Monofunctional Stealth Nanoparticle for Unbiased Single Molecule Tracking Inside Living Cells

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ABSTRACT: On the basis of a protein cage scaffold, we have systematically explored intracellular application of nanoparticles for single molecule studies and discovered that recognition by the autophagy machinery plays a key role for rapid metabolism in the cytosol. Intracellular stealth nanoparticles were achieved by heavy surface PEGylation. By combination with a generic approach for nanoparticle monofunctionalization, efficient labeling of intracellular proteins with high fidelity was accomplished, allowing unbiased long-term tracking of proteins in the outer mitochondrial membrane.

KEYWORDS: Stealth nanoparticle, cytosol, autophagy, mitochondria, monofunctionalization, single particle tracking

Localization and tracking of individual proteins provide powerful means for unravelling complex processes that would otherwise be averaged out by ensemble measurements. Detecting and tracking single molecules in living cells, however, requires labeling with bright and photostable probes. Fluorescent nanoparticles (FNP) such as quantum dots or dye-doped nanoparticles enable long lasting imaging with ultrahigh precision and have been successfully applied for tracking individual proteins in the plasma membrane of living cells. Biological applications moreover require highly specific FNP attachment to target proteins in a defined stoichiometry without affecting its functional integrity. While a broad spectrum of modification and targeting strategies was made available for extracellular labeling with FNPs, these approaches mostly proved insufficient for intracellular application, which therefore remained challenging. Recently, we developed a generic approach for efficient site-specific targeting of FNPs to HaloTag fusion proteins in the cytoplasm of living cells, which is based on an engineered HaloTag ligand termed clickHTL. By employing this approach for tracking of single molecules in the outer mitochondrial membrane, however, we observed highly restricted mobility of individual FNP-labeled proteins not seen in case of labeling with organic dyes. This strong effect of intracellular FNP labeling on protein mobility, which has not been observed for cell surface labeling, could be ascribed to unspecific attractive or steric repulsive interactions with structures or compounds of the cytosolic environment. Moreover, rapid binding to target proteins was required for successful protein labeling in the cytosol of living cells, suggesting that competing interactions with cellular components play a critical role. Indeed, even for microinjected FNPs, rapid clustering in the cytosol has been observed, pointing toward recognition by an active metabolic machinery.

Here, we aimed to systematically engineer inert FNPs to enable unbiased protein tracking on intracellular membranes with ultrahigh spatial and temporal resolution. As a scaffold, we chose the natural protein cage formed by the ferritin light chain (LCF), which by itself provides intrinsic biocompatibility by its proteinaceous surface. Moreover, these very stable and fully monodisperse protein cages offer versatile means for well-defined chemical and genetic modification of the core and surface properties, as well as monofunctionalization with the clickHTL to ensure labeling in a 1:1 stoichiometry. By systematically engineering the functional properties of these protein cages, we developed a monofunctionalized model nanoparticle exhibiting “stealth” properties within the cytosol as required for unbiased live cell single molecule studies (Figure 1).

Recombinant LCF was expressed in E. coli, purified to homogeneity, and labeled with Cy3 maleimide via cysteine residues fused to the N-terminus of each LCF subunit (Cy3LCF; degree of labeling (DOL): 3). Both, LCF and Cy3LCF formed stable and monodisperse protein cages as confirmed by dynamic light scattering and analytical size exclusion chromatography (Supporting Information Figure S1). Moreover, its protein repelling properties were confirmed by...
monitoring the interaction of bovine serum albumin (BSA) with immobilized LCF in real time (Supporting Information Figure S2). After microinjection into HeLa cells, however, Cy3LCF immediately aggregated (Figure 2A), as indicated by the formation of bright dots on a time scale of seconds. Taking the very high colloidal stability of LCF particles into account,

Figure 1. Strategy of LCF surface modification and functionalization. Fluorescence dyes (here: ATTO 647N highlighted in purple) and click HTL (red) conjugated to an (EEG)3 carrier peptide (blue) were coupled to cysteine residues engineered into the N-terminus of each subunit. PEG (orange) was coupled via surface lysine residues.

Figure 2. Metabolism of LCF and engineered LCF inside living cells probed by epifluorescence microscopy. (A) Rapid aggregation of Cy3LCF injected into HeLa cells. (B) Co-localization of Cy3LCF (red channel) with transiently expressed mEGFP-LC3B (green channel). (C) Apparent degree of degradation (DOD) after 1, 5, and 16 h (black, Cy3LCF; red, Cy3LCF-COOH; green, Cy3LCF-PEG750; blue, Cy3LCF-PEG2k). (D) HeLa cells 16 h after microinjection of modified LCF (top, Cy3LCF-COOH; middle, Cy3LCF-PEG750; bottom, Cy3LCF-PEG2k). Scale bar: 5 μm in all images.
we suspected cellular metabolism to be responsible for this effect. Indeed, we found that Cy3-LCF aggregates specifically colocalized with mEGFP-tagged LC3B (mEGFP::LC3B), a marker for autophagosomes (Figure 2B), indicating rapid recognition by the autophagy machinery. Importantly, the same behavior was also observed for the ferritin analog Dps (DNA protection during starvation protein; 9 nm in diameter; DOL: 3) from the Gram-positive bacterium *Listeria innocua*, which in its amino acid sequence is unrelated to human ferritin, suggesting generic recognition of intracellular nanoparticles by the autophagy machinery (Supporting Information Figure S3). To test this hypothesis, we microinjected Rhodamine B doped, core–shell PEG/silica nanoparticles (SiNPs; 12 ± 2 nm in diameter; ζ-potential: −4.3 ± 0.3 nm; Supporting Information Figure S4), into the same cell line, yielding particle aggregates colocalized with mEGFP::LC3B (Supporting Information Figure S3). However, the kinetics of SiNP degradation were substantially lower with respect to Cy3-LCF or Cy3-Dps, suggesting that nanoparticle PEGylation interferes with recognition by the autophagy machinery. In contrast, a homogeneous distribution of purified EGFP or Cy3 labeled BSA (DOL: 1) was observed 1 h after microinjection into HeLa cells (Supporting Information Figure S5), corroborating a selective autophagy response toward nanoparticles rather than a stress-induced response caused by the microinjection procedure.

In order to minimize LCF recognition by the autophagy machinery, we tested different surface modification strategies. Since the particles surface charge plays an important role for colloidal NP stability, the moderately negative ζ-potential of −2.2 ± 0.2 mV for the unmodified LCF was decreased by converting surface-exposed amines into carbonyl groups by reaction with succinic anhydride. Moreover, the viscoelastic surface properties of LCF were modified by attaching poly(ethylene glycol) (PEG) via lysine residues using NHS chemistry. As the surface coverage with PEGs also depends on its chain length, we tested PEG4k with an average of 13 ethylene glycol units, as well as PEG8k with an average of 42ethylene glycol units. A decreased ζ-potential for both the carboxylated (−7.5 ± 0.1 mV) and the PEGylated (PEG4k−6.1 ± 0.3 mV; PEG8k−6.5 ± 0.4 mV) LCF cage was obtained (Supporting Information Figure S1). The integrity of the modified LCFs was confirmed by analytical size exclusion chromatography (SEC) and dynamic light scattering, confirming a modification-dependent size increase of the LCF (Supporting Information Figure S1). Mass spectrometry of LCFPEG2k (Supporting Information Figure S6) revealed 8 PEG chains per subunit (altogether 192 chains/FNP; on average 1 chain/2.3 nm²). This result matches very well with the theoretical number of accessible amines observed in the LCF crystal structure (PDB: 3HX7).

Autophagy of native and modified Cy3-LCF was compared by microinjection into the cytoplasm of HeLa cells (Figure 2C,D and Supporting Information Figure S7). While the majority of nonmodified Cy3-LCF was fully aggregated after 1 h and degraded after 16 h, a slightly increased persistency of carboxylated Cy3-LCF was observed, indicating a decreased recruitment into autophagosomes, probably due to electrostatic repulsion. Yet, carboxylated Cy3-LCF clearly aggregated between 1 to 5 h and was almost fully degraded 16 h after microinjection. In contrast, surface PEGylation substantially increased the stability of Cy3-LCF and the majority of particles remained stable for at least 5 h. However, 16 h after microinjection small, isolated aggregates could be observed for Cy3-LCF grafted with PEG750, indicating that the short PEG is still not sufficient to fully protect the particle surface. Further stabilization was achieved by grafting PEG4k, as no significant particle aggregation could be observed up to 16 h. These results provide clear evidence that PEGylation of NP surfaces at high densities is required to protect cytosolic NPs against autophagy.

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of EEG was functionalized with clickHTL via a C-terminal cysteine residue (Supporting Information Scheme S3). Finally, the maleimide-activated clickHTL-peptide was coupled to cysteine residues present on the FNP surface (Supporting Information Scheme S4) and subsequently fractionated by AEX. Elution of bound LCFs through a linear salt gradient yielded 4 fractions of differently charged species (Figure 3A), corresponding to the unfunctionalized LCF as well as LCF with 1, 2, and 3 peptides attached. The elution profile was in excellent agreement with the theoretically expected binomial distribution, confirming an optimal yield of monofunctionalized LCF (Supporting Information Figure S10).

The kinetics of monofunctionalized LCF (mLCF) reacting with purified HaloTag fused to a dodecahistidine-tag (HaloTag-H12) immobilized via its His-tag on a PEG polymer brush was characterized in vitro by TIRFS-RIf detection. Specific, irreversible binding of mLCF to immobilized HaloTag-H12 was observed (Figure 3B). From the binding curves, a reaction rate constant of \(4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) was obtained for mLCF, that is, by 50% reduced compared to clickHTL alone. Interestingly, mLCF coated with PEG750 (mLCF-PEG750), PEG2k (mLCF-PEG2k), and PEG3k, where the clickHTL was reacted at the terminus of PEG3k as a spacer (mPEG3kLCF-PEG2k), yielded similar reaction rate constants as obtained for unmodified mLCF (Supporting Information Figure S11). These results confirmed robust reactivity of clickHTL irrespective of the coating and localization of the clickHTL on the LCF surface.

Figure 3. Purification and characterization of mono-clickHTL-functionalized LCF (mLCF). (A) AEX fractionation of LCF functionalized with clickHTL by means of a negatively charged carrier peptide (black, eluted protein fractions monitored at \(\lambda = 280 \text{ nm}\); blue, Gaussian fit for the quantification of fraction frequencies). (B) Binding kinetics of mLCF monitored by RIf (upper panel) and TIRFS (lower panel): (I) immobilization of His tagged HaloTag; (II) binding of 100 nM Cy3LCF monofunctionalized with clickHTL; (III) surface regeneration by imidazole (green curve). As a negative control, the same experiments carried out with 100 nM nonfunctionalized Cy3LCF is shown (red curve).

Figure 4. Specific targeting of mLCF to the actin-cytoskeleton and mitochondria. (A) Epifluorescence images of HeLa cells stably expressing Lifeact::mEGFP::HaloTag 1h after micro injection of AT647NmLCFPEG2k (green, Lifeact::mEGFP::HaloTag; red, AT647NmLCFPEG2k yellow, overlay). (B) Time-lapse imaging of AT647NmLCFPEG2k binding to mitochondria (green, Tom20::mEGFP::HaloTag; red, AT647NmLCFPEG2k). Scale bar: 5 \(\mu\)m in all images. (C) Kinetics of AT647NmLCFPEG2k binding to Tom20::mEGFP::HaloTag (data points from four independent measurements were fitted using a monoexponential). The functionalization half-life (\(t_{1/2}\)) averaged from independent experiments is shown in the inset.
targeting of AT647NmLCFPEG2k to intracellular HaloTag fusion proteins was possible using this approach. For probing specific and efficient protein labeling with mLCFPEG2k within the cytosol, we employed Lifeact, a polypeptide efficiently binding to F-actin,\(^*\) which was fused to mEGFP and the HaloTag (Lifeact::mEGFP::Halo). Upon microinjection of mLCFPEG2k labeled with ATTO 647N (\(^{\text{AT647N}}\)mLCFPEG2k DOL: 11), into HeLa cells stably expressing Lifeact::mEGFP::Halo, high colocalization of \(^{\text{AT647N}}\)mLCFPEG2k with the actin cytoskeleton was observed (Figure 4A), confirming efficient and specific reaction with the target protein. Furthermore, \(^{\text{AT647N}}\)mLCFPEG2k was efficiently targeted to Tom20::mEGFP::Halo, which is localized in the outer mitochondrial membrane (Supporting Information Figure S12 and Figure 4A). Time lapse imaging revealed rapid binding of \(^{\text{AT647N}}\)mLCFPEG2k to mitochondria. By quantifying the fluorescence increase specifically on mitochondria (Figure 4B,C and Supporting Information Movie 1), a functionalization time of \(\tau \approx 13\) min was obtained. In contrast, in control experiments no colocalization could be observed by using LCF without clickHTL (Supporting Information Figure S13). These results provide clear evidence that efficient and specific targeting of \(^{\text{AT647N}}\)mLCFPEG2k to intracellular HaloTag fusion proteins was possible using this approach.

The impact of labeling HaloTag::mEGFP::Tom20 with \(^{\text{AT647N}}\)mLCFPEG2k on its diffusion properties was evaluated by single particle tracking with \(^{\text{AT647N}}\)mLCFPEG2k microinjected at very low concentrations. Individual particles specifically colocalizing with mitochondria (Figure 5A) could be observed without significant photobleaching. Thus, individual Tom20 diffusing along mitochondria could be readily tracked up to several hundred frames (Figure 5B and Supporting Information Movie 2). Obtained trajectories followed the shape of mitochondria, confirming high fidelity targeting even at very low nanoparticle concentrations. From fitting to the longitudinal diffusion component of each trajectory (Supporting Information Figure S14A), an average diffusion coefficient of \(0.246 \pm 0.11\) \(\mu\text{m}^2/\text{s}\) was obtained (Figure 5C). The distribution of diffusion coefficients was in good agreement with reference measurements carried out using HTL-TMR labeling of HaloTag::mEGFP::Tom20 indicated by the failure to reject the null hypothesis of equal distribution (p-value = 0.81, kstest2, The Mathworks MATLAB 2013a).\(^{15}\)

The heterogeneity of observed diffusion coefficients is partly explained by the inability to precisely decompose the trajectories due to their finite observation lengths (Supporting Information Figure 15A,B) as well as the fact that mitochondria might be slightly tilted with respect to the focal plane. Further heterogeneity could stem from intrinsic mobility changes of Tom20 due to interaction. Despite these constraints, essentially unbiased tracking of proteins in intracellular membranes was achieved by labeling with \(^{\text{AT647N}}\)mLCFPEG2k. In addition to the diffusion properties, by analysis of the transversal diffusion component of each trajectory we were able to extract local mitochondrial radii as a geometrical parameter (Figure 5D and Supporting Information Figure S14) which are in good agreement with the radii observed by electron microscopy.\(^{37}\)

This was possible with much higher fidelity for \(^{\text{AT647N}}\)mLCFPEG2k compared to TMR due to the longer trajectories obtained with these highly photostable nanoparticles.

Taken together, we engineered a model nanoparticle and could show for the first time that specific and quantitative application of monofunctionalized nanoparticles is possible inside living cells. Rather than nonspecific nanoparticle aggregation, our results highlight that specific recognition by the autofagy machinery is a key mechanism responsible for clustering and clearance of nanoparticles in the cytoplasm. While autophagy has been frequently implicated in the downstream metabolism of endocytosed nanoparticles,\(^{38-41}\) direct recognition by the cytosolic autophagy machinery has not been described yet. Our results suggest that rather than
direct trafficking from endosomes to autophagosomes, nanoparticle release into the cytosol may cause induction of autophagy responses. Surprisingly, even protein-based nanoparticles, which are physico-chemically highly stable and by definition biocompatible, are efficiently autophagized in the absence of further protection. Yet, also inorganic nanoparticles were targeted to autophagosomes, suggesting a generic intracellular mechanism for metabolism of nanomaterials at this scale. Diffusion studies indicated the formation of a protein corona on the surface of nonmodified LCF particles, which have been suggested to determine biological trafficking and metabolism.32,34,35 As the composition of this protein corona is very likely responsible for autophagosomal targeting, further studies will be required to identify the molecular components involved in particle recognition in order to design evasion strategies more systematically. However, similar as for preventing macrophage uptake of synthetic nanoparticles in organismic applications,44 dense surface PEGylation efficiently reduced nanoparticle recognition by the autophagy machinery. Thus, despite distinct mechanisms, extra- and intracellular nanoparticle clearance mechanisms seem to share common principles of surface pattern recognition, which have probably evolved as part of the antiviral defense.45 By eluding autophagy, intracellular stealth FNPs with a moderate size and surface charge were obtained, which are key requisites for unbiased single molecule studies by minimizing repulsive or attractive interactions with the cellular environment. By establishing LCF monofunctionalization with a reaction rate-enhanced clickHTL, efficient and selective labeling of proteins within the cytoplasm was achieved, enabling versatile intracellular application. Upon fulfilling all these requirements, unbiased diffusion dynamics of a membrane protein within the outer mitochondrial membrane could be observed by a ~16 nm-sized NP. Thus, rather than the size, the surface properties of nanoparticles are the critical determinants, especially for cytosolic applications. These insights provide key guidelines for designing nanoparticles suitable for biophysical studies in the cytosol of living cells.

ASSOCIATED CONTENT

Supporting Information
Detailed description of materials and methods, supplementary figures, and movies. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Notes
The authors declare no competing financial interest.

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