**cRole of the protein corona derived from human plasma in cellular interactions between nanoporous human serum albumin particles and endothelial cells**

Mikhail V. Zyuzin,[†,#] Yan Yan,[‡,#] Raimo Hartmann,[†] Katelyn T. Gause,[‡] Moritz Nazarenus,[†] Jiwei Cui,[‡,] Frank Caruso[‡,*], Wolfgang J. Parak[†,*]

[†] FachbereichPhysik, Philipps Universität Marburg, 35037 Marburg, Germany

[‡] ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, and the Department of Chemical and Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

Centre for BioNano Interactions, School of Chemistry, University College Dublin, Belfield, Dublin 4, Ireland

 Key Laboratory of Colloid and Interface Chemistry of Ministry of Education, and the School of Chemistry and Chemical Engineering, Shandong University, Jinan, Shandong 250100, China

* corresponding authors: fcaruso@unimelb.edu.au, wolfgang.parak@physik.uni-marburg.de

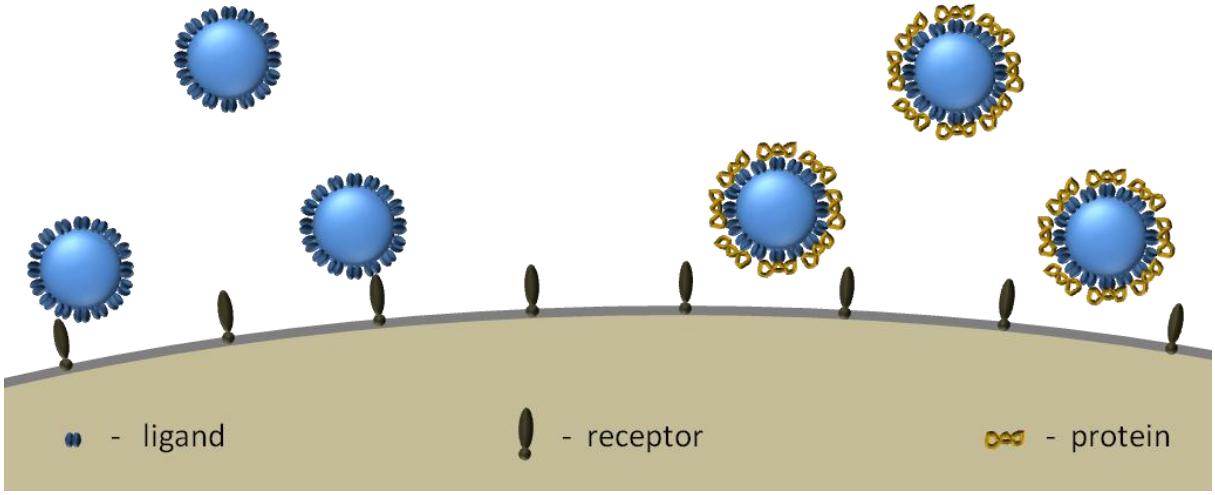
[#] both authors contributed equally to this work

## Abstract

The presence of a protein corona on various synthetic nanomaterials has been shown to strongly influence how they interact with cells. However, it is unclear if the protein corona also exists on protein particles, and if so, its role in particle-cell interactions. In this study, pure human serum albumin (HSA) particles were fabricated via mesoporous silica particle templating. Our data reveal that various serum proteins adsorbed on the particles. The presence of a corona from human plasma was shown to decrease particle binding to cell membrane, increase the residence time of particles in early endosomes, and reduce the amount of internalized particles within the first hours of cell exposure to particles. These findings reveal important information regarding the mechanisms used by vascular endothelial cells to internalize protein based-particulate materials exposed to blood plasma. The ability to control the cellular recognition of these organic particles is expected to aid the advancement of HSA-based materials for intravenous drug delivery.

Key words: Protein particles, Protein corona, cell particle interaction

**TOC**




The protein corona influences the interaction of protein particles with cells

## Introduction

It is known that proteins from cell culture medium bind to the surfaces of particles, forming a so-called protein corona.[1] In the literature, the formed protein interface is classified into a “hard” and “soft” corona, depending on the binding affinity and exchange rates of proteins to particles.[2] Generally, it is considered that a hard corona consists of proteins that adhere to the particle surface strongly enough to survive interactions with the cell membrane and thus are internalized with particles together upon endocytosis. However, another interpretation is available, which rather sees no clear differences between the hard and soft corona, but instead, dissociation constants of proteins.[3,4] For a general discussion, the reader is referred to recent reviews.[5-7] In any case, the protein corona has been shown to significantly affect colloidal stability,[8,9] cellular recognition,[10,11] and cellular/intracellular processing of particles.[12,13] For many different types of particles, ranging from the nano- to the micrometer scale, it has been shown that the presence of serum proteins in the cell culture medium is associated with slower rates of particle internalization.[14-17] Arguably, the effect of the protein corona on the cellular uptake of particles has been studied typically with particles that do not intrinsically comprise proteins, such as inorganic particles.[18] Thus, the existence of a protein corona can be understood to impact the particle properties and thus their interaction with the environment.

Mesoporous silica (MS) particle templating has proven to be a versatile method for engineering polymer particles with tunable size, shape, structure, composition, stiffness, and surface chemistry. Thus, it has been widely applied in the synthesis of polymer particles in drug and gene delivery.[19-24] In the case of particles composed of proteins, the adsorption of proteins from the media onto the surfaces of such particles and their influences on the cellular internalization has to be studied.

Human serum albumin (HSA) particles are already actively used in vitro and in vivo as drug and gene carriers due to the minimal cytotoxicity and good biocompatibility of HSA as a biomaterial building block.[25-30] Another remarkable advantage of HSA particles is the effective enzymatic degradation inside the cells,[31-33] allowing the release of a loaded drug.[34] Successful enabled delivery of different drugs across the blood-brain barrier was shown with HSA particles in several studies. Rapid in vitro release

of drugs from these particles took place within 3 h.[35,36] Particles based on HSA show enhanced uptake in solid tumors by binding of native albumin to a 60-kDa glycoprotein (gp60) receptor located on the cell surface as well as binding to an extracellular matrix glycoprotein, which is expressed in different cancers.[37,38] HSA particles possess a useful feature for targeted delivery of anticancer drugs. Specific albumin-receptor mediated uptake of these organic particles takes place across the vascular endothelium, where albumin binds to the albumin receptor on the surface of endothelial cells.[39]

Taking into account attractive abilities of HSA particles as drug delivery carriers in vivo, the uptake of HSA particles by endothelial cells under plasma-containing and plasma-free conditions is investigated. HSA is also largely abundant in the blood, and thus the corona formed around HSA particles is also likely to be abundant in HSA. A protein corona around HSA particles may be formed driven by reduction in overall free enthalpy, caused either by reduction in enthalpy or increase in entropy.[40] Presence or absence of serum in the culture medium may lead to differences in uptake of particles, explained by the formation of a protein corona. In order to get a direct link to involvement of the protein corona, HSA particles were incubated first with human plasma (HP), then the soft corona was removed by washing, and only the hard corona of HP sticking to the particle surface would remain. As control to these HSA-HP particles plain HSA particles were used. Differences in the intracellular distribution of HSA particles with and without protein corona in caveolae, early endosomes and lysosomes were studied, and their uptake was quantified.

**Results and Discussion**

HSA particles were synthesized with an average core diameter (± standard deviation) of $d_c$ = 758 ± 84 nm, as determined by transmission electron microscopy (TEM; see Figure 1A and Supporting Information Figure SI 1). The mean hydrodynamic diameter of the particles (± standard deviation), as measured in Milli-Q water with dynamic light scattering (DLS), was $d_h$ = 779 ± 98 nm (Figure SI 5). For the discussion about the meaning of $d_c$ and $d_h$ we refer to a previous publication.[41] The hydrodynamic diameter of the HSA particles was also probed in cell medium and in phosphate buffered saline (PBS, pH 7.4), and changes over time were analyzed. In cell medium and PBS, mean hydrodynamic diameters (± standard deviation) of $d_h$ = 589± 47 nm and $d_h$ = 857± 50 nm, respectively, were obtained (Figure SI 5). The increase and decrease in the absolute value of the hydrodynamic diameter of the particles under the presence of salt (PBS) and proteins (supplemented cell medium) can be explained by changes in colloidal stability due to reduction in electrostatic screening, and addition of electrostatic repulsion / steric repulsion, respectively. Within 24 h, there was no significant change in hydrodynamic diameter over time (Figure SI 5), indicating that the particles do not agglomerate when left in solution for 24 h. Additionally, hydrodynamic diameters are of the same order of magnitude as the particle diameter determined by TEM. Thus, the particles are individually dispersed and stable. Fluorescence microscopy was used to confirm the presence of both fluorophores, AF633 and SNARF-1 (see Figure 1B and Figure SI 2 for AF633 and Figure SI 3 for SNARF-1). As expected, fluorescence of SNARF-1-labeled HSA particles depended on pH, shifting the yellow emission under acidic conditions to red emission under neutral and slightly alkaline conditions (Figure SI 4).

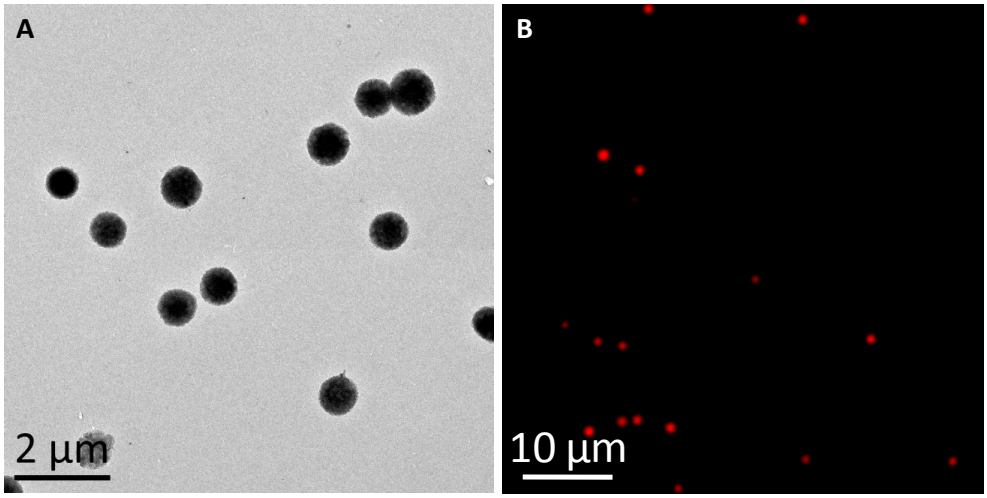



*Figure 1: A. TEM images of HSA particles, B. Fluorescence microscopy image of AF633-labelled HSA particles.*

In case of HSA-HP particles the presence of a hard corona was verified by gel electrophoresis (Figure 2).

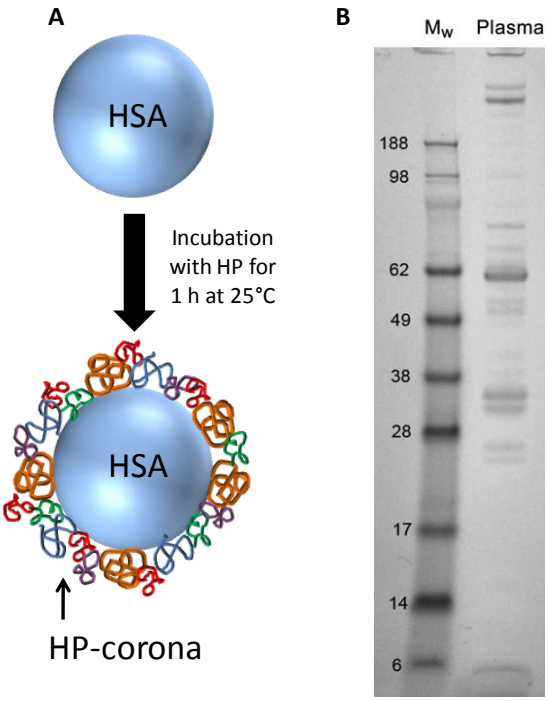



*Figure 2: A. Scheme of human plasma (HP) corona formation, B. Image of a SDS-PAGE gel of the proteins forming the protein corona of HSA-HP particles. A molecular weight ladder is shown in the left lane.*

Literature data indicate that endothelial cells can uptake albumin via caveolae-mediated endocytosis.[42-44] Endocytosis also typically involves endosomes and lysosomes. In order to investigate the internalization pathways of HSA and HSA-HP particles, thus their colocalization with caveolae, early endosomes, and lysosomes was probed. For colocalization experiments, human umbilical vein endothelial cells (HUVECs) were incubated with HSA particles fluorescently-labeled with AlexaFluor 633-succinimidyl ester. After determined times of incubation, cells were fixed and caveolae (Figure SI

6), early endosomes (Figure SI 9), and lysosomes (Figure SI 12) were stained with fluorescently-labeled antibodies. Colocalization of HSA particles with stained organelles was analyzed using Manders' correlation coefficients $m_1$ and $m_2$.[45] In Figure 3, colocalization of HSA particles with caveolae, endosomes, and lysosomes is shown (*i.e.* coefficient $m_1$).

The colocalization data with caveolae suggest that caveolae-mediated endocytosis plays an important role in the internalization of HSA particles, though typically there is not only one, but there may be several pathways of internalization.[46]. Note, that caveolae (which are lipid raft forming protein/lipid mixtures) are not only present close to the membrane of cells, but upon internalization there is fission from the cell membrane, with possible later fusion with early endosomes/cavesomes.[47,48] Thus, the colocalization of particles to caveolae does not provide information about the precise location of particles inside cells. They however clearly indicate involvement of caveolae in particle internalization. The colocalization of particles with caveolar compartments increases over time. Over time, more particles adhere to the cell membrane and therefore colocalized with caveolae. In fact, studies of similar particles have been demonstrated, that particles can reside attached to cells for extended periods of time, before they are endocytosed to acidic vesicles, which can be followed by changes in the local pH around the particles.[49,50] This would be in agreement with the present data, which show colocalization of HSA particles with caveolae, either at the cell membrane, or already after caveolae with the particles have pinched-off the membrane, but before presence in acidic endosomes.

HSA particles were not predominantly found in early endosomes. Since early endosomes of cells can be considered as the transition points for particles between the surface (either on the extracellular outside, or already in the early stage of endocytosis engulfed by caveolae), and lysosomes of cells, particles reside there for the certain period of time and then leave these compartments. Colocalization of the HSA particles with early endosomes, did not clearly raise over time. Thus localization with caveolae seems to be the rate-limiting step, not colocalization with endosomes.

Furthermore, with increasing incubation time, there is significant colocalization with lysosomes, indicating that particles are collected in lysosomal compartments. Colocalization with lysosomes increased over time, indicating that there is accumulation of HSA particles in lysosomes and that this might be the final location of the HSA particles inside cells.

It is known from previous studies that upon endocytosis particles undergo a cascade of transition events, in which they are subsequently present in vesicular compartments with increasing size.[51] In the present study this is indicated by transition from caveolae to early endosomes and then to lysosomes. Particles accumulate at or close to the cell membrane, they reside there and may be engulfed by caveolae. Continuously particles are then transferred to early endosomes. This however is the rate-limiting step. Even if with time more particles are accumulated on or close to the surface of cells, the amount of particles in early endosomes does not significantly increase over time. Early endosomes can be considered as transient transport to lysosomes, where the particles accumulate with time and remain.

HSA-HP particles showed similar colocalization with caveolae (Figure SI 8) and thus, caveolae-mediated endocytosis is likely to also be significantly involved in the uptake of HSA particles by HUVECs.

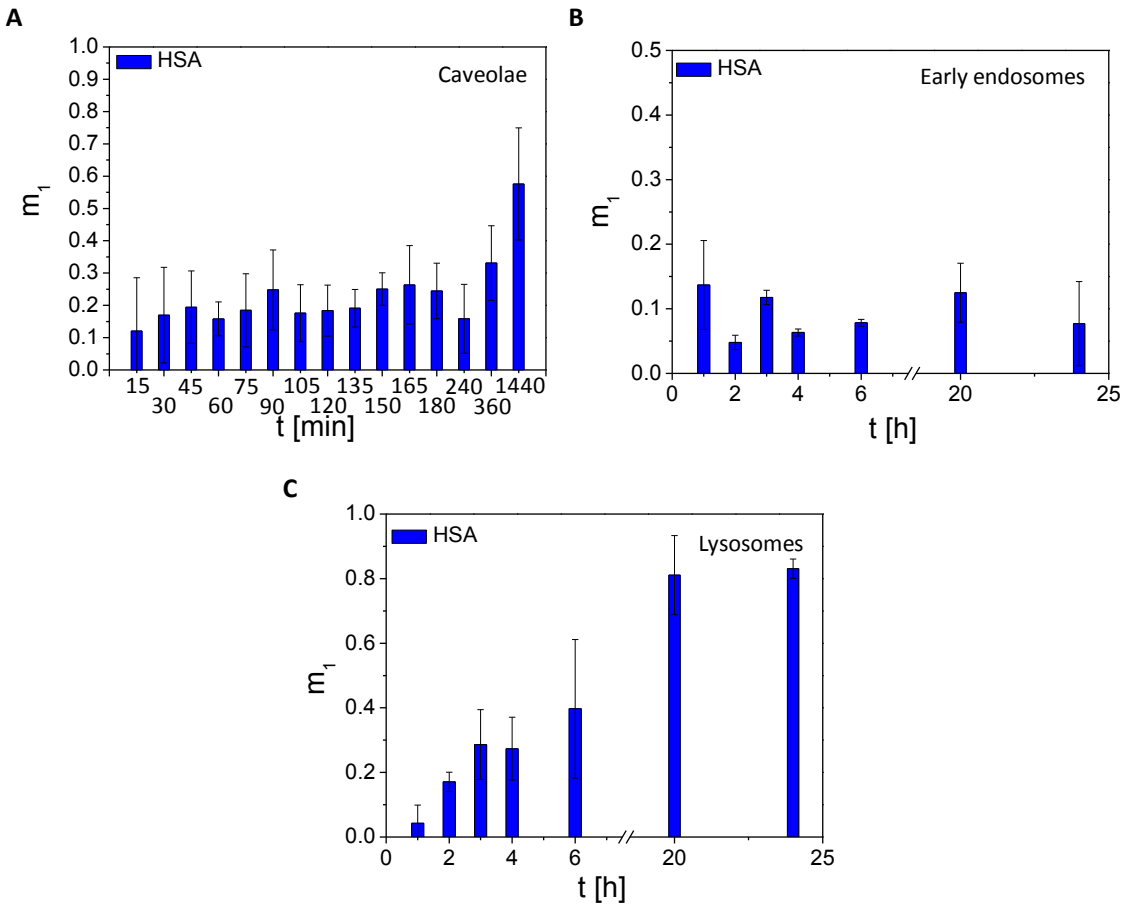



*Figure 3: Colocalization coefficients $m_1$. A. HSA particles colocalized with caveolae of HUVECs, B. HSA particles colocalized with early endosomes of HUVECs, C. HSA particles colocalized with lysosomes of HUVECs. Data are presented as mean values ± standard deviations.*

As indicated, the particles appear to reside in early endosomes only after endocytosis during their transition to lysosomes. To investigate particle transport by early endosomes, the transition times of the particles were determined as the mean time interval in which colocalization between particles and early endosomes was observable (Figure 4).

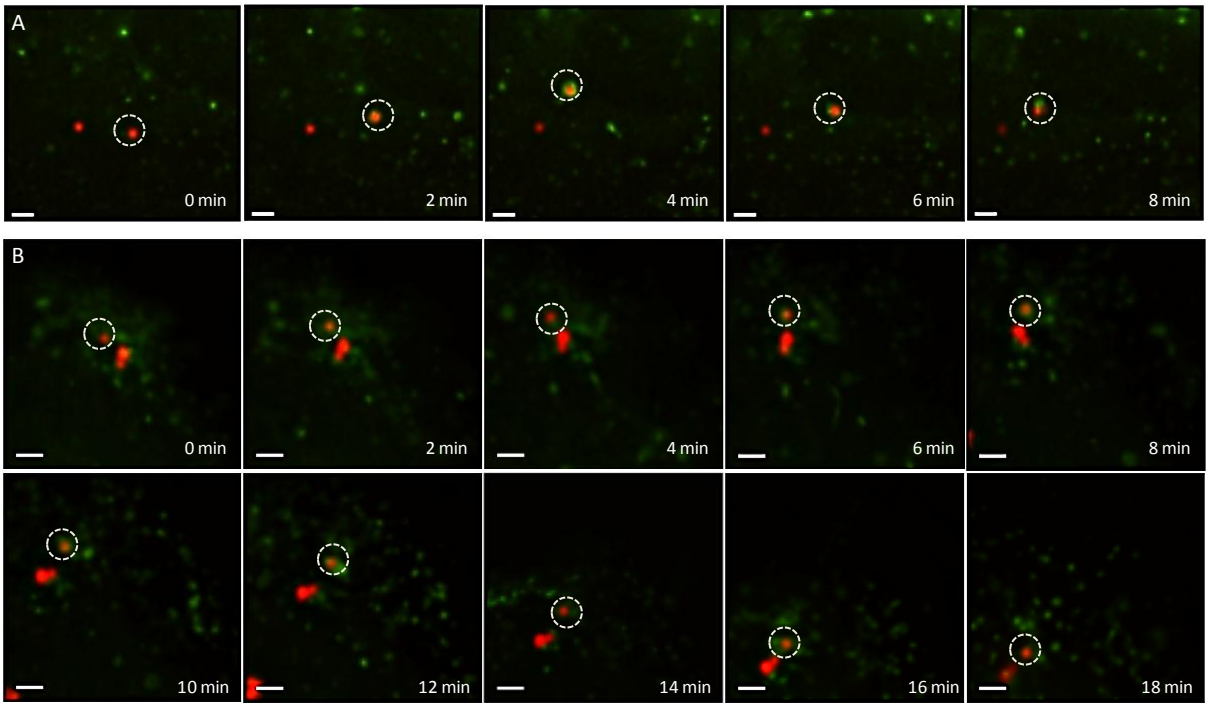



*Figure 4: Fluorescence micrographs (time series) of particles residing in early endosomes. A) HSA particles. B) HSA-HP particles. This experiment was repeated twice; about 40 HSA/HSA-HP particles were analyzed. The scale bar corresponds to 2 µm. Note that the movement of the particles slow enough that a rate of one image per each two min is enough to trace location of the particles.*

For HSA and HSA-HP particles, the mean transition times, calculated as the average ± standard deviation from *ca.* 40 data points (Table SI 2) are $\langle t_{HSA} \rangle = 7.8 \pm 3.4$ min and $\langle t_{HSA\text{-}HP} \rangle = 10.1 \pm 3.7$ min. A t-test showed that the different dwelling times are statistically significant, see the Supporting Information § 2.2. These data suggest that the protein corona formed from incubation in human plasma plays a role on how long particles colocalize with early endosomes. While from these data no precise "kinetics" can be deduced, that fact that HSA-HP particles reside longer than HSA particles in early endosomes, which are considered as transient stage towards transport to lysosomes, that HSA-HP particles are transported to the lysosomes slower than the HSA particles.

The number of cell-associated particles over time was also quantified using fluorescence flow cytometry, whereby the amount of fluorescence originating from each cell was quantified to determine the relative amount of associated particles. Note, that (apart from autofluorescence) cells without internalized particles or cell fragments do not contribute to the fluorescence signal. The fluorescence data show that the number of particles associated with cells increases over time (Figure SI 17). As the fluorescence channels in flow cytometry do not allow lateral resolution, it is not possible to distinguish between particle fluorescence within or outside cells. Non-associated particles (which are fluorescent, but much smaller than a cell) can be excluded by appropriate gating (*i.e.* according to the scattering signal small objects are excluded; Figure SI 15).However, cells with internalized particles and cells with particles adhered to the cell membrane cannot be distinguished, as both have similar fluorescence and scattering signal.[49,52] As internalized particles in lysosomes reside in the acidic environment, their intracellular presence can be determined using the pH-sensitive SNARF-1 fluorescent-label.[49,53] According to previous studies using SNARF-1-labelled particles, fluorescent particles are transported to and accumulate inside acidic intracellular organelles, such as late endosomes/lysosomes (Figure SI 22). Time-dependent cell-associated fluorescence in the yellow and

red spectral region, originating from particles in acidic and alkaline environment, respectively, is shown in Figure 5.

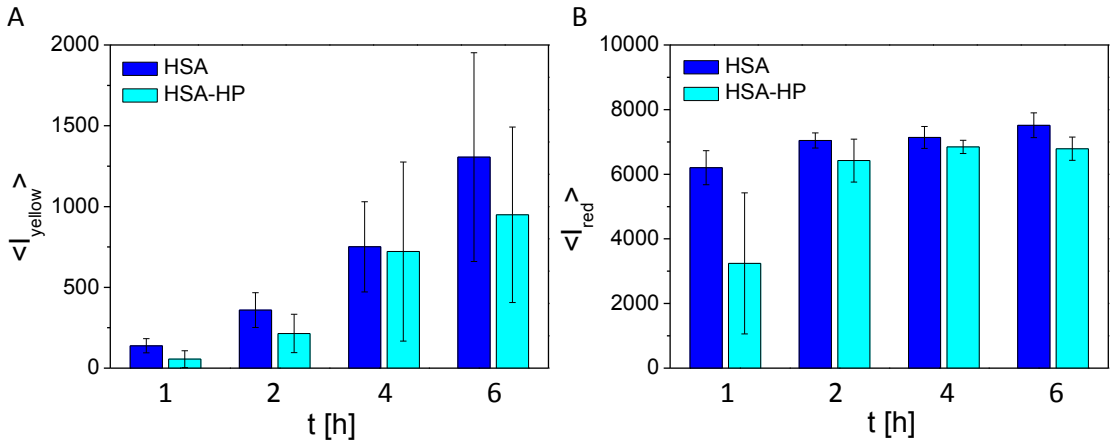



*Figure 5: Mean yellow fluorescence intensity A. <$I_{yellow}$>, B. <$I_{red}$>, originating from HUVECs after incubation with SNARF-1-labelled HSA and HSA-HP particles for different time periods. Data are presented as mean values ± standard deviations.*

From the data shown in Figure 5, one can derive two findings. First, up to incubation times of 6 h, HSA particles are internalized by HUVECs to a higher amount than HSA-HP particles, as indicated by the temporal increase of yellow fluorescence originating from particles in acidic intracellular vesicles. This is compatible with the findings of the colocalization studies. As HSA-HP particles have longer transition times in early endosomes their transport to lysosomes seems slower. Second, while HSA particles seem to fully attach to the outer cell membrane after 1 h, HSA-HP particles seem to bind slower, as indicated by the red fluorescence originating from particles in the neutral/slightly alkaline cell medium. While the data are not quantitative but rather indicative, they point out the significant differences in uptake behavior between HSA and HSA-HP particles.

The obtained results suggest that protein corona derived from human plasma affects interaction of HSA particles with cells; in particular, it slows down the internalization of these particles with endothelial cells. HSA particles are often used for specific (receptor-mediated) uptake, which is activated by the binding of albumin to the specific receptor (gp60) located in caveolae of endothelial cells.[54,55] In the case of HSA-HP particles, proteins probably "shield" the original material of particles. That is, the protein corona establishes a barrier that screens interactions between ligands and their receptors on the cell plasma membrane and thus internalization is less efficient.

**Conclusions**

It is well studied that nanoparticles adsorb surrounding proteins to form a protein corona. Such an interface has been shown to be critical for defining nanoparticle-cell interactions. This phenomenon has been widely observed in many type of particles, mostly synthetic particles. The current study shows that a protein corona also forms on pure protein particles. In particular, even when the protein comprising the particle is also the major component of the surrounding milieu, the protein corona concept is still valid. Furthermore, our data has shown that the presence of a protein corona also plays an important role in mediating protein particle-cell interactions. The presence of additional proteins

derived from human plasma can slow down particle binding to the extracellular membrane, increase the dwelling time in early endosomes, and reduce the amount of internalized particles within the first hours of cell exposure to particles. Thus, it has been shown that before bringing such particle systems into a clinical use, it is important to perform preliminary tests dedicated to the influence of the environment in vivo on particle behavior. This should aid the prediction of possible effects of such materials in vitro and in vivo.

**Materials and Methods**

MS particles with an average size of 1 µm were prepared according to a previously reported method.[56] HSA particles were produced using MS particles as template, which was later removed.[57] The HSA particles could be labeled by standard bioconjugate chemistry with AlexaFluor633 dye (AF633), or pH-sensitive seminaphtharhodafluor (SNARF-1). Details can be found in the Supporting Information § 1.3. HSA particles were characterized by transmission electron microscopy (TEM), fluorescence microscopy, and dynamic light scattering (DLS). For the formation of a hard corona, HSA particles were incubated in human plasma (HP) (*i.e.*, HSA-HP particles) and after rinsing, the existence of a corona was verified by gel electrophoresis. Cells were incubated with fluorescently-labeled HSA and HSA-HP particles. Colocalization with caveolae, early endosomes, and lysosomes was investigated by immunostaining. The uptake of particles by cells was studied using flow cytometry. SNARF-1-labeled particles attached to the outer cell membrane could be distinguished from endocytozed particles due to their changes in fluorescence properties upon internalization.[49]

**Supporting Information Available:** Detailed protocols and raw data are available free of charge *via* the Internet at http://pubs.acs.org.

**Acknowledgements**

This work has been supported by DAAD (grant 56265460 to FC and WJP) and the German Research Society (DFG grant PA 794/25-1 to WJP). This research was also conducted and funded by the Australian Research Council (ARC) Centre of Excellence in Convergent Bio-Nano Science and Technology (Project Number CE140100036) and funded by the ARC under the Australian Laureate Fellowship (FL120100030) scheme.

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Zyuzin, MV;Yan, Y;Hartmann, R;Gause, KT;Nazarenus, M;Cui, J;Caruso, F;Parak, WJ

Title:
Role of the Protein Corona Derived from Human Plasma in Cellular Interactions between Nanoporous Human Serum Albumin Particles and Endothelial Cells

Date:
2017-08

Citation:
Zyuzin, M. V., Yan, Y., Hartmann, R., Gause, K. T., Nazarenus, M., Cui, J., Caruso, F. & Parak, W. J. (2017). Role of the Protein Corona Derived from Human Plasma in Cellular Interactions between Nanoporous Human Serum Albumin Particles and Endothelial Cells. BIOCONJUGATE CHEMISTRY, 28 (8), pp.2062-2068. https://doi.org/10.1021/acs.bioconjchem.7b00231.

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