Fluorescent Nanodiamonds Encapsulated by Cowpea Chlorotic Mottle Virus (CCMV) Proteins for Intracellular 3D-Trajectory Analysis

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Long-term tracking of nanoparticles to resolve intracellular structures and motions is essential to elucidate fundamental parameters as well as transport processes within living cells. Fluorescent nanodiamond (ND) emitters provide cell compatibility and very high photostability. However, high stability, biocompatibility, and cellular uptake of these fluorescent NDs under physiological conditions are required for intracellular applications. Herein, highly stable NDs encapsulated with Cowpea chlorotic mottle virus capsid proteins (ND-CP) are prepared. A thin capsid protein layer is obtained around the NDs, which imparts reactive groups and high colloidal stability, while retaining the opto-magnetic properties of the coated NDs as well as the secondary structure of CPs adsorbed on the surface of NDs. In addition, the ND-CP shows excellent biocompatibility both in vitro and in vivo. Long-term 3D trajectories of the ND-CP with fine spatiotemporal resolutions are recorded; their intracellular motions are analyzed by different models, and the diffusion coefficient are calculated. The ND-CP with its brilliant optical properties and stability under physiological conditions provides us with a new tool to advance the understanding of cell biology, e.g., endocytosis, exocytosis, and active transport processes in living cells as well as intracellular dynamic parameters.

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Fluorescent Nanodiamonds Encapsulated by *Cowpea Chlorotic Mottle* Virus (CCMV) Proteins for Intracellular 3D-Trajectory Analysis

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Keywords: Nanodiamond, virus capsid protein, intracellular trajectory, diffusion coefficient, mean square displacement.

Abstract Long-term tracking of nanoparticles to resolve intracellular structures and motions is essential to elucidate fundamental parameters as well as transport processes within living cells. Fluorescent nanodiamond (ND) emitters provide cell compatibility and very high photostability. However, high stability, biocompatibility, and cellular uptake of these fluorescent NDs under physiological conditions are required for intracellular applications. Herein, highly stable NDs encapsulated with *Cowpea chlorotic mottle virus* capsid proteins (ND-CP) are prepared. A thin capsid protein layer is obtained around the NDs, which imparts reactive groups and high colloidal stability, while retaining the opto-magnetic properties of the coated NDs as well as the secondary structure of CPs adsorbed on the surface of NDs. In addition, the ND-CP shows excellent biocompatibility both *in vitro* and *in vivo*. Long-term 3D trajectories of the ND-CP with fine spatiotemporal resolutions are recorded; their intracellular motions are analyzed by different models, and the diffusion coefficient are calculated. The ND-CP with its brilliant optical properties and stability under physiological conditions provides us with a new tool to advance the understanding of cell biology, e.g., endocytosis, exocytosis, and active transport processes in living cells as well as intracellular dynamic parameters.
Nanodiamonds (NDs), which consist of an all-carbon sp$^3$-scaffold, exhibit both exciting opto-magnetic properties and excellent biocompatibility. Point defects in the lattice of NDs, such as nitrogen-vacancy (NV) centers, provide stable fluorescence without bleaching or blinking, even after several months of continuous irradiation.\textsuperscript{1} In addition, the emission wavelength of NDs is size-independent and tunable from the visible to the near-infrared region by using different defects (e.g. SiV, GeV etc.).\textsuperscript{2} Hence, NDs are widely used in bioimaging\textsuperscript{3-5}, and drug delivery\textsuperscript{6-10}. In addition, it has been proven that NDs can act as cellular biomarkers for long-term intracellular tracking.\textsuperscript{11-13} Furthermore, NDs containing negatively charged NV centers can also serve as single-spin sensors to detect many essential properties in the biological microenvironment, such as temperature\textsuperscript{14-16}, magnetic fields\textsuperscript{17, 18}, electron spins\textsuperscript{19, 20}, and strain\textsuperscript{21} with nanoscale resolution.

For most of these applications, appropriate surface functionalization of NDs is required because the poor colloidal stability of unmodified NDs under physiological conditions leads to uncontrolled aggregation in aqueous media\textsuperscript{22}. Surface functionalization, like harsh chemical treatments or air oxidation, improves surface uniformity and provides new binding sites for the attachment of drug molecules, dyes, targeting groups, or antibodies.\textsuperscript{23, 24} Furthermore, surface coating is crucial to avoid foreign body interactions of the particles \textit{in vivo}\textsuperscript{25} and allows the nanoparticles to accumulate and remain at the target sites for a
prolonged time period. Different surface coatings have been developed for this purpose, such as silica\textsuperscript{26}, hyperbranched polyglycerol (HPG)\textsuperscript{27}, poly(L-DOPA)\textsuperscript{7}, insulin\textsuperscript{28}, and human serum albumin (HSA), which was modified with polymers\textsuperscript{6}, among others. In addition to synthetic common protein coatings, hybrid virus-like particles have recently gained attention due to their straightforward preparation, high biocompatibility, and unique properties and their application as drug delivery vehicles.\textsuperscript{29}

Viruses are evolutionary optimized carrier systems. Uptake and diffusion pathways of viruses to their host cells are still barely understood.\textsuperscript{30} However, it is very important to elucidate virus cell interactions and their trafficking inside cells in order to identify new therapeutic interventions or to mimic such processes for improved drug delivery of virus-like nanoparticle transporters. \textit{Cowpea chlorotic mottle virus} (CCMV) has gained great interest in recent years as tools in nanotechnology as well as for the development of targeted drug delivery vehicles due to their perfectly defined structure, high stability, good biocompatibility, homogeneity, self-assembly, and low toxicity.\textsuperscript{31} The size of the native CCMV is about 30 nm in diameter and can be disassembled into capsid proteins (CP). The genome can be removed by centrifugation under high salt concentrations at neutral pH.\textsuperscript{32} The thus obtained CPs reassemble into either empty capsids at lower pH or virus-like particles (VLPs) at neutral pH using templates such as negatively charged inorganic nanoparticles,\textsuperscript{33, 34} negatively charged polymers,\textsuperscript{35} enzymes,\textsuperscript{36} and organic
aggregates\textsuperscript{37} among others. Furthermore, by controlling the pH and ionic strength of the assembly buffer, CCMV can be reassembled into a variety of geometries such as tubes, multilayered structures, and dumbbells.\textsuperscript{38-41} Despite their broad applications, CCMV cell uptake and diffusion inside cells is still barely understood.

Here, we report the preparation and characterization of fluorescent nanodiamonds encapsulated within \textit{cowpea chlorotic mottle virus} capsid proteins (ND-CP) as model system for fluorescence imaging and single particle tracking applications inside living cells. We believe that our strategy could also be applicable to other viruses, which could give new insights into their intracellular pathways, which would be of fundamental interest as well as for the design of improved drug delivery systems.

A schematic outline of the preparation of ND-CP is displayed in Figure 1. Firstly, CPs were isolated from the \textit{CCMV} and stored in a capsid storage buffer according to our previous work\textsuperscript{34}. Before encapsulation of the NDs, the CPs were transferred into a coat protein buffer. Then NDs in a polyvinylpyrrolidone (PVP) solution were added to the CPs and the mixture was stirred at 4 °C overnight.
Figure 1. Schematic illustration of the preparation of *cowpea chlorotic mottle virus* capsid protein encapsulated fluorescent nanodiamonds (ND-CP).

Free CPs and encapsulated ND-CPSs were separated and removed by fast protein liquid chromatography (FPLC) equipped with a UV-vis detector. As shown in Figure 2A, the NDs coated with CP both in PBS buffer and Tris buffer eluted at a lower volume ($V \approx 8 \text{ mL}$) than the native CCMV virus ($V \approx 9.5 \text{ mL}$)\(^{33}\), suggesting that NDs were successfully coated. The successful coating of NDs with CPs was also confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S2), which shows that the band of purified ND-CP appeared at the same position as native CCMV. To test whether the CPs retained their native structure after adsorption to the ND surface, the secondary structure was investigated by circular dichroism (CD) spectroscopy. As shown in Figure 2B, only native CP and ND-CP showed a signal in CD, and both spectra were very similar, indicating that the native structure of CP was not affected. Subsequently, the hydrodynamic sizes of the ND-CP were characterized by dynamic light scattering (DLS) (Figure 2C). The average hydrodynamic diameters changed from $35 \pm 2 \text{ nm}$ in water before coating to $46 \pm$
3 nm for NDs in Tris buffer containing PVP, and 52 ± 5 nm for ND-CP in Tris buffer, respectively. Furthermore, ND-CP showed a monomodal distribution in DLS. Uncoated NDs and ND-CP were characterized in terms of their distribution, shape, and morphology by transmission electron microscopy (TEM). Whereas bare NDs were prone to significant aggregation and displayed a heterogeneous distribution (Figure 2D), non-aggregating, homogeneous single particles were observed for ND-CP (Figure 2E). The histogram analysis of TEM images of ND and ND-CP showed a size increase from 15.9 ± 10.6 nm to 23.2 ± 11.2 nm, respectively (Figure S3). From the TEM images (Figure S4), we determined a shell thickness of 3.03 ± 0.96 nm ($n = 36$) of ND-CP, which corresponds nicely to the diameter of the $CCMV$ capsid. To further assess the structure of the ND-CP, the liquid mode of atomic force microscopy (AFM; Figure S5) was applied. The topographic image of ND-CP showed a narrow distribution with no significant aggregation. The height-sensor images recorded by AFM were converted into deformation images using the NanoScope Analysis 1.8 software to visualize the CP coating. In addition to the height profile images, other nanomechanical properties were simultaneously recorded. In particular, the deformation of the sample caused by the probe was analyzed to obtain in-depth information on the structure of the coated NDs. As the ND core is much harder than the CP shell, the deformation of the CP shell under the same stress conditions could be detected with a high intensity. The deformation image revealed clearly that all ND-CP possessed a core-shell structure.
Figure 2. (A) FPLC of ND and ND-CP in Tris buffer (50 mM Tris, 50 mM NaCl, 10 mM KCl, pH 7.2), ND-CP in PBS buffer (PBS, 150 mM NaCl, pH 7.2); (B) CD spectra of ND, CP and ND-CP; (C) DLS diagram of uncoated NDs in water, ND+PVP and ND-CP in Tris buffer; (D) TEM image NDs (scale bar: 500 nm); (E) TEM images of ND-CP at different magnifications.

To prove that the optical properties of NDs were retained, the influence of the CP shell on the charge state of the NV centers in NDs was investigated (Figure 3A). Spectra measurements were performed on a custom-built confocal microscope with an excitation
laser at 532 nm and 100 µW power in front of the objective (oil, NA = 1.35) (Figure S1). The spectra of ND-CP revealed that the CP proteins on the surface of NDs did not affect the fluorescence, and the zero phonon lines of NV centers were clearly visible without any shift or background noise. NV centers in NDs are very sensitive to the surface states and their charge state can switch to neutral or positive, which is optically nonactive. These results demonstrate that the CP coating did not affect the charge properties of NV centers. These remained in the optical and spin active state (NV\(^-\)), which is essential for applications of ND-CP in bioimaging, tracking, and nanoscale sensing. The biocompatibility of ND-CP was investigated using HeLa cell line as well as in a chorioallantoic membrane (HET-CAM) ex vivo model\(^{42}\). As depicted in Figure 3B, the ND-CP showed low cytotoxicity upon treating cells at high concentrations of up to 200 µg/mL. It is worth noting that the HET-CAM method represents a potential alternative to animal experiments to assess toxicity and is widely applied to replace animal experiments.\(^{42}\) In our HET-CAM assay, we observed hemorrhage from blood vessels that started within 2–4 seconds after applying 1% sodium dodecyl sulfate (SDS) as positive control. Lysis occurred after 27 seconds, and we observed coagulation within 24 hours. The negative control (phosphate buffered saline) and two concentrations of NG-CP (50 µg/mL, 100 µg/mL) did not show any irritation (such as hemorrhage, vascular lysis, or coagulation) within 5 minutes to 24 hours after application (Figure S6). A summary of the results is shown in Table S2. These in ovo results verify the high biocompatibility of ND-CP.
To track the motion of the ND-CP inside living cells, HeLa lung cancer cells were incubated with 20 µg/mL ND-CP for 4 hours. The measurements were performed on a custom-built confocal microscope with an excitation laser at 532 nm (Figure S1). As depicted in Figure 3C, ND-CPs were efficiently taken up by Hela cells at concentrations of 20 µg/mL. To track ND-CP nanoparticles, the spectrum of the selected spots inside living cells was first measured to confirm the presence of ND-CP in the focus volume (Figure 3D). Then, the fluorescence intensities of the tracked ND-CPs were recorded simultaneously to ensure that the same ND-CP was being tracked (Figure 3F). A representative trajectory of single particles is shown in Figure 3E for 43 minutes and two other trajectories are depicted in Figure S7.

Figure 3. (A) Normalized emission spectra (ex. 532 nm) of ND and ND-CP. NV− zero
phonon lines are visible in both spectra. (B) Cell viability of ND-CP in HeLa cells. (C) X-Y axis confocal microscopy images of ND-CP taken up into HeLa cells at 20 µg/mL for 4 hours incubation. (D) Emission spectra (ex. 532 nm) of ND-CP in HeLa cell. (E) The trajectory of tracked ND-CP spot 1 in the intracellular space of HeLa cell. (F) Real-time counts of fluorescence of the tracked ND-CP spot 1 with continuous refocusing (upper panel) and regional enlarged view showing three refocusing steps (lower panel).

The high photostability of NDs provides the opportunity to study intracellular dynamic parameters of ND-CP inside living cells. We performed a set of proof of principle experiments by recording 3D trajectories of three different ND-CP spots. Trajectory 1 was observed for 43 minutes containing 520 points, whereas Trajectories 2 and 3 were recorded for 7 minutes with 88 and 81 points, respectively. For trajectories 2 and 3, long-time trajectory measurements did not succeed due to interference from other ND-CPs that could not be distinguished during the refocusing process. With increasing tracking time, the probability of interference from nearby ND-CPs increases as well, thus limiting the maximum tracking time for individual ND-CPs. The obtained data points allowed calculating the mean square displacement (MSD), which is a measure of the deviation of the position of NP-CP nanoparticles with respect to a reference position over time. The results for each NP-CP spot are presented in Figure 4A (see MSD analysis for calculations details in SI). Subsequently, the diffusion behavior of the ND-CPs was obtained by fitting
the MSD data with a power-law (Equation S2), where the power index $\alpha$ reflects the diffusion behavior of the tracked nanoparticle with $\alpha < 1$, $\alpha = 1$, and $\alpha > 1$ to identify the dynamic movements of ND-CPs inside cells.$^{43, 44}$ Within the complex and highly heterogeneous, crowded environment in the cell’s cytoplasm, normal diffusion, anomalous diffusion, confined diffusion or directed motion of nanoparticles may occur.$^{45}$ In normal diffusion, nanoparticles diffuse completely unrestricted, whereas directed motion represents an active process that has been observed when small vesicles are transported by molecular machines along microtubules.$^{46, 47}$ Confined diffusion has been observed for trapped particles or particles whose free diffusion is confined by cytoskeletal elements.$^{48}$ However, the origin of anomalous diffusion is commonly traced back to the macromolecular crowding in the interior of cells, but its precise nature is still under discussion.$^{45, 49}$

According to Saxton$^{50}$, we focused on data with $t_{\text{lag}} < t_{\text{total}} / 4$, where $t_{\text{total}}$ is the total time of the trajectory. Our experiments showed a confined diffusion at the short lag time, which was also observed for single-walled-carbon-nanotube (SWNT)-labeled kinesins in COS-7 cells before.$^{51}$ Previously, this behavior was allocated to the existence of mechanical obstacles such as microtubule-microtubule intersections that exist in the complex cytoskeletal network of cells$^{51, 52}$. We have distinguished normal and anomalous diffusion of ND-CPs by determining the cumulative distribution function (CDF) of the square
displacements at a given $t_{\text{lag}}$.\textsuperscript{43, 53} To provide the CDF computation, the probability distribution function (PDF) of the square displacements was first calculated. The PDF and CDF were analyzed only for trajectory 1 because of the largest number of points, as shown in Figure 4B. The CDF data were fitted with single and double exponential functions, where the double function has fast and slow mobility components. The double fit has a much smaller residual than the single exponential fit. The ratio between the fast and slow mobility parts in the double fit confirms that the diffusion behavior of the ND-CP in HeLa cells is indeed a combination of normal (i.e., MSD $\propto t_{\text{lag}}$) and anomalous (i.e., MSD $\propto t_{\text{lag}}^\alpha$, with $\alpha$ deviating from one) diffusion. The diffusion coefficient ($D_0$) calculated from the single exponential fit is equal to $2.09 \times 10^{-4}$ $\mu$m$^2$/s. The double exponential fit gives two diffusion coefficients $D_1 = 2.95 \times 10^{-5}$ $\mu$m$^2$/s and $D_2 = 2.93 \times 10^{-4}$ $\mu$m$^2$/s. We also calculated the lag-time-dependent diffusion coefficient of the ND-CP in HeLa cell from the MSD data for each of the three recorded trajectories (for more details see SI). We obtained the diffusion coefficients of $1.25 \times 10^{-4}$ $\mu$m$^2$/s, $3.07 \times 10^{-4}$ $\mu$m$^2$/s, and $1.39 \times 10^{-3}$ $\mu$m$^2$/s for trajectory 1, 2, and 3, respectively (Figure 4C). They are comparable to the values obtained from the single and double exponential fits of the CDF data. The nominal diffusion coefficient of the ND-CP in the HeLa cell was then determined to be the average of these three values, that is, $6.07 \times 10^{-4}$ $\mu$m$^2$/s.

Interestingly, the recorded intracellular trajectories of ND-CPs are in line with previous
reports on the diffusion of adeno-associated virions in the cytoplasm of a living HeLa cell, where intracellular trajectories have also been classified as anomalous diffusion and normal diffusion. It has been speculated that anomalous diffusion occurred due to nonspecific interactions between the virions and obstacles inside the cytoplasm such as organelles, which could also be the origin of the anomalous diffusion of ND-CPs. Therefore, ND-CPs consisting of a fluorescent nanodiamond core and capsid protein corona could represent valuable tools to elucidate the infection pathway of the virions over the cytoplasm.

Figure 4. (A) Mean square displacement (MSD) vs. lag time ($t_{\text{lag}}$) for three independent trajectories. The segments of the MSD profiles at short and intermediate lag times are analyzed using a power law relation. The power indices are indicated. (B) Probability density function (PDF, upper panel, note the linear scale of the x axis) and cumulative density function (CDF, lower panel) distributions of the square displacements at $t_{\text{lag}} = 2\Delta t$
= 10 s. The CDF distribution is fitted with single and double exponential functions. The residuals of the fitting are shown on the top of the lower panel. (C) Time-dependent diffusion coefficient (D) of the three trajectories (see (A) for the legends). The nominal D values are calculated as the average values at intermediate lag times, as indicated by the black horizontal lines. In these ranges of the lag times, the power indices in (A) are close to one, implying normal diffusion.

In conclusion, we have developed a virus-inspired NDs hybrid system consisting of a negatively charged fluorescent nanodiamond core stabilized by a corona of virus capsid proteins. ND-CP showed excellent stability in PBS and Tris buffer. Both the NDs and CPs retained their unique features, such as photophysical properties and 3D structure facilitating cellular uptake. Furthermore, they showed good biocompatibility up to 100 μg/mL in vitro and in vivo, which is essential for bioapplications, such as bioimaging and intracellular trajectory analysis. The estimation of intracellular motions of ND-CP revealed confined diffusion at the beginning, which then changed to normal diffusion (i.e., MSD ∝ t_{lag}), which has also been observed for adenovirus-like particles. We believe that ND-CP represent a useful tool to study the intracellular motions over long time periods and with spatiotemporal details in living cells. In combination with optically detected magnetic resonance technique, one could also envision simultaneous elucidation of intercellular magneto-, electro-, and temperature-information at the nanoscale, which will allow
acquiring quantitative information on the intracellular pathways of virions. The trajectory analysis of the ND-CP have the potential to enhance the conceptual understanding of cell-virus interactions, host adaption, and immune regulation, and elucidate the interactions between eukaryotes and viruses during infection.

Conflicts of interest

The authors declare no competing financial interest.

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Supplementary Information

**Fluorescent Nanodiamonds Encapsulated by Cowpea Chlorotic Mottle Virus (CCMV) Proteins for Intracellular 3D-Trajectory Analysis**

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1. Experimental section

**Materials:** Nanodiamonds with 35 nm average diameter were purchased from FND Biotech (Taiwan), polyvinylpyrrolidone (MW: 10 kDa) was purchased from Sigma-Aldrich, Quartz glass cuvette were purchased from Hellma-analytics, Quartz SUPRASIL (QS). Amicon Ultra centrifuge filter with 10K MWCO from Pall MicrosepTM Advance Centrifuge Device with Omega Membrane were used for particle preparation. Dulbecco’s Modified Eagle’s Medium (DMEM, 1x), Dulbecco's phosphate-buffered saline (DPBS, 1x), fetal bovine serum (FBS), Penicillin Streptomycin (Pen Strep) were purchased from gibco. All solvents and chemicals were purchased from commercial sources and were used without further purification.

**Preparation of CCMV**

Pots are filled with soil and the Cowpea beans are planted in the soil. The plants are allowed to grow for ten days and watered regularly. At this point primary leaves are present. The leaves are dusted with carborundum. The inoculation solution is prepared with a purified virus. In both cases a mixture of sap from ground leaves, water and purified virus solution (0.5 mg virus) is prepared. The leaves are inoculated by smoothly rubbing. By using a household sprayer, the plants are immediately sprayed with water after inoculation to prevent leaf dehydration. Seven days after inoculation, the leaves are harvested by cutting them from the stem. The plant material is cut into pieces and blended in cold homogenization buffer (0.2 M NaAc, 0.01 M ascorbic acid, 0.01 M Na2EDTA). Two layers of cheesecloth are boiled in water containing EDTA and rinsed with MilliQ water. The homogenate is filtrated to remove the larger plant debris. The homogenate is kept at T
= 4 °C for an hour to allow the proteins to precipitate. The homogenate is subjected to low-speed centrifugation to precipitate the leaf tissue. The pellet is discarded and the supernatant is added to 10 % (w/v) solid PEG (MW = 6000 g/mol). The mixture is stirred for 1 h at T = 4 °C. The precipitate is pelleted by low-speed centrifugation. The supernatant is discarded and the bottles are drip-dried thoroughly to remove the PEG solution (Hebert, T. T., Precipitation of plant viruses by polyethylene glycol. Phytopathology 1963, 53, 362.) The pellet is suspended in cold virus buffer (0.1 M NaAc, 1 mM Na2EDTA, 1 mM NaN3) with the help of a glass stick or a pipette. The resuspension is cleared of undissolved material by low-speed centrifugation.

The pellet is discarded and the supernatant is mixed with cesium chloride. Once completely dissolved, the mixture is subjected to a density gradient centrifugation. The obtained solution is dialyzed against virus buffer at T = 4°C, and then stored at T = 4 °C. The presence and purity is checked by SDS-PAGE and FPLC. Typical yields of CCMV are 200–300 mg per kg of cowpea tissue. During the whole process, the virus solution is kept cold either in an ice bath or in the cold room (T = 4°C).

**Preparation of CP**

RNA in a suspension of CCMV (1 mL 10 mg/mL) is precipitated by dialysis against RNA buffer (0.05 M Tris-HCl, 0.5 M CaCl₂, 1 mM DTT) at 4 °C. The white precipitate containing RNA is centrifuged down. The supernatant is removed and the pellet discarded. The supernatant is afterwards dialyzed against cleaning buffer (0.05 M Tris-HCl, 0.5 NaCl, 1 mM DTT). The dissociated protein without RNA is obtained and associated into spherical particles by dialysis against capsid storage buffer (0.05 M NaAc, 1 M NaCl, 1 mM NaN₃).
Preparation of ND-CP

In a typical experiment, NDs solution (400 μL, 0.2 mg/mL; H₂O) is added to a solution of CCMV coat protein (100 μL, 15 mg/mL; pH 7.2; 250 mM Tris, 500 mM NaCl) and allowed to incubate overnight at 4 °C. The reaction mixture is subsequently resulting CP-NDs are purified using preparative FPLC.

Purification of ND-CP by fast protein liquid chromatography (FPLC)

FPLC analysis were performed on a GE Healthcare ÄKTApurifierTM system equipped with a Superose 610/300 GL column from GE Healthcare and a fractionating device. Injection of 500 µL pre-filtered samples which are injected on a 24 mL superpose-6 column. Compound elution is monitored using a UV-vis spectrometer at 260 nm, 280 nm. Fractionation are collected separately

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE samples are prepared by mixing 10 µL of sample with 9 µL of sample buffer (125 mM Tris-HCl, 20% (v/v) glycerol, 5% (w/v) sodium dodecyl sulfate, 0.02% (w/v) bromophenol blue, pH 6.8) and 1 µL 2-mercaptoethanol. The mixture was heated at 99 °C for 5 minutes to denature the protein, after which the mixture was used to fill the wells of 4-15% stain free pre-cast poly acryl amide gels (Bio-Rad). Precision Plus Protein™ Unstained Protein Standard was added in a separate well. Electrophoreses was conducted at 100 V for 5 minutes followed by 200V for approximately 20 minutes. Gels where activated with UV for 5 minutes on a stain-free enabled UV transilluminator and imaged
with a Gel Doc™ EZ system with Image Lab software (Bio-Rad).

**Dynamical light scattering (DLS)**

DLS analysis was performed using a Nanotrac (Anaspec) instrument. Microtrac FLEX Operating software was used at 25°C with laser wavelength of 780 nm and a scattering angle of 90°. The observed size and standard deviation of the nanoparticles were calculated by taking an average of 5 measurements.

**Transmission Electron Microscopy (TEM)**

4 µL 0.1 mg/mL solution of ND-NGs in MilliQ was placed onto an oxygen treated carbon coated copper grid. After 10 minutes the solution was removed using a filter paper and grids were stained with uranyl acetate 4% for 1.5 minutes. The grids were washed three times with MilliQ water and dried before measuring. A Jeol 1400 transmissions electron microscope was used to obtain bright field images. And Image J software was used to process the data.

**Atomic Force Microscopy (AFM)**

Atomic force microscopy was conducted in liquid state with a Bruker Dimension FastScan BioTM atomic force microscope, which was operated in PeakForce mode. AFM probes with a nominal spring constant of 0.25 N m⁻¹ (FastScan-D, Bruker) were used. The samples were diluted with MilliQ water to a concentration of 0.05 mg mL⁻¹. Sample solution (30 µL) was added onto a freshly cleaved mica substrate (circular, 15 mm) and incubated for at least 10 minutes to allow deposition of the structures. Remaining solution
was removed and 300 µL MilliQ water was applied onto the mica surface, forming a droplet for measuring in liquid. Samples were scanned with scan rates between 1 and 2 Hz and scan sizes between 0.5 and 2 µm. Images were processed with NanoScope Analysis 1.8.

**Cytotoxicity measurements**

HeLa cells were seeded with DMEM (contains 10% FBS, 1% MEM NEAA, and 1% PenStrep) on a 96 well half-area flat bottom microplate (50 µL/well, $1.3 \times 10^5$ cells/mL), the plate was then incubated at 37 °C, 5% CO$_2$ overnight. Next day, different concentrations of ND-CP samples were added to the cells and incubated again for overnight. Next day after removing the ND-CP containing medium and washing each well three times with DPBS, luminescent cell viability assay (CellTiter-Glo) was added to the cells as instructed by the manufacturer’s protocol. To measure the luminescence, a Promega GloMax plate reader was utilized.

**Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM)**

Fertile eggs were purchased from LSL Rhein-Main Geflügelvermehrungsbetriebe GmbH & Co.KG, Germany. The eggs were wipe-cleaned carefully, then incubated at 37 °C for 3 days. The pointy end of the eggs were placed downward during the incubation. From the first day of incubation, we started to count the so called Embryo development day (EDD). On the EDD 3, using a syringe and needles- about 6 mL egg albumin was removed from each egg. Afterward, a small portion of the eggshell was carefully cut with a surgical scissor and removed from the wide end of the eggs. By employing a thin parafilm tape, the open area was sealed again. The eggs were then incubated again for 7 more days till EDD 10.
The eggs were inspected frequently (at least once every 24 hours) to remove any nonviable egg.

On the EDD 10, eggs with healthy embryo were separated and treated with ND-CP, positive and negative controls (1% SDS and PBS). During the material dropping, eggs were always placed near a light bulb to simulate the incubator temperature. The samples were dropped directly on the chorioallantoic membrane of three eggs, 10 μL of each sample. Before adding the materials and after 5 minutes of application of the samples, high resolution photographs were taken. We also recorded videos continuously for 5 minutes during the application of ND-CP, SDS, and PBS on CAM of each eggs to detect any exhibition of hemorrhage, coagulation, or vascular lysis. After adding the materials- eggs were placed in the incubator again. Next day, after about 24 hours, photograph was taken of the material application sites.

We used the Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) method as a potential alternative to animal experiments. Fertile eggs are widely available, and the egg hatching temperature (37-38 °C) easy to achieve- these makes the HET-CAM a nice alternative experiment platform compared to the animal models. The vascular network formation of the chorioallantoic membrane (CAM) is suitable for studying tumor growth, drugs delivery, tissue xenograft, wound healing, as well as toxicological study. It was reported that the CAM is not innervated. Additionally, the chick embryo advances to a functional brain on EDD 13. Thus, we can consider the HET-CAM model as a humane alternative of animal testing. After adding the materials to the CAM, following three category of irritation reactions can be detected on the CAM: hemorrhage, vascular lysis, and coagulation. The seconds needed for each type of irritational reaction after applying
the materials can be used directly to determine the irritation score (IS) from the following equation.\(^8\)

\[
IS = \frac{5 \times (301 - mth)}{300} + \frac{7 \times (301 - mtl)}{300} + \frac{9 \times (301 - mtc)}{300}
\]

Here, mth = mean time to haemorrhage (in seconds), mtl = mean time to start vascular lysis (in seconds), mtc = mean time to start coagulation (in seconds). When no irritation component (such as hemorrhage, lysis, or coagulation) was observed within 5 minutes after sample application, the contribution of respective irritation component was considered zero to calculate the total irritation score (IS).

Table S1. Classification of irritant based on the irritation score (IS) \(^8\)

| Classification         | Irritation Score (IS) |
|------------------------|-----------------------|
| Maximum                | 21                    |
| Severe irritant        | > 9                   |
| Non-to-moderate irritant | 0 - 9                |

**Custom-built confocal microscopy for bioimaging and Intracellular tracking**

The confocal setup is a conventional custom-built setup, driven by the software Qudi, which could perform a variety of basic measurement functionalities.

The key hardware of the setup consists of an oil-immersion objective lens (Olympus, 1.35, 60\(\times\)), a 532 nm continuous-wave laser, a spectrometer (Princeton Instruments, Acton SP 300i), an avalanche photo-diode (APD, Excelitas, SPCM-AQRH), optical filter for
confocal imaging is a bandpass of 709 ± 83.5 nm. For compatibility with the ibidi cell-chamber, the recommended ibidi immersion oil by ibidi was used for the objective of the confocal microscope.

On the detection arm, a motorized flip-mirror was used to conduct fluorescence to the APD or the spectrometer, for confocal imaging and tracking, or for spectral measurements respectively. Fast tracking by CCD camera was applied in some of the literatures, which can reveal real-time marker positions on the focal plane in milliseconds. However, there is a sacrifice of losing the position information on the Z-axis. With the positioning excellence of the 3-D piezo scanners on the scanning confocal microscope, as in our case, it takes a few seconds to perform a tracking action but with the advantage that high precision of 3-D trajectories of the cellular marker can be achieved.

Within the fluorescence image, a measurement loop was carried out repeatedly on single bright and photo-stable ND-CP particles. Firstly, a fluorescence scan was performed in a small-range of 100 μm × 100 μm on the X-Y plane, and then zoom in to one single cell and find out the ND-CP fluorescence and measurement the spectrum to prove it is NV center. A 600 nm × 600 nm × 2 μm cube was taken as an optimiser to catch the tracking ND-CP particles. Every 5 second the optimiser was used to find out the best focal point to collect maximum fluorescence counts in the well-aligned scanning confocal setup in the 600 nm × 600 nm × 2 μm. The real-time fluorescence intensity was recorded and the maximum intensity should be kept similar in the whole process. Otherwise the process was given up and to look for other points.
Mean square displacement (MSD) analysis and the calculation of diffusion coefficient ($D_0$)

The mean square displacement (MSD) data of an intracellular trajectory allows understanding of the diffusion behavior of the tracked object and calculation of the diffusion coefficient. In this work, we recorded three trajectories, labeled as “Trajectory 1”, “Trajectory 2”, and “Trajectory 3”, which contain 520, 88, and 81 position vectors, respectively. Each position vector was recorded every 5 seconds, so the tracking times are about 43 minutes, 7 minutes and 7 minutes for
“Trajectory 1”, “Trajectory 2”, and “Trajectory 3” individually. The MSDs of the trajectories were calculated as

\[
\text{MSD}(t_{\text{lag}}) = \text{MSD}(n\Delta t) = \frac{1}{N-n} \sum_{j=1}^{N-n} |\mathbf{r}((n + j)\Delta t) - \mathbf{r}(j\Delta t)|^2 ,
\]

where \(t_{\text{lag}}\) is the lag time, \(\Delta t\) is the measurement interval (5 s) of the trajectories, \(n\) is the number of intervals contained in the lag time, \(N\) is the total number of position vectors in the trajectory, and \(\mathbf{r} = [x, y, z]\) is the position vector of the tracked particle. As shown in Figure 4A, the MSDs of the three trajectories show different magnitudes and lag time dependencies. To identify the diffusion behavior of the ND-CP, we fitted the MSD data with a power-law as

\[
\text{MSD}(t_{\text{lag}}) = A \tau_{\text{lag}}^\alpha ,
\]

where \(A\) and \(\alpha\) are fitting parameters. In particular the power index \(\alpha\) reflects the diffusion behavior of the tracked object, with \(\alpha < 1\), \(\alpha = 1\), and \(\alpha > 1\) indicating confined diffusion, normal diffusion, and directed motion, respectively.\(^9,10\) According to Saxton\(^11\) et al., we focused on data with \(t_{\text{lag}} < t_{\text{total}} / 4\), where \(t_{\text{total}}\) is the total time of the trajectory. Furthermore, we divided the MSD data into two regions, corresponding to short and intermediate lag times, respectively. Through segmental fitting with Equation S2, we obtained the characteristic power indices of the three trajectories, as indicated in Figure 4A. The power indices are smaller than one at short lag times and become close to one at intermediate lag times, suggesting different diffusion behaviors of the ND-CP at different lag time scales. Similar MSD results have been reported for tracking of single-walled-carbon-nanotube (SWNT)-labeled kinesins in COS-7 cells\(^12\) and the confined diffusion behavior at short lag times could be explained by the existence of mechanical obstacles in the cell\(^12,13\).
Another way to distinguish normal and anomalous diffusion (either confined diffusion or directed motion) is by calculating the cumulative distribution function (CDF) of the square displacements ($\Delta r^2$) at a particular $t_{\text{lag}}$. Here we focused on “Trajectory 1”, the longest trajectory recorded in this work, and $t_{\text{lag}} = 2\Delta t = 10$ s. The probability distribution function (PDF) of the square displacements were first calculated (Figure 4B, top panel), based on which the CDF was computed (Figure 4B, bottom panel). We further fitted the CDF data with single and double exponential functions as follows,

$$\text{CDF}(r^2, t_{\text{lag}}) = 1 - \exp\left(-\frac{r^2}{r_0^2}\right),$$  \hspace{1cm} (S3)

where $r_0^2$ is the MSD at $t_{\text{lag}}$, and

$$\text{CDF}(r^2, t_{\text{lag}}) = 1 - \left[w\exp\left(-\frac{r^2}{r_1^2}\right) + (1-w)\exp\left(-\frac{r^2}{r_2^2}\right)\right],$$ \hspace{1cm} (S4)

where $r_1^2$ and $r_2^2$ are the MSDs at $t_{\text{lag}}$, corresponding to the fast and slow mobility components, respectively. The contributions of these two components to the CDF are weighted with factors $w$ and $(1-w)$ respectively. Whereas Equation S3 implies normal diffusion, Equation S4 covers both normal ($w = 0$ or 1) and anomalous ($w \rightarrow 0.5$) diffusion. The $r_i^2$ ($i = 0, 1, 2$) are related to the diffusion coefficient $D_i$ as $r_i^2 = 6D_i t_{\text{lag}}$. From the fitting, we obtained $r_0^2 = 1.2540 \times 10^{-2}$ μm$^2$, $r_1^2 = 1.7715 \times 10^{-3}$ μm$^2$, $r_2^2 = 1.7590 \times 10^{-2}$ μm$^2$, and $w = 0.2616$. As a result we obtained the following diffusion coefficients (at $t_{\text{lag}} = 10$ s): $D_0 = 2.09 \times 10^{-4}$ μm$^2$/s, $D_1 = 2.95 \times 10^{-5}$ μm$^2$/s, and $D_2 = 2.93 \times 10^{-4}$ μm$^2$/s. A $w$ value of 0.2616 and the much smaller residual of the double exponential fit than that of the single exponential fit (Figure 4B, top of the bottom panel) confirming that the diffusion behavior of the ND-CP in the HeLa cells is indeed a combination of normal and anomalous diffusion.
We also calculated the lag-time-dependent diffusion coefficient of the ND-CP in HeLa cell according to

\[
D(t_{\text{lag}}) = \frac{\text{MSD}(t_{\text{lag}})}{6t_{\text{lag}}},
\]  

(S5)

Applying Equation S5 to the three trajectories at intermediate lag times, where the power indices are close to one (Figure 4A), we obtained the following three diffusion coefficients, \(1.25 \times 10^{-4}\), \(3.07 \times 10^{-4}\), and \(1.39 \times 10^{-3}\) μm²/s (Figure 4C), which are comparable to the values obtained from the single and double exponential fits to the CDF data at \(t_{\text{lag}} = 10\) s. The nominal diffusion coefficient of the ND-CP in the HeLa cell was then determined to be the average of these three values, that is, \(6.07 \times 10^{-4}\) μm²/s.

2. Tables and Figures

Table S2. Irritation Score (IS) for different concentrations of ND-CP, compared to 1% SDS (positive control) and PBS (negative control).

| Eggs with sample (n = 3)       | Classification     | Irritation score (IS) |
|--------------------------------|--------------------|-----------------------|
| 1% SDS                         | Severe irritation   | 11.36±0.02            |
| PBS                            | No irritation       | 0.00                  |
| ND-CP (100 μg/mL)              | No irritation       | 0.00                  |
| ND-CP (50 μg/mL)               | No irritation       | 0.00                  |
Figure S2. SDS-PAGE images of ND-CP: 1, standard protein ladder; 2, ND-CP in Tris buffer; 3, ND-CP in PBS buffer; 4, CP in Tris buffer.

Figure S3. (A) The size distribution histogram of NDs. (B) The size distribution histogram of ND-CPs.
Figure S4. (A) TEM images of ND-CP; (B) The shell thickness distribution histogram of ND-CP.

Figure S5. (A) AFM images of ND-CP in liquid state in height sensor; (B) AFM images of ND-CP in deformation sensor.
Figure S6. Photographs of HET-CAM test results for 1% SDS (positive control), PBS (negative control) and ND-CP at 50 µg/mL and 100 µg/mL.
Figure S7. (A) The trajectory of tracked ND-CP spot 2 in intracellular space of HeLa cell and Real-time counts of fluorescence of the tracked ND-CP spot 2; (B) The trajectory of tracked ND-CP spot 3 in intracellular space of HeLa cell and Real-time counts of fluorescence of the tracked ND-CP spot 3.

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