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Original Article

Nanoprobing the acidification process during intracellular uptake and trafficking

Simone Lerch, PhD[a,1], Sandra Ritz, PhD[a,b,1], Karina Bley[a,1], Claudia Messerschmidt, PhD[a], Clemens K. Weiss, PhD[a,c], Anna Musyanovych, PhD[a,d], Katharina Landfester, PhD[a], Volker Mailänder, PhD[a,e,*]

[a]*Max-Planck-Institute for Polymer Research, Mainz, Germany*
[b]*Institute of Molecular Biology (IMB) gGmbH, Mainz, Germany*
[c]*University of Applied Sciences Bingen, Bingen, Germany*
[d]*Fraunhofer ICT-IMM, Mainz, Germany*
[e]*3rd Department of Medicine (Hematology, Oncology, and Pneumology), University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany*

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Abstract

Many nanoparticular drug delivery approaches rely on a detailed knowledge of the acidification process during intracellular trafficking of endocytosed nanoparticles (NPs). Therefore we produced a nanoparticular pH sensor composed of the fluorescent pH-sensitive dual wavelength dye carboxy seminaphthorhodafluor-1 (carboxy SNARF-1) coupled to the surface of amino-functionalized polystyrene NPs (SNARF-1-NP). By applying a calibration fit function to confocal laser scanning microscopy (CLSM) images, local pH values were determined. The acidification and ripening process of endo/lysosomal compartments containing nanoparticles was followed over time and was found to progress up to 6 h to reach an equilibrium pH distribution (maximum pH 5.2 [±0.2]). The SNARF-1-NP localization in endo/lysosomal compartments was confirmed by transmission electron microscopy (TEM) and quantitative co-localization analysis with fluorescent endolysosomal marker Rab-proteins by confocal laser scanning microscopy (CLSM). The herein described nanoparticular pH-sensor is a versatile tool to monitor dynamic pH processes inside the endolysosomal compartments.

From the Clinical Editor: In this interesting article, the authors elegantly designed a nanoparticular pH sensor with fluorescence probe with the capability to measure intracellular and intravesicular pH changes. The application of this method would enable the further understanding of nanoparticle uptake and intracellular physiology.
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Key words: Nanoparticle; Intracellular trafficking; Acidification; pH responsiveness; Rab-family

For the last decade, polymeric nano-sensors have been developed as probes for real-time imaging and dynamic monitoring of various ions, such as H^+, Ca^{2+}, Mg^{2+}, K^+, Na^+, and Cl^-, which are important for cellular metabolism.[1-4] Nanoparticle (NP) based sensor systems have several advantages in comparison to highly invasive methods, like e.g. microelectrode probing or the use of unconjugated fluorescent probes: i) due to their small size and inert material they are physically and chemically less invasive than macroscopic probes, ii) the local concentration and therefore signal strength of the chemical probe can easily be tuned due to the high surface-to-volume ratio[5] and iii) surface functionalization with targeting agents may guide them towards specific sites at or in cells. Surface properties as well as multiple functionalization, such as additional incorporation of a drug,[6] magnetic resonance imaging active inorganic labels[7] or antibodies,[8] e.g. as delivery targets, can be introduced without difficulty. NPs have emerged as promising tools to study mechanisms innate to cells such as endocytotic uptake machineries as well as to function as novel delivery systems for drug transport and for addressing specific cell or tissue types. Most NPs are taken up by cells via various endocytotic mechanisms and follow the endo/lysosomal pathway.[9] The pH of these compartments is lowered during the maturation of the vesicles – from early to late endosomes to lysosomes – to trigger the release of receptor-bound

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* Corresponding author at: Max-Planck-Institute for Polymer Research, Mainz, Germany.

E-mail address: mailaend@mpip-mainz.mpg.de (V. Mailänder).

[1] Authors contributed equally.

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ligands,[10] and to digest debris or nutrients. This acidification can be used to trigger the degradation of biocompatible polymeric nanocapsules and to subsequently release incorporated markers or drugs.[11] Intracellular pH changes also occur in the development of tumor cells, e.g. the pH of some tumors is lowered due to an increased P-glycoprotein activity.[12]

Since changes in local ion concentration, such as H^+, play an important role in metabolic processes, ion transport, cell signaling and cell growth,[13] it is essential to have probes to distinguish between different pH values with a high spatial resolution. So far, knowledge on the accurate interactions of NPs with endosomal compartments and the time scales of NPs trafficking in cells is sparse.[14] Understanding the acidification kinetics in the endo/lysosomal system is important to tailor nanoscale systems for pH-triggered drug release. Currently, some studies attempt to combine a novel sensor-NP with an ability for pH-sensitive drug delivery and the quantitative monitoring of local NPs in endocytotic vesicles.[15-18]

Here we present the synthesis of pH-sensitive NPs and their use as nano-sensors for intracellular and intravesicular pH monitoring. Amino-functionalized polystyrene NPs were synthesized by an emulsifier free emulsion copolymerization approach.[19,20] With this technique, additional functionalization[21] as well as the incorporation of markers and drugs[22] is well established. The dual-wavelength pH-sensitive dye carboxy SNARF-1 (seminaphthorhodafluor-1) was bound to the particle surface by a succinimidyl ester (NHS)-mediated coupling reaction. Using this strategy leads to NPs with an accessible probe dye on the particle surface. Ratiometric imaging of the protonated and deprotonated forms of SNARF-1 (emission maxima at λ_{pr} = 580 nm and λ_{dep} = 640 nm) enables that the readout of the NP pH-sensor is not sensitive to local dye concentration. However, the surface charge of NPs and distance between dye molecules on the surface could influence the measurable pH range, as has been shown.[23] Additionally, contact with the intracellular environment may influence the fluorescence properties of dye-conjugated NPs.[24] Therefore, the ratiometric signal of the dye-coupled NPs was carefully calibrated in cells. Based on the ratiometric pH quantification, a mode for the acidification process of SNARF-1-NPs after endocytosis was established and visualized as pseudo-colored confocal laser scanning microscopy (CLSM) images showing the pH distribution in different endosomal structures. It was also readily possible to distinguish between intra- and extracellularly located NPs. The localization of SNARF-1-NPs in different endo/lysosomal structures was mapped by transmission electron microscopy (TEM) imaging and co-localization studies with Rab proteins.

Materials and methods

Materials

Styrene (Merck, Darmstadt, Germany) was purified using a nitrogen pressured alumina flash column. All other chemicals were used without further purification: 2-aminoethyl methacrylate hydrochloride (AEMH, Sigma-Aldrich, St. Louis, USA; 90%), 2,2'-azobis(2-(2-imidazolin-2-yl)propane) dihydrochloride (VA-044, Wako Chemicals, Neuss, Germany), pH-sensitive dye 5′ (and 6′) carboxy-10-dimethylamino-3-hydroxy-spiro[7*H*-benzo[*c*]xanthene-7,1′(3′*H*)-isobenzo-furan]-3′-one (carboxy SNARF-1), SNARF-1 acetate succinimidyl ester (SNARF-1-NHS), SNARF-1 acetoxymethyl ester acetate (SNARF AM ester; all Invitrogen, Karlsruhe, Germany), fluorescamine (Sigma Aldrich, St. Louis, USA; >98%), sodium borate (Merck, Darmstadt, Germany) and hexylamine (Sigma Aldrich, St. Louis, USA; 99%). Demineralized and ultrapure water was used throughout the experiments.

Synthesis of amino-functionalized polymeric NPs

Positively charged amino-functionalized particles were synthesized using soap-free emulsion copolymerization in the presence of the co-monomer AEMH following the procedure of Ganachaud et al.[19] Prior to polymerization, 200 mL of ultrapure water was degassed with argon for 30 min under magnetic stirring in a three-necked round bottom flask equipped with a condenser and septa. 15 mL of styrene was added and stirred for 5 min before 682.5 mg ($4.1 \cdot 10^{-3}$ mol) AEMH dissolved in 2 mL ultrapure water was added with a syringe. After additional 5 min 284.4 mg ($8.8 \cdot 10^{-4}$ mol) of the initiator VA-044 dissolved in 3 mL ultrapure water was added. The polymerization proceeded under argon atmosphere and continuous stirring at 500 rpm for 24 h at 55 °C. After synthesis, the particle dispersion was dialyzed (molecular weight cut-off [MWCO] 14,000 $g \cdot mol^{-1}$, Carl Roth, Karlsruhe, Germany) for 3 days under repeated exchange of demineralized water. It was further purified with demineralized water via repetitive centrifugation/redispersion at 22,000 min^{-1} for 45 min.

Characterization of amino-functionalized polymeric NPs

After purification, the colloids' average particle size and size distribution were measured by angle-dependent dynamic light scattering (ALV/CGS3 compact goniometer system with a He/Ne laser [632.8 nm]) at 20 °C. The ζ-potential measurements were performed in KCl solution ($1 \cdot 10^{-3}$ $mol \cdot L^{-1}$) using a Zeta Nanosizer (Malvern Instruments, UK). The amount of amine groups on the particle surface was determined with fluorescence titration following the procedure published by Ganachaud et al.[19] In brief, the calibration curve was plotted from hexylamine solutions of given concentrations in sodium borate buffer (pH = 9.5, 0.1 $mol \cdot L^{-1}$) and freshly prepared fluorescamine solution in acetone (0.3 $g \cdot L^{-1}$). 25 μL of the colloidal dispersions with a solid content of 1 weight% (wt.%) was added to 725 μL of borate buffer together with 250 μL of fluorescamine solution. After 30 s vigorous mixing, 100 μL of the dispersion was placed in a 96-well plate (Corning Incorporated 3603) and fluorescence emission of the fluorescamine at λ_{em} = 470 nm was followed by a Tecan Infinite M1000 Plate Reader (Tecan Group Ltd., Maennedorf, Switzerland) using an excitation wavelength of λ_{ex} = 410 nm. The amount of amine groups was found to be $1.5 \cdot 10^{-5}$ $mol \cdot mL^{-1}$ or 2.5 amine groups per nm^2.

Coupling the pH-sensitive dye onto the particles

The pH-sensitive dye SNARF-1-NHS was coupled onto amino-functionalized polystyrene colloids forming an amide bond between the amine group of the nanoparticle and the carboxylic acid function of the dye (Figure 1, *A*). 0.11 mg of SNARF-1-NHS dissolved in 440 μL DMSO (c = 0.25 $mg \cdot mL^{-1}$) was added to a dispersion of amino-functionalized colloids with a

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