EUROPEAN SYNCHROTRON RADIATION FACILITY

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Experiment Report Form

	Experiment title: Probing the protein corona around colloidal metal nanoparticles in cellular environments using nanofocused X-ray synchrotron radiation	**Experiment number**: MA-4868
Beamline: ID16A	**Date of experiment**: from: 02/07/2021 to: 08/07/2021	**Date of report**: 16/11/2021
Shifts: 12	**Local contact(s)**: **Dmitry Karpov**	*Received at ESRF:*

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Report:

1. Abstract: Inorganic nanoparticles interact with biomolecules, such as proteins, after being exposed into cellular environment. This so-called protein corona is of great interest in current science since it affects the biophysical properties and the cellular interactions of the initial nanoparticles.[1] Recently published work on quantum dots surrounded by fluorescence-labeled proteins shows the separately degradation of the protein corona inside cells.[2] To overcome common problems with fluorescent probes, like possible labeling detachment, quenching and a spatial resolution in a scale of several hundred nanometers, we've acquired X-Ray fluorescence (XRF) maps of mammalian cells treated with three different systems of gold nanoparticles (AuNPs) and gadolinium-labeled proteins at ID16A.

2. Experimental Details: Gold nanoparticles with a size of 12 nm were synthesized by an adjusted Turkevich method, using citrate buffer to reduce and stabilize gold(III)chloride.[3] Gd-labeled proteins were either covalently bound to the nanoparticles surfaces (**A**), adsorbed by forming a protein corona (**B**) or a ligand exchange with polyethylene glycol (PEG) was conducted prior forming a protein corona (**C**). The model proteins, consensus tetratrico peptide repeat protein (CTPR), bearing gadolinium nanoclusters were designed, expressed and kindly provided by collaborators.[4] The resulting hybrid particles were characterized by TEM, UV-Vis absorption spectroscopy, ICP-MS and gel electrophoresis prior cellular treatment.
Mouse embryonic fibroblasts (3T3-cells) were seeded on poly-L-lysine pre-treated silicon nitride windows and incubated in appropriate growth medium overnight (37°C, 5% CO_2). Then, the cells were treated with AuNPs ($c_{Au} \approx 10$ µg/mL) surrounded by a protein corona of Gd labeled CTPR. Following 24 hours of incubating the medium was removed again, membranes were washed in 1 mL PBS, 1 mL fresh growth medium added, and cells were let to recover in particle free medium for 0, 30, 60 or 120 minutes. Then, the membranes were taken out, immersed in 150 mM ammonium-acetate buffer (pH 7.1), blotted with filter paper and manually plunge-freezed in liquid ethane.[5] The freeze-dried membranes were transferred to holders that were 3D-printed inhouse for storage and transport, and kept under cryogenic conditions in liquid nitrogen till measurement.
XRF maps at ID16A were acquired under cryogenic conditions using two six elements silicon drift diode detectors. The beam energy was set to 17 keV, focused at 50 x 50 nm^2, $1.55*10^{11}$ phs/s. The fine cellular mapping was derived by a step size of 70 nm with 50 ms dwell time.

3. Results: XRF imaging at ID16A allowed us to visualize and quantify different elements of interest inside cryofixed mammalian cells (Fig. 1). AuNPs surrounded by covalently bound proteins (**A**) suffer from intense aggregation after 24 h in cellular environment, as seen in our XRF maps. Moreover, the ratio between gold and gadolinium decreased by a factor of 10 within these 24 hours compared to the initial elemental composition of the particles. This suggested the disintegration of the ordered protein corona, probably during sample preparation. In case of citrate stabilized AuNPs surrounded by a hard protein corona (**B**), similar aggregation

behavior was observed after 24 h in cellular environment. The gold to gadolinium ratio increased by a factor of two within the first 24 h treatment. Again, these results could indicate the dynamic exchange of secreted cellular proteins with the preformed Gd labeled CTPR protein forming the initial corona.

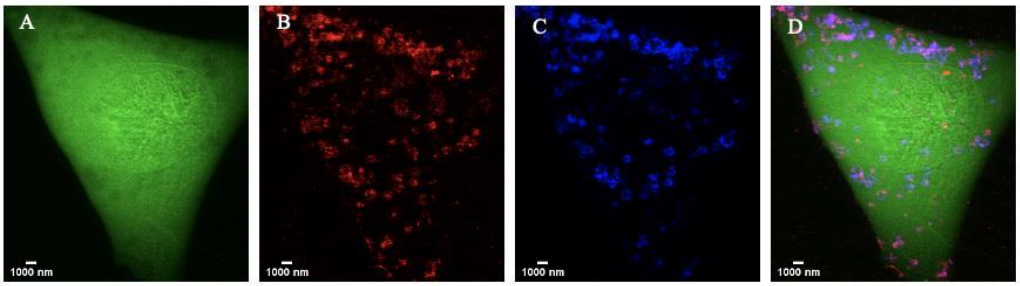

Fig. 1: 3T3 cell treated with pegylated AuNPs surrounded by Gd labeled CTPR protein, 30 min recovery time. A) signal originated from potassium, B) gold signal, C) gadolinium signal, D) merged channels.

Overall, the XRF maps of cells treated with pegylated AuNPs surrounded by a labeled protein corona showed the presence of vesicular transport of the hybrid particles, indicated by accumulation of gold and gadolinium in nearly spherical structures in sizes smaller then 1 µm (compare Fig. 1B and 1C). The analysis was focused on colocalization as well as elemental amounts of gold and gadolinium. The Pearsons' correlation coefficient (PCC) slightly decreased in the mean value, starting within the first 30 minutes, implying a decomposition of the protein corona (Fig. 2A). Such result would prove the hypothesis of a time dependent degradation process after the particles are taken up by the cells. However, these variations were not statistically significant, proved by students t-test ($p > 0.05$). We also showed that the correlation of both elements was dependent on the localization inside the cells. The particles were taken up at the outer areas, i.e., cell membrane and transported inside vesicles in direction of the nuclear area. During this process the PCC decreased (Fig. 2B), whereas the ratio Au to Gd increased (Fig. 2C). Both findings support the hypothesis of the time dependent intracellular protein corona disintegration.

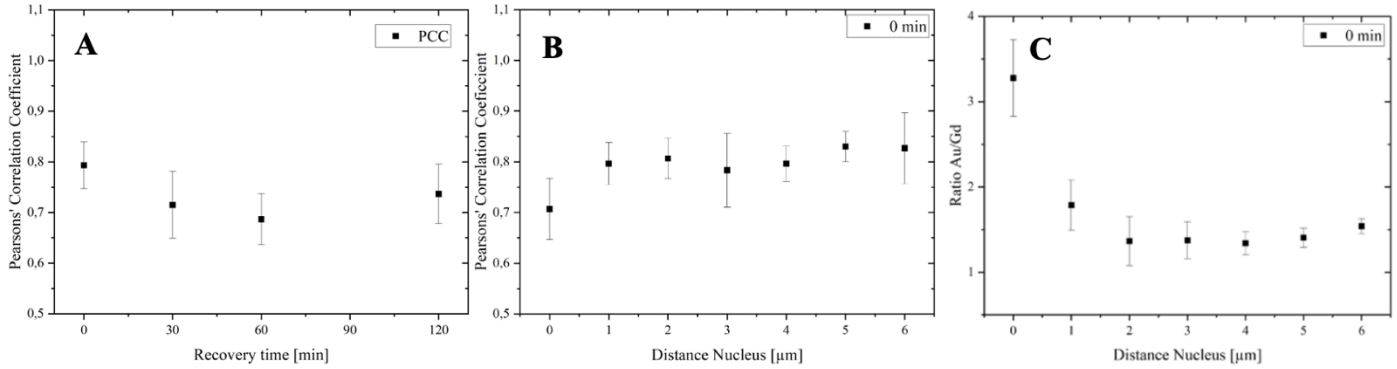

Fig. 2: Time dependent PCC of whole cell area (A), PCC (B) and elemental ratio (C) at one time point dependent on distance to nuclear area.

4. Conclusion and future work: XRF mapping at ID16A shows the possibility of intracellular probing small nanoparticle protein interactions with sub-cellular resolution at relatively low elemental concentrations. The overall analysis supports the hypothesis of the time dependent protein corona disintegration after cellular internalization. However, the small population of these studies, i.e., three cells per condition, suffer from a huge intrinsic cellular variability and thus not allow to determine proper statistic. Currently we are establishing a label for the nanoparticle surrounding ligand to probe the individual intracellular disintegration of gold nanoparticles, stabilizing ligands and protein corona at the same time, which is crucial to understand for future nanomedical applications. Building on the results obtained at ID16A, we are confident to probe our improved system in future XRF mapping and/or tomography experiments. Thereby we will focus on shorter time points and more cells per condition, which is feasible by faster acquisition times due ongoing upgrades at synchrotron facilities.

5. References

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