by the Science Foundation Ireland (SFI) Principal Investigator Award (agreement no. 12/IA/1422). P.M.K. gratefully acknowledges the Irish Research Council for funding under the Embark scheme (RS/2011/106). We also acknowledge the small collaborative project NanoTransKinetics (NMP4-2010-EU-US-266737) funded by the European Union Seventh Framework Programme, and the Helmholtz-Gemeinschaft Deutscher Forschungszentren eingetragener Verein Virtual Institute grant Nano Tracking (agreement no. VH-VI-421). This work was based on work supported by NanoSolutions (NMP-L6-2012-309329) and NAMDIATREAM (NAnotechnological toolkit for Multi-modal disease DIAgnostics and TREATment Monitoring; NMP4-LA-2010-246479), both funded by the European Commission 7th Framework Programme, as well as Celtic Alliance for Nanohealth (CAN) funded by European regional development fund - Ireland Wales Territorial Co-operation Programme 2007-2013 (INTERREG 4A). We greatly acknowledge Mass Spectrometry Resource and Conway Core Technologies based at the Conway Institute of Biomolecular and Biomedical Research (University College Dublin) and the Advanced Microscopy Laboratory at the Centre for Research on Adaptive Nanostructures and Nanodevices (Trinity College Dublin) for access to advanced Electron Microscopy facilities

### **Author Contributions**

P.M.K. designed and performed the experiments, collected analysed and interpreted the data, and contributed to the writing of the manuscript. C.Å. constructed a theoretical framework to describe the experimental results and created the pairwise distributions, interpreted the data, and contributed to the writing of the manuscript. E.P. contributed to the biological interaction experiments. A.O'C performed the advanced electron microscopy analysis. J.C. contributed to TEM imaging. J.F. contributed to particle counting and image analysis. Z.K contributed to the design of the experiments, oversaw the acquisition and interpretation of the microscopy images and contributed to the writing of the manuscript. K.A.D conceived and designed the experiments, interpreted the data, and wrote the paper. All authors discussed the results and commented on the manuscript.

### **Additional information**

Supplementary information accompanies this paper at [www.nature.com/naturenanotechnology](http://www.nature.com/naturenanotechnology). Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>.

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### **Figure Legends**

**Scheme 1. Three dimensional structure of transferrin (Tf) and transferrin receptor-transferrin (TfR) complex.** Transferrin receptor (green) binds two transferrin molecules (blue and red). The epitope implicated in transferrin receptor binding (Leu122-Asp125) is highlighted in yellow, whilst the epitope recognised by the monoclonal antibody (Pro142-Pro145) is highlighted in white. It can be observed that spatially these epitopes are close together and are both orientated on the receptor binding side of the protein.

**Scheme 2. Schematic representation of Differential Centrifugal Sedimentation (DCS) to measure immunogold labelling.** The addition of proteins or immunogold labels increases the net density and the hydrodynamic radius of the particles. The net effect for the polystyrene nanoparticles is a reduction in the sedimentation time in a disc centrifuge, causing them to reach the detector faster. The diameter reported by the instrument is incorrect as it assumes the density of the unmodified material, but the measured (apparent) diameter can nevertheless be related to the amount of substance attached (see Supplementary Discussion 1 for details).

**Figure 1. Immunogold labelling and epitope mapping of transferrin-coated 220 nm polystyrene nanoparticles.** **a**, DCS distributions in apparent diameter for transferrin-coated polystyrene particles incubated with increasing concentrations of monoclonal (mTf) and polyclonal (pTf) immunogold labels, respectively. The colour shifts from grey (polystyrene), orange (transferrin coated polystyrene) and then progressively changing in colour to indicate the increase in concentration of label. Arrows also show the direction of increasing concentration, and only the top 40% of the peak is shown for clarity (the full DCS distributions are available in Supplementary Fig. S7). **b**, Corresponding peak positions, relative to the apparent diameter of the transferrin-coated polystyrene nanoparticles, extracted from measurements such as those in panel a. Error bars represent the typical precision of a DCS measurement for this system (see Supplementary Fig. S34); errors at the saturation points for mTf and pTf represent the variation between 3 and 2 independent replicates, respectively. Immunogold labels without antibody (BSA) and with anti-R-Phycoetherin (cAb) serve as controls, showing no significant shifts and thus indicating lack of non-specific interaction with the labels. Monoclonal (mTf) and polyclonal (pTf) immunogold labels show significant shifts and saturation, albeit at different final shifts, indicating a lower abundance of epitopes recognised by mTf. Double refers to transferrin-coated polystyrene particles saturated with monoclonal (mTf) immunogold labels and subsequently titrated with polyclonal (pTf) labels. It is possible to reach the same shift in this way as that achieved by titration of the polyclonal (pTf) alone. Dotted lines and square represent those conditions imaged in panel c. **c**, Representative TEM images for mTf, pTf and the double titration illustrating the immunogold-labelling of the transferrin-coated nanoparticles using these different antibodies. The condition represented by the TEM of the control (cAb) is marked with a square.

**Figure 2. Number and spatial organisation of epitopes exposed on the surface of transferrin-coated 220 nm polystyrene nanoparticles.** **a**, Representative electron microscopy images for low (25 nM), medium (100 nM) and high (250 nM) concentrations of immunogold labels, highlighting that more epitopes are labelled when using the polyclonal (pTf) than the monoclonal (mTf) antibody. **b**, DCS titration curve (from Fig. 1b) together with counted numbers of immunogold labels per particle for both the monoclonal (mTf) and polyclonal (pTf) antibodies. Errors represent the variance in the number of immunogold labels counted by TEM for each point. Dashed line indicates that  $76 \pm 22$  is for the 10 nM concentration. The TEM inset and single data point represent the saturation conditions for 80 nm polystyrene nanoparticles. **c**, Three-dimensional reconstruction of immunogold label positions on a 220 nm transferrin-coated polystyrene particle. The positions of the labels are determined using STEM imaging with a varied focal plane (See Supplementary Figs S22-24 for details). **d**, (Bar chart) Distribution of distances between polyclonal (pTf) immunogold labels (300 nM) on a polystyrene nanoparticle. Immunogold labels were identified in two dimensions from images such as those shown in panel d, the distances between them,  $r$ , calculated and their distribution determined. The result represents the average over 18 polystyrene particles (normalising the distances with the nanoparticle radius,  $R$ ) with error bars representing the standard error of the mean (see Supplementary Figure S29 for convergence). (Solid line) Distribution expected for a completely random arrangement (Supplementary Discussion 2). The close agreement between the experimental results and the random arrangement suggests no clear spatial organisation of the proteins on the nanoparticle surface.

**Figure 3. Interaction of transferrin-coated 220 nm polystyrene nanoparticles with transferrin**

**receptor. a,** Binding of transferrin receptor to the transferrin-coated polystyrene nanoparticles measured using DCS. Error bars represent duplicate independent measurements. The results show that the receptor is capable of interacting with the transferrin molecules on these particles and that this interaction only occurs if the transferrin is iron-bound (*holo*) and not iron free (*apo*). **b,** If the transferrin-coated polystyrene nanoparticles are incubated with TfR and subsequently labelled with the monoclonal (mTf) immunogolds there is a reduction in the number of immunogold labels bound as determined by DCS. Error bars represent the typical precision of the DCS for this system. **c,** Electron microscopy images were taken of samples blocked with medium (50 nM) and high (200 nM) amounts of transferrin receptor prior to immunogold labelling (Supplementary Fig. S26 shows a larger subset of these images). The results again show how prior binding by the receptor blocks immunogold labelling, though only to a minor extent. **d,** The mean number of immunogold labels counted for each of these blocked conditions as a function of number of particles counted shows that the averages for these samples are robust. In addition a clear difference can be observed between the sample which is blocked with a high amount of TfR (light grey) and the particles which have not been blocked (black). Error bars represent the standard deviation over the number of particles sampled.

**Figure 4. Immunogold labeling of plasma-derived corona on 220 nm polystyrene nanoparticles. a,** DCS titration curves for monoclonal and polyclonal immunogold labels against transferrin (mTf and pTf, respectively) and IgG (mIgG and pIgG, respectively). Immunogold labels without antibody (BSA) and with anti-R-Phycoerythrin (cAb) serve as controls, and show some nonspecific binding. Nevertheless, binding of the specific immunogold labels is significantly stronger, with the transferrin polyclonal antibodies exhibiting a stronger binding than IgG to the plasma-derived coronae, in line with the relative abundance of these proteins determined by mass spectrometry (Supplementary Table S4). The error bars represent the standard deviation over 3 independent samples. **b,** Corresponding DCS distributions in apparent diameters. Arrows show direction of increasing concentration and the colour shifts from grey (unlabelled) progressively to indicate the increase in concentration of label. Only the top 40% of the peak is shown for clarity (the full DCS distributions are available in Supplementary Fig. S10). Interestingly, the widths of the distributions are larger than corresponding widths measured for the simple transferrin model system (Fig. 1a). **c,** Representative TEM images for the saturation point (250 nM) of each condition. The number of immunogold labels follow the trend shown in panel a.

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