Protein Interactions

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Superparamagnetic Nanoparticles as a Powerful Systems Biology Characterization Tool in the Physiological Context**

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Recently, functionalized superparamagnetic iron oxide nanoparticles (SPIONs) have been utilized for protein separation[1] and therapeutic delivery of DNA and drugs.[2] The development of new methods and tools for the targeting and identification of specific biomolecular interactions within living systems is of great interest in the fields of systems biology, target and drug identification, drug delivery, and diagnostics.

Magnetic separation of organelles and proteins from complex whole-cell lysates allows enrichment and elucidation of intracellular interaction partners for a specific immobilized protein or peptide on the surface of SPIONs. This was previously shown for streptavidin-coated magnetic iron oxide beads.[3,4] However, certain binding processes can be energy-dependent and therefore only occur within the intact cell. Our attempt to target and isolate mitochondria by using multifunctionalized nanoparticles relies on 1) efficient cellular uptake, 2) formation of an affinity complex between the multifunctional particles and the organelle import machinery, and 3) efficient cell disruption and magnetic separation.

Polymer and peptide surface derivatization (Figure 1a) of aminopropyltriethoxysilane (APS)-coated SPIONs (APS-SPIONs) was accomplished in a fixed-bed reactor by previously described methods.[5] Coupling of the peptide(s) on the magnetic particles was accomplished with a heterobifunctional polyethylene glycol (PEG) cross-linker. Three different

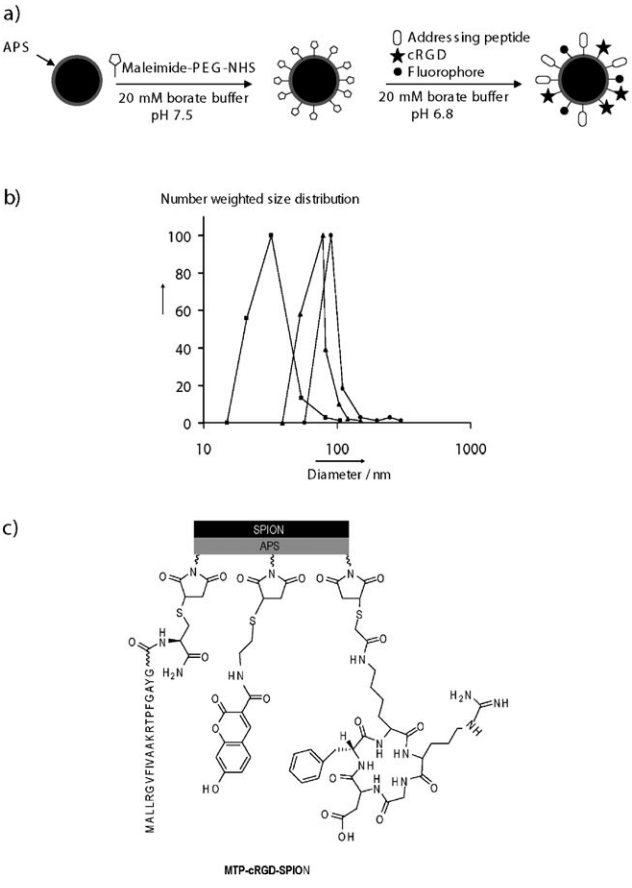

Figure 1. Surface derivatization and characterization of mitochondrial targeted APS-SPIONs. a) Two-step surface functionalization of APS-SPIONs. b) Photon correlation spectroscopy number-weighted hydrodynamic size distribution of subsequently functionalized SPIONs. ■ APS-SPIONs, ▲ MTP-SPIONs, ● MTP-cRGD-SPIONs. c) Addressing peptide bound on the surface of PEG-APS-SPIONs.

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nanoparticle groups were manufactured: 7-hydroxycoumarin-labeled SPIONs, fluorescently labeled mitochondrial targeting peptide derivatized SPIONs (MTP-SPIONs), and cyclic RGD (cRGD, containing the Arg–Gly–Asp motif) in parallel with the fluorescently labeled MTP-SPIONs (MTP-cRGD-SPIONs). The cyclic pentapeptide cRGD is well known to enhance receptor-specific uptake and targeted gene delivery when the complex sizes are on the < 100 nm scale.[6]

The MTP we employed was an N-terminal 20-residue sequence from the protein mitochondrial 3-oxoacyl-coenzyme A thiolase containing the sequence MALLRGVFIVAAKRTPFGAYGC, where the GC residues were engineered into the peptide to allow for a spacer (glycine) and a C-terminal cysteine for facile conjugation to the functionalized SPIONs. Reaction conditions were optimized in terms of concentration, incubation time, and recirculation cycles so that the final compounds showed sufficient colloidal stability and did not show any changes in zeta potential or particle size distribution upon further recirculation. As a consequence, 99% of the addressing peptide was bound on the surface of the PEG-APS-SPIONs (Figure 1c).[5]

We observed the initial zeta potential of (33.3 ± 0.9) mV for APS-SPIONs to decrease significantly after coupling to the heterobifunctional PEG to (-20.8 ± 0.6) mV. Subsequent peptide derivatization resulted in a further change of zeta potentials to (-10.6 ± 0.4), (-9.4 ± 0.4), and (-13.9 ± 0.4) mV for cRGD-SPIONs, MTP-SPIONs, and MTP-cRGD-SPIONs, respectively.

The mean particle size increased from (23.8 ± 0.6) nm for APS-SPIONs to (64.5 ± 0.6) nm for MTP-SPIONs and (76.9 ± 0.5) nm for MTP-cRGD-SPIONs (Figure 1b). HeLa cells were cultured and incubated in the presence of the three SPION preparations mentioned above (Figure 2a). The iron content in control samples without SPIONs measured below the detection limit of the assay. After 2 hours, APS-SPIONs were extensively taken up into the cells, whereas the peptide-derivatized SPIONs showed significantly lower cellular uptake (maximum p value $= 0.006$, two-sample t test between APS-SPIONs and cRGD-SPIONs). Internalization of the MTP-cRGD-SPIONs increased after 6 hours, at which time the differences between the four groups were most prominent and barely changed up to 10 hours. After incubation for 24 hours, the uptake of functionalized SPIONs by the HeLa cells was similar in all three groups, which indicated that phagocytosis had become the predominant mechanism of particle uptake, potentially because of agglomeration.[7]

Confocal microscopy images are shown in Figure 2b. Coumarin (left-hand column) shows the internalized differently derivatized SPIONs, whereas Mitotracker (central column) shows the stained mitochondria in live cells. Overlay (right-hand column) shows the superimposition of the two previous columns. MTP-cRGD-SPIONs are nicely co-localized at mitochondria, whereas cRGD-SPIONs show reduced co-localization. In fact, cRGD-SPIONs, once internalized, are detected in the cytoplasm, nucleus, and partly mitochondria. MTP-SPIONs were only poorly internalized.

After incubation the cultures were disintegrated and the nanoparticles were recovered by magnetic separation from the whole-cell lysate. Nonbinding and only weakly binding

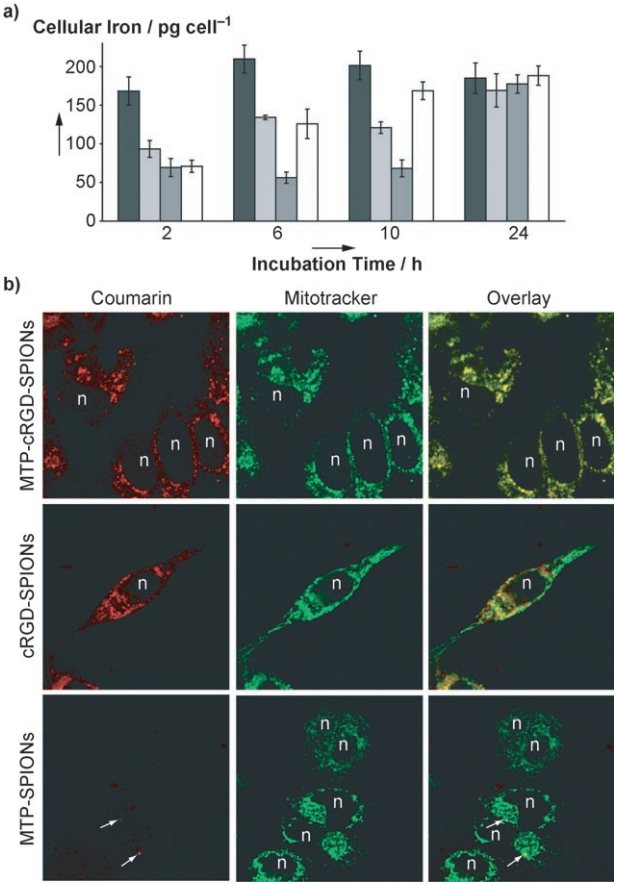

Figure 2. In vitro uptake of SPIONs and co-localization with mitochondria. a) Cellular uptake of functionalized SPIONs over time; black: APS-SPIONs, light gray: cRGD-SPIONs, dark gray: MTP-SPIONs, white: MTP-cRGD-SPIONs. b) Confocal microscopy images of internalized SPIONs after incubation for 10 h: MTP-cRGD-SPIONs (top), cRGD-SPIONs (middle), MTP-SPIONs (bottom). The fluorescent signal of hydroxycoumarin is in the red channel and the Mitotracker signal is displayed in the green channel. The overlay is obtained by merging the images from both channels. The region where the nucleus is located is indicated (n).

proteins and other components were removed by washing. To identify interaction partners, the recovered proteins were analyzed by separation with SDS-PAGE followed by in-gel tryptic digestion and identification by liquid chromatography–ESI tandem mass spectrometry. The identification of tryptic digest peptides revealed 59 unique proteins (see the Supporting Information). Apart from integral mitochondrial proteins (Hsp60, Hsp75, ATP synthase subunits, mitochondrial malate dehydrogenase), we found plasma membrane receptors, cytoplasmic chaperones, chaperonins involved in actin and tubulin folding, cytoskeletal elements, and components of protein translation machinery, as well as cytosolic proteins involved in glycolysis and gluconeogenesis.

In combination with the fluorescence images, the mass spectrometry data confirm that targeted nanoparticles are actively transported to the mitochondria by the same pathway, as the fused post-translational sequence directs the import of the mitochondrial 3-oxoacyl-coenzyme A thiolase.[8] Therefore, the targeting peptide must have remained

bound to the SPIONs and was able to actively direct the transport of the material to the mitochondria.

Analysis of the APS-SPIONs, without mitochondrial targeting, resulted in the identification of albumins, keratins, and actins (data not shown), which indicates that these particles may adsorb serum proteins prior to cell uptake as well as interact with proteins within the cell after endocytosis. When cell lysate instead of living cells was incubated with MTP-cRGD-SPIONs, no specific proteins could be identified.

The proteins identified by mass spectrometry to be interacting with the MTP-cRGD-SPIONs were further correlated to known cellular interaction pathways by using the STRING database21. We found that 48 proteins formed a network that consisted of 308 interactions (Figure 3). cRGD-

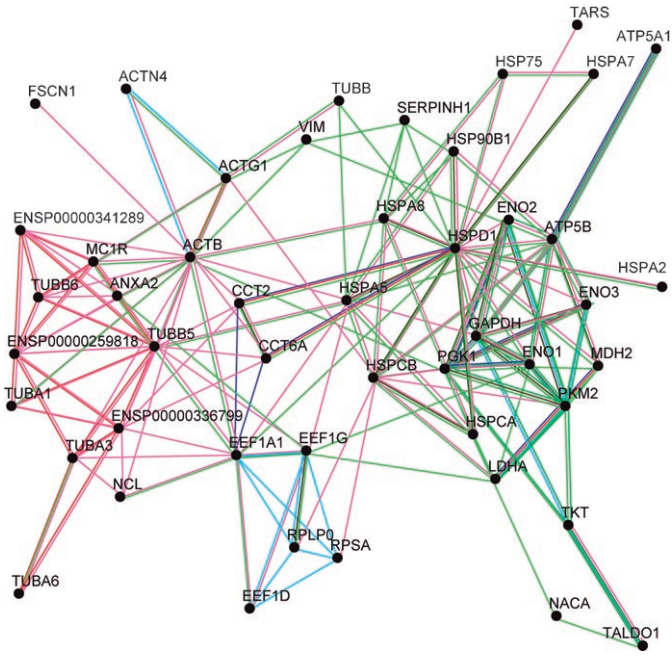

Figure 3. Evidence view of the protein interaction network in STRING. Different line colors represent the types of evidence for the association. Green: neighborhood, red: homology, blue: co-occurrence, brown: co-expression, magenta: experiments, light blue: databases, and light green: text mining.

SPIONs showed merely unspecific interactions in the cell and 74 proteins consisting of 461 interactions were identified. cRGD-SPIONs demonstrated low affinity toward mitochondria and more important interaction with nucleus proteins compared to MTP-cRGD-SPIONs. Detailed mass spectrometry results are shown in the Supporting Information.

Our data confirmed the presence of annexin-I (ANX1) and annexin-II (ANX2), which have been reported to play a role in regulating traffic within the endocytotic pathway.[9] In addition, nucleolin (NUC) was also identified. This protein is reported to be localized in the plasma membrane and is involved in the cellular uptake of peptides rich in basic amino acids.[10]

Translocation of the particles toward the mitochondria most likely occurred by an ATP-dependent interaction of the immobilized MTP with multiple Hsp70/Hsp90 (HSPA2, HSPA5-8/HSP90AA1, HSP90AB1) complexes.[11] Mitochondrial chaperonin Hsp60 (HSPD1) and cytoplasmic actin 2 (ACTB) both display 19 direct interaction partners (Figure 3). From our data, the identified chaperonin TCP complex (CCT2 and CCT6A) represents a link between the central transport complexes, which are Hsp60 and the cytoskeletal constituents. One may hypothesize that actin and tubulin, which were also observed within the interaction network, were synthesized and folded in situ through the specific activity of Hsp70s and the TCP complex. We can imagine that these were then assembled as cytoskeletal tracks directing the SPIONs toward the mitochondria.

We demonstrated that a part of the cellular pool containing glycolytic enzymes (GAPDH, PGK1, ENO1-3, and PKM2) was co-purified with the magnetic nanoparticles. The role of this glycolytic multiprotein complex is the channeling of pyruvate to the mitochondria. These proteins have been shown to be associated with the cytosolic side of the outer mitochondrial membrane in *Saccharomyces cerevisiae*.[12] The identification of threonyl-tRNA synthetase (TARS) in our sample indicates the interaction of the yeast glycolytic enzyme enolase (ENO1) with premitochondrial lysyl-tRNA synthetase. Enolase is known to have two or more different functions. It is involved in the glycolysis and transport of lysyl-tRNA toward mitochondria.[13] Furthermore, enzymes of the pentose phosphate pathway (TALDO1 and ENSP00000296289) were identified.

In summary, the combination of functionalized SPIONs and their ability to be recovered by using a magnetic column coupled with mass spectrometry has allowed us to explore a complex intracellular pathway with a peptide that is known to target mitochondria. Other applications of this technology include organelle-specific drug delivery, study of complex cellular signaling pathways and metabolism, and imaging of specific pathologies, such as malignant neoplasia.

Experimental Section

SPION synthesis, characterization, and coating (APS-SPIONs), in addition to PEG functionalization and peptide coupling, were performed as previously described and the magnetic-bed reactor setup was also as used before.[5] Synthesis of the MTP and 7-hydroxycoumarin thiol, determination of particle size and zeta potential, the cell culture setup, and iron quantification, as well as live cell imaging, magnetic enrichment of particle-associated proteins, and protein identification, are described in detail in the Supporting Information.

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