Intracellular Trafficking of Fluorescent Nanodiamonds and Regulation of Their Cellular Toxicity

Neeraj Prabhakar, Meraj H. Khan, Markus Peurla, Huan-Cheng Chang, Pekka E. Hänninen, and Jessica M. Rosenholm

†Pharmaceutical Sciences Laboratory, Faculty of Science and Engineering, Åbo Akademi University, Tykistökatu 6A, Biocity, FI 20520 Turku, Finland
‡Laboratory for Biophysics, Cell Biology and Anatomy, Faculty of Medicine, University of Turku, Tykistökatu 6A, Biocity, FI 20520 Turku, Finland
§Electron Centre for Biotechnology, Åbo Akademi and University of Turku, Tykistökatu 6A, Biocity, FI 20520 Turku, Finland
∥Institute of Atomic and Molecular Sciences, Academia Sinica, Roosevelt Rd., Sec. 4, Taipei 10617, Taiwan

Supporting Information

ABSTRACT: In this paper, cellular management of fluorescent nanodiamonds (FNDs) has been studied for better understanding in the design for potential applications of FNDs in biomedicine. The FNDs have shown to be photostable probes for bioimaging and thus are well-suited, for example, long-term tracking purposes. The FNDs also exhibit good biocompatibility and, in general, low toxicity for cell labeling. To demonstrate the underlying mechanism of cells coping the low but potentially toxic effects by nondegradable FNDs, we have studied their temporal intracellular trafficking. The FNDs were observed to be localized as distinct populations inside cells in early endosomes, lysosomes, and in proximity to the plasma membrane. The localization of FNDs in early endosomes suggests the internalization of FNDs, and lysosomal localization, in turn, can be interpreted as a prestage for exocytosis via lysosomal degradation pathway. The endocytosis and exocytosis appear to be occurring simultaneously in our observations. The mechanism of continuous endocytosis and exocytosis of FNDs could be necessary for cells to maintain normal proliferation. Furthermore, 120 h cell growth assay was performed to verify the long-term biocompatibility of FNDs for cellular studies.

INTRODUCTION

Fluorescent nanodiamonds (FNDs) are a promising class of carbon-based nanomaterials. The FNDs have exhibited potential applications in multidisciplinary sciences, especially in biomedicine. They have been studied for their potential applications, for example, for drug delivery, as nanosensors, bioimaging, and several other areas of biomedicine. The FNDs have shown to possess unique optical properties for bioimaging, because they contain high density of negatively charged nitrogen vacancies (NV−) rendering them with optical properties that make them exploitable as photostable fluorescent markers for single photon, multiphoton, and stimulated emission depletion (STED) microscopy, as well as small animal bioimaging. A single NV− has an optical absorption maxima at 580 nm and broad emission range of 670–800 nm, corresponding to nearly optimal spectral range in view of bioimaging needs and requirements. The biocompatibility of FNDs in cells has been thoroughly studied on different cell lines (in vitro) and in animal studies (in vivo). In vitro studies have reported that FNDs do not appear to significantly affect the cell differentiation, cell cycle progression, protein expression, or proliferation. In vivo toxicity studies have been performed on rabbits, mice, zebrafish, and Caenorhabditis elegans (C. elegans). Mohan et al. reported on a comprehensive study of in vivo toxicity and imaging of FNDs in C. elegans. Experimental observations with C. elegans have shown no detrimental effects on reproduction potential and longevity. Puzyr et al. conducted a long-term study for 3–6 months in mice by substituting water in diet and replacing it with nanodiamond colloids to investigate the effects on mice health. The experimental results have shown that nanodiamonds neither induce mortality nor affect the normal internal organ growth. However, based on mouse model studies, the level of toxicity can be dependent on dosage, surface functionalization, and routes of administration.

Cellular internalization of FNDs is reported to be driven predominantly by energy-dependent clathrin-mediated endo-
RESULTS AND DISCUSSION

Intracellular Trafficking of FNDs. An early endosomal marker (EEA1) was used as an internalization “coordinate” marker for FNDs in cells. The FND colocalization with early endosomes (2–48 h) was studied by immunofluorescence microscopy (Figure S1). Initial observation after 2 h internalization suggested that FND uptake in cells was mainly confined around early endosomes (Figure 1a). Nonfunctionalized FNDs were seen to be internalized as smaller aggregates (Figure S2). The FNDs were observed to be localized in early endosomes, and some early endosomes can also be seen without FNDs. Localization in early endosomes was again observed at 6 h. Confined in large endosomal aggregates at 6 h was observed (Figure 1b). The aggregates were approximately 1.5–2 μm in size as observed by fluorescence microscopy (Figure 1b). After 6 h, we started to observe two distinct populations of FNDs in single cells. A major FND population mainly aggregated in early endosomes, whereas a second widely dispersed population of FNDs was observed lying outside of the early endosomes. These two distinct FND populations were again observed after 24 h (Figure 1c). The size of early endosome-confined FNDs was approximately 2.5–3 μm in size at 24 h (Figure S3a). The majority of FND aggregates at this time was no longer bound to early endosomes. In addition to the aggregates, a distinct and scattered FND population was observed in proximity to the plasma membrane. The FNDs localized near the cell membrane were generally scattered and less aggregated as compared to the early endosome-bound FNDs (Figure S3b,c). After 48 h, the FNDs localized with early endosomes at 48 h were less than that observed at 24 h. The size of early endosome-confined FNDs at 48 h was slightly larger than that observed during 6 and 24 h (Figure 1d). However, compared with overall internalized FND population, the early endosome-confined FNDs represent a smaller fraction. There are also reported cases of FND endosomal escape. There are possibilities of endosomal escape of FNDs over time and the notion that FND endocytosis eventually gets slower at longer time points (48 h) as compared to earlier time points (6 and 24 h). The early endosomal localization of FND at 48 h appears to be recently endocytosed FNDs. We also observed a population of FNDs in the form of large-size aggregates that was not confined to early endosomes. Scattered
FNDs were again observed close to the cell membrane (Figures 1d and S4).

In a classical endocytosis process, the endosomes mature from early endosomes to late endosomes and finally fuse to lysosome-based degradation pathway. Therefore, we investigated the full lysosomal localization of nondegradable FNDs to study their subsequent fate (Figure S5). We used live-cell LysoTracker dye to label and track lysosomes. After 2 h incubation of live cells with FNDs, the particles were mainly seen outside of lysosomes (Figure 1e). The observation suggests that FNDs were localized in compartments other than lysosomes. The FNDs were observed to be mostly spread across the cells. Significant observation was made at 6 h incubation of cells with FNDs (Figure 1f). We observed a population of FNDs colocalizing with lysosomes. Another FND population was aggregated but not colocalized with lysosomes and lysosomes without any FNDs (Figure 1f). The observation suggests that only a part of FND population were inside of lysosomes. Therefore, a significant population was localized in other cellular compartments. At 24 h time point, we observed again three clearly distinct populations of FNDs in cells (Figure S6): aggregated FNDs colocalized with lysosomes, FNDs not colocalized with lysosomes, and a scattered population of FNDs in proximity to the plasma membrane. We had observed a similar localization of FNDs in proximity to the plasma membrane with EEA1-labeled cells (Figure 1c) at 24 h. The scattered FND population in proximity to the plasma membrane were associated neither with EEA1 nor with lysosomes (Figure 1g), thus, suggesting that these scattered FNDs could be ready to be exocytosed from cells. At 48 h (Figure 1h), some FNDs were colocalized with lysosomes forming large aggregates measuring up to 2–3 μm in size. Some lysosomes can be seen with few FNDs. Another consistent observation was the localization of FNDs in proximity to the plasma membrane (Figure S7). In summary, the FNDs were observed to be localized and present in different cellular compartments, such as the FNDs confined to early endosomes, the FNDs confined to lysosomes, and the scattered FNDs localized in proximity to the plasma membrane.

**FND Management by Cells.** The result of FND colocalization with early endosomes and lysosomes (Figure 1) can be interpreted as one population of FNDs that are localized either with early endosomes (Figure 1b–d) or lysosomes (Figure 1f–h) at 6, 24, and 48 h time points. However, another significant FND population was mainly located close to the plasma membrane. The FND localization (2–48 h) to EEA1 could be interpreted as continuous endocytosis of FNDs in cells (Figure 1a–d). However, continuous endocytosis of FNDs would finally lead to the accumulation of FNDs in cells. The FND accumulation could be speculated to cause adverse effects on cell proliferation and perhaps lead to cellular toxicity. However, we observed no adverse effects on cell growth in 120 h experiment of culturing cells with FNDs (Figure S8). The FNDs have previously been reported to be nontoxic.\(^4,7,12\) The consistent observation of FND localization with lysosomes at 6–48 h time points suggests that FNDs have to be exocytosed from cells. Therefore, it is logical to argue that exocytosis and endocytosis of FNDs are simultaneously occurring processes in cells. The cells could be using this mechanism to avoid possible toxicity induced by nondegradable FNDs.

The demonstration of FND exocytosis was performed with coculture cell studies. The FND-labeled eGFP MDA-MB-231 cells were cocultured with cancerous (HeLa and nonGFP MDA-MB-231) and noncancerous cell lines (HSF and MEF). The eGFP-expressing MDA-MB-231 cells were first cultured with FNDs for 6 h to facilitate FND internalization. The culture medium was then changed to one not-containing FNDs and HeLa; nonGFP MDA-MB-231, HSF, or MEF cells were seeded to the coculture. After 48 h of coculturing, a significant fraction of FNDs were exocytosed from GFP MDA-MB-231 and internalized by cells in the coculture (Figure 2a–d).

**Figure 2.** Observation of FND exocytosis in cocultured cells. FNDs (red) exocytose from eGFP MDA-MB-231 cells were internalized (arrow) to other cells in coculture. (a) HeLa cell, (b) HSF, (c) nonGFP MDA-MB-231 cells, and (d) MEF cells.

**Distribution of Vesicle-Bound and Peripheral FNDs.** FNDs were earlier observed by fluorescence microscopy to form two visibly distinct populations: vesicle-confined FND (endosomal, lysosomal) and scattered FNDs that were mainly located in proximity to the plasma membrane (Figure 1). Electron microscopy verified (Figure 3a,b) that after 24 h, FNDs were mostly localized in the vesicular space (green arrow) or near the plasma membrane (orange arrow). There was a progressive aggregation of FNDs seen in the vesicular space (Figure 3c,d) at 48 h internalization. Vesicles were mostly packed with FNDs, and the size of the aggregates was approximately 1–2 μm as observed using a transmission electron microscope (Figure 3d).

In one individual observation, after 72 h of FND internalization, we observed an FND-packed vesicle that may lead to exocytosis of FNDs from the cell (Figure 3e). The vesicles were localized on the extreme edge of the plasma membrane. We suspect it to be off-loading the vesicular content in the extracellular space. However, other FNDs remained confined to the vesicular space (Figure 3f). Also during longer investigation time points (96 and 120 h), we had similar observations of FNDs being mostly confined to the vesicular space in an aggregated manner (Figure 3g–j). We did not observe the interaction of FNDs with cellular organelles such as nuclei, mitochondria, or Golgi (Figure S9). The FNDs localized in the vesicular spaces were aggregated and distinct from the empty vesicle of control cells (Figure 3k,l). The dark spots present in the TEM images were observed to be seen with both FND-containing cells and control cells. The dark spots are mostly spherical and easily distinguishable from FNDs (Figure S10). The TEM imaging of FNDs in cells confirms the presence of two distinct FND populations: one being
aggregated and confined to the vesicular space and the other being mostly dispersed near the plasma membrane.

**CONCLUSIONS**

The fluorescence microscopy study presented suggests that significant populations of cellular FNDs were localized within early endosomes and lysosomes at the same time (6, 24, and 48 h). These observations imply that cells could be regulating the FND population by continuous endocytosis and exocytosis. This could be a rational mechanism for the cells to overcome the potential toxic effects of nondegradable FNDs. The observations with fluorescence microscopy in combination with TEM can be concluded as the presence of distinct FND populations in cells. The FNDs were mainly localized with: (1) early endosome-confined FNDs, (2) lysosome-confined FNDs, and (3) FNDs localized in proximity to the cell membrane. The vesicle-bound FNDs (early endosomal and lysosomal) were mostly aggregates. The sizes of the vesicular-bound FND aggregates were roughly up to 2 μm, and the fusion of vesicles to even larger multivesicular bodies was observed. The FNDs localized in proximity to the plasma membrane were more dispersed and less aggregated. The proximal FNDs are suggested to be those being exocytosed from the cells. The exocytosis of FNDs was demonstrated with coculture studies. In summary, this study proposes new insights for understanding the FND interaction with human cells and could explain possible mechanisms for management of nondegradable FNDs.

**EXPERIMENTAL SECTION**

**Fluorescent Nanodiamonds.** The FNDs (~100 nm in diameter) were produced by radiation damage of synthetic type-Ib diamond powders (micron + MDA, element six) with 40 keV He⁺, followed by vacuum annealing at 800 °C for 2 h, air oxidation at 450 °C for 1 h, and acid wash in concentrated H₂SO₄/HNO₃ (3:1, v/v) at 100 °C for 3 h. They are well-established as bright and photostable and are reported for various biological applications. The material synthesis, characterization, and imaging potential have been reported by Chang et al., 2008.

**Live Cell Imaging of Lysosomal-Localized FNDs.** The MDA-MB-231 (human breast adenocarcinoma) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2mM l-glutamine, and 1% penicillin–streptomycin (v/v). The FND particles (10 μg/mL) were prepared in 1 mL of cell growth media. The MDA-MB-231 cells were incubated with FNDs (10 μg/mL) at different time points (2, 6, 24, and 48 h). The medium (0.5 mL) was collected from the plate and mixed with 0.6 μL of LysoTracker Green (Thermo Fisher Scientific Inc, USA) and added back to the media drop-by-drop to the plate. The cells were finally incubated for 20 min in 37 °C incubator. Before imaging, regular DMEM with supplements [10% fetal bovine serum, 2mM l-glutamine, and 1% penicillin–streptomycin (v/v)] were added to the cells. The live cell microscopy setup consisted of a Leica TCS SP5 STED (Leica Microsystems, Germany) instrument, LASAF software (Leica application suite), photomultiplier tube (PMT), and 63× water objective. The cells were maintained at 37 °C, 5% CO₂ during live cell microscopy measurements. LysoTracker Green and the FNDs were excited using an argon laser of 488 nm.

**Immunofluorescence Microscopy of EEA1-Bound FNDs.** The MDA-MB-231 cells (human breast adenocarcinoma) were cultured as described above. The FND particles (10 μg/mL) were prepared in 1 mL of cell growth media. The cells were fixed at 2, 6, 24, and 48 h to investigate early endosomal localization. The cells were fixed in 4% paraformaldehyde (PFA) for 10 min. The cells were permeabilized using 0.1% Triton X-100 for 10 min and blocked with horse serum. A 1° anti EEA1 antibody (Thermo Fisher Scientific Inc, USA) was prepared (1:100) in PBS (10% horse serum). Antibody conjugation was performed overnight at +4 °C. The cells were washed three times with PBS; Alexa 488 secondary antibody (Sigma-Aldrich, US) in PBS was added to the cells at RT for 1 h. The cells were mounted over coverslips with VECTASHIELD (4',6-diamidino-2-phenylindole). The microscopy setup consisted of a Leica TCS SP5 STED (Leica
Microsystems, Germany) instrument, LASAF software (Leica application suite), PMT, and 100x oil objective. Alexa 488 and the FNDs were excited using an argon laser of 488 nm.

**Electron Microscopy of Subcellular Localized FNDs.** The MDA-MB-231 cells were incubated with FNDs (10 μg/mL) for respective time points (24–120 h). Then, the cells were fixed with 4% PFA for 10 min after 48 h coculturing. The cocultured cells (mouse embryonic fibroblasts), and MEF cell lines without FND incubation such as HeLa (human cervical cancer cells), MDA-MB-231 nonFND (human breast adenocarcinoma), HSF (human skin fibroblasts), and MEF (mouse embryonic fibroblasts) cells. The cells were fixed with 4% PFA for 10 min after 48 h coculturing. The cocultured cells were imaged using a confocal microscope. The microscope setup consisted of a Leica TCS SP5 STED (Leica Microsystems, Germany) instrument, LASAF software (Leica application suite), PMT and 100x oil objective. The GFP and the FNDs were excited using an argon laser of 488 nm.

**Exocytosis of FNDs (CoCulture Studies).** The MDA-MB-231 eGFP-expressing cells were incubated with 10 μg/mL FNDs for 6 h. Then, these cells were cocultured with different cell lines without FND incubation such as HeLa (human cervical cancer cells), MDA-MB-231 nonFND (human breast adenocarcinoma), HSF (human skin fibroblasts), and MEF (mouse embryonic fibroblasts) cells. The cells were fixed with 4% PFA for 10 min after 48 h coculturing. The cocultured cells were imaged using a confocal microscope. The microscope setup consisted of a Leica TCS SP5 STED (Leica Microsystems, Germany) instrument, LASAF software (Leica application suite), PMT and 100x oil objective. The GFP and the FNDs were excited using an argon laser of 488 nm.

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**ASSOCIATED CONTENT**

1. Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00339.

Temporal localization of FNDs with lysosomes, early endosomes, early aggregated internalization of FNDs in cells, early endosomal localization of FNDs at 24 and 48 h, lysosomal localization of FNDs at 24 and 48 h, cell viability assay for 120 h with FND-internalized cells, and TEM of vesicle bound FNDs and control cells (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: jessica.rosenholm@abo.fi (J.M.R.).

**ORCID**

Huan-Cheng Chang: 0000-0002-3515-4128
Jessica M. Rosenholm: 0000-0001-6085-1112

**Notes**

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