Supporting Information

Assessing the Stability of Fluorescently Encoded Nanoparticles in Lysosomes by using Complementary Methods

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Abstract: Nanoparticles (NPs) are promising tools in biomedical research. In vitro testing is still the first method for initial evaluation, however, NP colloidal behavior and integrity, in particular inside cells (i.e. in lysosomes), are largely unknown and difficult to evaluate due to the complexity of the environment. Furthermore, while the majority of NPs are usually labelled with fluorescent dyes for tracking purposes, the effect of the lysosomal environment on the fluorophore properties, as well as the ensuing effects on data interpretation, is often only sparsely addressed. In this work, we have employed several complementary analytical methods to better understand the fate of fluorescently encoded NPs and identify potential pitfalls that may arise from focusing primary analysis on one single attribute, e.g. fluorophore detection. Our study shows that in a lysosomal environment NPs undergo significant changes which ultimately leads to dye quenching and distorted fluorescence signals.

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Experimental Procedures

Section S1: Materials and methods

Gold NPs synthesis and functionalization

Citrate stabilized AuNPs, with a core diameter of approximately 17 nm, were synthesized through citrate reduction, as reported by Ernust and Turkevich.1 AuNPs were further functionalized with a polymer mixture in order to obtain COOH poly(vinyl alcohol) (PVA)/PVA functionalized NPs, as previously reported.2 This was achieved through ligand exchange, PVA (Mw=82 Mw=000, Calbiochem, EMD Bioscience, Inc. La Jolla, CA, USA) and carboxyl-modified PVA (COOH PVA Kuraray Specialties Europe GmbH, Hattersheim am Main, Germany) were used to obtain COOH PVA/PVA coated particles. For this study polymers were fluorescently encoded with an ATTO590 NHS (Sigma-Aldrich, Buchs, Switzerland) dye, via ester bond formation in the case of the PVA and COOH PVA polymers. The carboxyl groups of the ATTO590 NHS dye were activated using 2% w/v EDAC (1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDAC, Sigma-Aldrich, Buchs, Switzerland). For the conjugation of the dye to COOH PVA and PVA, the reaction mixtures’ pH was adjusted to 5. For the COOH PVA polymer, DMSO was used, instead of PBS as the reaction solvent. This solvent substitution step occurred during the study, and only affects polymers that were used in the fluorescence based studies. Following the conjugation, polymers were purified by gel filtration chromatography, using a PD-10 desalting column (Sephadex G-25 medium, exclusion limit M r = 5000, GE Healthcare Life Sciences) and PBS as eluent. In the case of the COOH PVA polymer that was conjugated in DMSO, dialysis was used for purification (Slide-A-Lyzer™ Dialysis Cassettes, 7K MWCO, 3 mL, Thermo Scientific). Finally conjugated polymers were freeze-dried prior to being stored at −20 °C. Hetero-functionalized AuNPs were obtained, via a two-step functionalization reaction, where particles were first homo-functionalized with the above-mentioned polymers. Prior to the functionalization of citrate AuNPs, fluorescently conjugated polymers were dissolved in PBS by sonication (30 min). The amount of polymer was calculated to provide an Au/PVA ratio of 15. Gold NPs were dropwise added to the dissolved polymer, and kept on a shaker overnight at room temperature. The following day, particles were centrifuged twice for 1 h at 10000 x g to remove any unattached polymer, and the now homo-functionalized NPs, were used for further functionalization. Re-dispersed homo functionalized NPs particles were dropwise added to dissolved unconjugated polymer. The COOH PVA hetero-functionalized particles were obtained following the same procedure of over-night incubation and purification by centrifugation.

Characterization of NPs

Citrate stabilized and functionalized COOH PVA AuNPs were characterized with regard to their optical properties, size, and zeta potential. Optical properties were characterized using a UV –Vis spectrometer (Jasco Europe S. R. L., Milano, Italy), where the spectra of nanoparticles were measured in water (for citrate stabilized AuNPs) and PBS (for COOH PVA AuNPs). The ATTO590 dye attached to the polymer was quantified using a fluorescence plate reader with excitation wavelength centered at 560 nm and emission filter centered at 615 nm (Wallac 1420 VICTOR3 reader, PerkinElmer, Boston, MA). The size of the NPs was measured in terms of the core diameter (d_c) and hydrodynamic diameter (d_h). The diameter and size distributions of NPs were obtained via transmission electron microscopy (TEM), using a Tecnai Spirit transmission electron microscope (FEI, Frankfurt, Germany), operating at 120 kV. The images were recorded at a resolution of 2048 x 2048 pixels (Veleta CCD camera, Olympus, Volketswil, Switzerland) and Image J was used for image analysis. Hydrodynamic diameters were measured using dynamic light scattering in depolarized light.
Macrophage cultures

The J774 A.1 macrophage cell line was obtained from ATCC, Manassas, USA. The cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Gaithersburg, MD, USA) supplemented with 1% glutamine (Gibco, Gaithersburg, MD, USA), 1% Penicillin Streptomycin (Gibco, Gaithersburg, MD, USA) and 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA). J774 A.1 macrophages were cultured at 37 °C and 5% CO2. After reaching 80% confluency, cells were sub-cultured by gentle removal of cells from the flask bottom with the cell scraper (Starstedt, Nümbrecht, Germany). Following that, cells were re-suspended in fresh media.

**Nanoparticle exposure**

J774 A.1 cells (5x10^5 cells per well) were seeded in 8-well (0.8 cm²/well) chambers (Corning, New York, USA) and exposed on the following day to COOH PVA AuNPs (20 µg/mL diluted in 250µL of complete medium). After 24 h exposure (5 min, 30 min and 24 h for a short exposure), cells were fixed, permeabilized, stained with 1:50 Phalloidin Alexa 488 (Thermo Fisher Scientific) and 1:50 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Aldrich, Buchs, Switzerland), and mounted with anti-fading mounting media (Dako, Agilent, Santa Clara, California, United States). AuNPs uptake was assessed with an inverted Zeiss confocal laser scanning microscope 710 (LSM, Axio Observer.Z1) (Carl Zeiss, Jena, Germany) and a CytoViva hyperspectral microscope (CytoViva, Inc., Auburn, AL, USA) outfitted with a Dolan-Jenner DC-950 light source. UPL Florite 100× objective, and SPECIM V10E imaging spectograph with a PCO pixelfly detector. Flow cytometry measurements were performed on a BD LSRFortessa™ cell analyser (BD Biosciences, Allschwil, Switzerland). For LSM image processing Image J software was used, for FACS data FlowJo Software (Ashland, OR, USA) and for Cytoviva HSI data, Cytoviva ENVI software was used.

**ICP-OES quantification of cellular uptake**

Cells were incubated with AuNPs for 24 h and after incubation washed with phosphate buffered saline (PBS; Life Technologies). Upon completion of the study, cells were kept at -80 °C until further analysis. NP adherence to/uptake by cells was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) on an Optima 7000 DV (Perkin Elmer). The thawed cells were treated with a 2:1 mixture of HNO3 (Sigma-Aldrich, Buchs, Switzerland) and H2O2 (Reacto Lab SA) at 300 µL per sample, and digested for several hours. The samples were briefly sonicated at 50 °C prior to the addition of 400 µL of HCl (Honeywell AG, Volketswil, Switzerland) and digested overnight. Finally, the samples were diluted 10 fold with MiliQ water. The treated samples were then measured at a wavelength of 242.795 nm (gold). A standard curve of aqueous gold solutions (Fluka, Volketswil, Switzerland) was recorded to quantify the amount of cell-associate gold.

**UV-Vis spectroscopy and DDLS of NPs in artificial lysosomal fluid**

The artificial lysosomal fluid (ALF) was prepared as previously reported. [3] Briefly, sodium chloride (3.210 g), sodium hydroxide (6.000 g), citric acid (20.800 g), calcium chloride (0.097 g), sodium phosphate heptahydrate (0.179 g), sodium sulfate (0.039 g), magnesium chloride hexahydrate (0.106 g), glycerin (0.059 g), sodium citrate dihydrate (0.077 g), sodium tartrate dihydrate (0.090 g), sodium lactate (0.085 g), sodium pyruvate (0.086 g), formaldehyde (1.000 ml) were dissolved in 0.2L of MiliQ water in order to obtain a 5x concentrated stock solution that was later diluted with MiliQ water and nanoparticles during incubation. All chemicals were obtained from Sigma Aldrich, Buchs, Switzerland.

Nanoparticles were incubated with ALF for 24 h at 37 °C and the change in size was measured by DDLS in a time resolved manner (measurements were recorded every 3 min over 24 h) an for LSM image processing Image J software was used, for FACS data FlowJo Software (Ashland, OR, USA) and for Cytoviva HSI data, Cytoviva ENVI software was used. For LSM image processing Image J software was used, for FACS data FlowJo Software (Ashland, OR, USA) and for Cytoviva HSI data, Cytoviva ENVI software was used.

**IDLS of NPs in artificial lysosomal fluid**

The artificial lysosomal fluid (ALF) was prepared as previously reported. [3] Briefly, sodium chloride (3.210 g), sodium hydroxide (6.000 g), citric acid (20.800 g), calcium chloride (0.097 g), sodium phosphate heptahydrate (0.179 g), sodium sulfate (0.039 g), magnesium chloride hexahydrate (0.106 g), glycerin (0.059 g), sodium citrate dihydrate (0.077 g), sodium tartrate dihydrate (0.090 g), sodium lactate (0.085 g), sodium pyruvate (0.086 g), formaldehyde (1.000 ml) were dissolved in 0.2L of MiliQ water in order to obtain a 5x concentrated stock solution that was later diluted with MiliQ water and nanoparticles during incubation. All chemicals were obtained from Sigma Aldrich, Buchs, Switzerland.

Nanoparticles were incubated with ALF for 24 h at 37 °C and the change in size was measured by DDLS in a time resolved manner (measurements were recorded every 3 min over 24 h) and UV-Vis spectroscopy measurements were taken at the beginning of incubation (0 h) and at the end (24 h). As a control experiment, identical measurements were performed with AuNPs in PBS. The method of choice to characterize the particle size (hydrodynamic radius), was DDLS, which has previously shown to have an advantage over conventional DLS, when it comes to preforming measurements in a complex system, as other entities such as proteins present in media, can interfere with signal and the overall quality of data. [4]

**CryoEM of NPs in artificial lysosomal fluid**

Following the same procedure for NPs incubation in ALF (24 h, 37 °C), 5 µL of sample solution was first deposited on a carbon coated copper grid (Lacey carbon film grids, Plano Gmbh). Following this, excess liquid was carefully blotted away with filter paper (Whatman qualitative filter paper, grade 1) before plunging the sample into a liquid ethane bath cooled by liquid nitrogen. The vitrified specimens were then kept for storage in liquid nitrogen and analyzed the following day.

All grids were investigated with a FEI Tecnai F20 cryo-transmission electron microscope with an operating tension of 200 kV. Images were recorded under low-dose conditions with an UltraScanTM 1000 CCD sensor (Gatan, Inc.) with an image resolution of 2048x2048 pixels.

**Isolation of crude lysosomal fraction**

J774 A.1 cells were seeded in a T-25 flask (25 cm²) at the density 2x10⁶ cells/cm², and on the following day exposed to COOH PVA Au NPs (20 µg/mL diluted in 5 mL of complete medium). Cells were incubated with nanoparticles for 24 h, while one flask only contained media and served as a negative control. After incubation, cells were de-attached from the flask using a cell scraper, and centrifuged (200 x g for 3 min), in order to remove media and non-uptaken particles. Samples for flow cytometry (BD LSRFortessa™ cell analyzer (BD Biosciences, Allschwil, Switzerland)) were aliquoted and transferred in flow cytometry buffer (phosphate-buffered
saline (PBS) (Gibco) supplemented with, 1% BSA (Sigma-Aldrich, Buchs, Switzerland) and 0.1% sodium azide (Sigma-Aldrich, Buchs, Switzerland). The remaining cells were used for the isolation of crude lysosomal fraction. The cells were re-suspended in PBS containing a protease inhibitor cocktail (Sigma-Aldrich, Buchs, Switzerland) in a dilution recommended by the manufacturer. The cells were then mechanically disrupted by using a syringe (0.6 mm needle) and the efficiency of cell disruption was confirmed via trypan blue staining (Sigma-Aldrich, Buchs, Switzerland). Following their destruction, cells were centrifuged (1000 g for 10 min at 4 °C) and the supernatant containing crude lysosomal fraction was analyzed by UV-Vis spectroscopy and dynamic light scattering (DLS).

Quantification of poly(vinyl alcohol) polymer

The functionalized nanoparticles were incubated in ALF and PBS for 24 h at 37 °C. After incubation, AuNPs were centrifuged at 10,000 x g for 1 h, making sure that most of the NPs were present in the pellet. Supernatant, potentially containing free polymer, was collected and dialyzed (Slide-A-Lyzer™ Dialysis Cassettes, 7K MWCO, 3 mL, Thermo Scientific) against MiliQ water in order to remove all compounds from ALF that might interfere with an assay. The assay for polyvinyl alcohol is based on the formation of a characteristic blue complex with a KI/I₂-Boric acid mixture, which can be used for its quantification via UV-Vis. The method of choice for quantification was standard addition; since particles are composed of a complex polymer mixture (dye conjugated and unconjugated COOH PVA/PVA polymers) hence the matrix effect is highly significant and would interfere with classical measurements.

Stability of COOH PVA polymer in ALF

The COOH PVA polymer (1 mg/mL) (Kuraray Specialties Europe GmbH, Hattersheim am Main, Germany) was dissolved in PBS and ALF, which was followed by DLS measurements (3 x 300 sec measurements at 10°, 60°, 90° and 120°). All measurements were performed at 37 °C.

Results and Discussion

Section S2: NP stability in cell culture media

The stability of functionalized AuNPs was assessed over 24 h at 37 °C, where the size changes were measured in a time resolved manner with DDLS. The DDLS has previously shown to have an advantage over conventional DLS, when it comes to performing measurements in a complex system, as other entities such as proteins present in media, can interfere with signal and the overall quality of data.
Section S3: Fluorescence of a dye incubated in different media

Fluorescence of a free ATTO590 dye and on functionalized NPs

Free ATTO590 fluorescent dye, and nanoparticles functionalized with ATTO590 dye, were incubated with PBS, ALF and cRPMI in order to track and assess fluorescence of free and attached dye at the beginning of incubation and after 24 h, at 37 °C. The fluorescence of NPs, with attached dye, was assessed for a series of NP concentrations (5, 10, 20, 50 µg/mL). The fluorescent signal was measured at the beginning of incubation and after 24 h, via plate reader (excitation wavelength centered at 560 nm and emission filter centered at 615 nm (Wallac 1420 VICTOR3 reader, PerkinElmer, Boston, MA)). Incubation and measurements were performed in black 96 well plates recommended for fluorescence measurements (OptiPlate-96 Black, Black Opaque 96-well Microplate, PerkinElmer, Boston, MA).

Figure S2. Fluorescence of ATTO590 dye in PBS, ALF and complete media (cRPMI) at the beginning of incubation and after 24 h. It can be observed that upon incubation in ALF, the fluorescent signal drops, even at a beginning, comparing to cRPMI and PBS. This can be contributed to the chemical composition of ALF (previously described) and pH (4.5). The fluorescent signal stays constant over 24 h for cRPMI, while for ALF a slight decrease is observed. Even though the fluorescent signal in ALF is lower, it still does not show complete quenching. It can be also observed that, after 24 h, the fluorescence of the dye incubated in PBS increases, which could be contributed to evaporation of the liquid.

Figure S3. Fluorescence of ATTO590 dye attached to NPs in PBS, ALF and complete media (cRPMI), at the beginning of incubation and after 24 h. The fluorescence was measured at the following NPs concentrations: 5, 10, 20 and 50 µg/mL. Taking into account that the fluorescent dye is attached to NPs, it is no surprise that with the increase of NP concentration, the fluorescent signal also increases. As in the previous case (Figure S2) even at the very beginning, the fluorescent signal of NPs incubated in ALF is lower than for any other media. The same can be observed for the dye alone, as the fluorescent signal doesn’t vary noticeable after 24 h, with the exception of NPs incubated in PBS and cRPMI (only for the highest concentration). The data from fluorescence measurements, when AuNPs are present, should be treated with care since AuNPs can interfere with the fluorescent signal in the mentioned wavelength range.
Section S4: NPs uptake and fluorescence after short-time exposure

**Figure S4.** Fluorescence of functionalized NPs as observed by LSM. Functionalized NPs (20 µg/mL) were casted onto microscopic slide and imaged via LSM using settings for ATTO590 detection. NPs were imaged at the edge of the drop and the acquired signal is shown in red. White box indicates the edge of the drop.

**Figure S5.** NPs uptake and fluorescence after short-time exposure. J774.A.1 mouse macrophages were exposed to 20 µg/mL of COOH PVA ATTO590 AuNPs for 5 min, 0.5 and 4 h, and fluorescence was detected by LSM and flow cytometry. LSM: Cells were stained with the nucleus stain DAPI (blue), F-actin stain phalloidin Alexa 488 (green) and particles are labelled with the ATTO590 dye (red). DF HSI: Dark field hyperspectral imaging microscopy of NPs inside J774.A.1 mouse macrophages. The image was formed, based on spectral information of each pixel, where due to the intensity of light scattering, AuNPs give the brightest signal. White box indicates the area where some NPs were detected. ICP OES: Following the incubation and acid digestion, the amount of cell-associated gold was measured at 242.795 nm. The graph shows the fraction of the uptake relative to the amount of the gold used for the exposure, and the inset graph shows the uptake at the shorter time points.
Section S5: NP stability after transfer from PBS to water

In order to access the stability of NPs in ALF, it was necessary to transfer NPs from PBS to MiliQ water, to avoid the influence the salts present in PBS. To achieve that, NPs were centrifuged 2x at 10000 \times g for 1 h and re-suspended in water. Following centrifugation it was necessary to check whether the particles remain stable. DLS measurements were performed before (PBS) and after (water) centrifugation.

Figure S6. NP stability after centrifugation. Hydrodynamic radius of functionalized nanoparticles was measured by DLS (60 measurements for each sample) in PBS (before centrifugation) and in water (after centrifugation). The data does not show aggregation of NPs after centrifugation and transfer to water. Size increase of 2-5 nm could be contributed to different behaviour/conformation of COOH PVA/PVA polymer in different ionic force environment.

Section S6: NP stability in artificial lysosomal fluid (ALF) and phosphate buffer pH 4.5

Scattering intensity of functionalized nanoparticles incubated in ALF

Figure S7. Colloidal stability of AuNPs. DDLS (depolarized dynamic light scattering) data for NPs in PBS (left) and ALF (right). The figure shows the scattering intensity over 24 h of incubation in PBS (left) and ALF (right). While the scattering intensity remains unchanged for NPs incubated in PBS. It significantly increases after approx. 5 h, when NPs were incubated in ALF, indicating NP size increase, and the sudden drop is an indication for NP sedimentation.
Stability of functionalized AuNPs in a low pH buffer

In order to investigate whether the pH itself contributes the most to the NPs instability in ALF, AuNPs were incubated under the same conditions as in ALF (pH 4.5) (24 h at 37 °C) in phosphate buffer (pH 4.5). DDLS measurements were performed in a time resolved manner as previously described.

Figure S8. DDLS (depolarized dynamic light scattering) data for AuNPs in phosphate buffer pH 4.5. The figure shows the hydrodynamic radius and scattering intensity over 24 h. This demonstrates that aggregation is a consequence not only of the pH, but also the presence of other molecules present in the ALF (e.g. small organic molecules such as formaldehyde and salts).

Section S7: Polymer stability and dissociation in ALF

Polymer stability in ALF

The COOH PVA polymer (1 mg/mL) (Kuraray Specialties Europe GmbH, Hattersheim am Main, Germany) was dissolved in PBS and ALF, which was followed by DLS measurements (3 x 300 sec measurements at 10°, 60°, 90° and 120°). All measurements were performed at 37 °C.

Figure S9. Stability of COOH PVA polymer in ALF. 1 mg/mL of COOH PVA polymer was dissolved in PBS (left) and ALF (right). After incubation at 37 °C, an image of both samples was taken. The increased turbidity of the sample incubated in ALF (right), suggests that the polymer conformation changed, which might result in the formation of larger structures.

Polymer dissociation from NPs in ALF

| Sample  | PBS | ALF |
|---------|-----|-----|
| COOH PVA| 2.1[a] | 2.3[a] |

Table S1. Quantification of the polymer loss in PBS and ALF. Functionalized AuNPs were incubated in both PBS and ALF over 24 h at 37 °C. Following the incubation, particles were centrifuged and the concentration of polymer was measured in supernatant. Polymer quantified in supernatant was regarded as polymer that had de-attached from NPs during incubation. It can be observed, that for both solvents, no significant polymer loss occurred.

[a] Polymer amount in µg.
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Author Contributions

A.M.M. designed and performed the experiments, and drafted the manuscript. L.R.L. performed SERS experiments. C.A.M. performed the CryoTEM imaging experiments. S.B. supervised the light scattering experiments. L.R.L. and A. P. F. were involved in the study design, supported the technical aspects of the study, and helped to revise the manuscript. B.R.R. was the project leader, was involved in planning of the study as well as in the experimental work, and made substantial input for the manuscript writing. All authors read and approved the final manuscript.