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Supporting Information

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Keywords**:** ratiometric nanothermometer, luminescence, organic radical nanoparticles, excimer emission, in-vivo sensing, *Caenorhabditis elegans*

The data that support the findings of this study are available from the corresponding author upon reasonable request.

1. Materials, reagents, and solvents

Tris(2,4,6-trichlorophenyl) methyl radical (TTM) and tris(2,4,6-trichlorophenyl) methane (TTM-αH) were synthesized as previously reported.[1,2] Tetrahydrofuran (THF, HPLC grade from Chem-Lab.) and ultrapure MilliQ water were used for the preparation of ONPs using the re-precipitation method.[3]

2. Equipment and measurements

Absorbance measurements were performed using a V-780 UV-Visible/NIR Spectrophotometer from JASCO. We used quartz glass cuvettes with path lengths of 1 cm, as they are transparent in the UV-visible-NIR range. The range of wavelengths recorded was from 250 nm to 850 nm. First, a cuvette with the solvent (blank) was placed inside the spectrophotometer to obtain its absorption spectra, which will be later subtracted from the absorbance values obtained from the sample, to obtain the real absorbance values of the ONPs.

A Cary Eclipse Fluorescence Spectrophotometer coupled with a temperature controller was used to measure the fluorescent emission intensity of prepared suspensions. The fluorimeter had a multi-holder with four cavities for measuring quartz glass cuvettes. One cuvette of 1 cm path length was filled with water, containing the temperature probe to have a precise value of the temperature inside the cuvettes. For the ONPs suspensions, 1 cm path length cuvettes were also used while stirring with a magnetic stir bar, whereas for the *C. elegans* a 1 cm path length sub-micro cuvette was used because the worms precipitated at the bottom at higher volumes; so low volumes were preferable to concentrate all the worms in a small volume. An excitation wavelength of 377 nm was used for all measurements. First, an emission scan from 400 nm to 850 nm at 298 K and 278 K was recorded to obtain the maximum fluorescence intensities wavelengths of the monomer and excimer bands. Then, the samples were heated from 278 K to 328 K with a heat ramp of 0.8 K min^{-1} and then cooled down from 328 K to 278 K. Fluorescence intensity at the maximum of the monomer and excimer bands was recorded every 0.5 K. Between both temperature ramps, another complete scan was performed at 328 K. The ratio between the maximum emission of the monomer band and that of the excimer band Q (T), was fitted with an empirical equation (LIR, Equation 1). This fitting was

numerically derived with respect to temperature, dQ(T)/dT, to estimate the absolute sensitivity (S_a) values for each temperature using Equation S1, and from these values it is possible to calculate the relative sensitivity (S_r).

Dynamic Light Dynamic Light Scattering (DLS) measurements were performed with a Zetasizer Nano ZS from Malvern Instruments at RT, for colloidal characterizations and to study their stability at different temperatures. This equipment allows to measure the hydrodynamic size and the Z-potential of the ONPs. In each measurement, the equipment performs 3 replicates.

3. Statistical Analysis

For the calculation of sensitivity, it was used an empirical Equation 1, and its derivative Equation S1 and 2. Sample size for dialysis measurements is n=3 and for confocal fluorescence intensity in C. elegans n=6 and p was calculated using the normal distribution function and cumulative normal distribution table. The software used for analysis and graphical representation was OriginPro. Confocal image quantification was performed using ImageJ/Fiji software, and 3D representation images were deconvoluted using the software Huygens Professional Software v17.10.0p8 (Scientific Volume Imaging B.V., Leiden, The Netherlands) and Imaris x64 v7.2.1 software (Bitplane, Zurich Switzerland) with Surpass Mode.

4. Preparation of TTM radical doped ONPs suspensions

A 2 mM solution of molecular components containing 20 % w/w of TTM radical and 80 % w/w of TTM-αH was prepared in THF (2 mL). The resulting red solution was filtered through a PTFE filter of 220 nm and a diameter of 1.3 cm to remove possible impurities. Then, to prepare the ONP suspension, 0.5 mL of the latter solution (unless elsewhere indicated) was slowly dropped at a speed of 1 mL h^{-1} with a 1 mL Hamilton syringe (Ref: CAL81330), and a syringe pump (KDS 100 Legacy Single Syringe Infusion Pump - 220 VAC) into MilliQ water (9.5 mL, unless elsewhere indicated) at RT, under vigorous stirring (1000 rpm) and the resulting 20 % TTMd-ONP suspension was stored at 277 K in the dark. For the preparation of ONP suspensions with 15 % and 25 % w/w of TTM radical, the same protocol was used. In all cases, the resulting TTMd-ONPs suspensions have 5 % v/v of THF in water (unless elsewhere indicated). For some experiments the remaining TTF solvent was removed by dialysis, as follows: 10 mL of ONPs suspension were dialyzed in 2 L of MilliQ water. Water was changed approximately every 8 hours during 48 hours (three times per day). After dialyzing the ONPs suspensions, different amounts of KCl salt were added to yield aqueous ONPs suspensions with different ionic strengths corresponding to 50, 100, and 150 mM

KCl concentrations. PBS solutions at different *pH* were also prepared adding to the dialyzed ONPs suspensions KCl to achieve a concentration of 100 mM and Na_2HPO_4 and NaH_2PO_4, as reported in **Table S1**. Finally, the *pH* was adjusted by adding 2 N HCl or NaOH aqueous solution until the desired *pH* value was obtained. The *pH* values were measured with a *pH* meter HI 5521 (Hanna Instruments).

Table S1. Amounts of mono- and bi-basic sodium phosphate salts used for the preparation of PBS solutions at different *pH*. KCl in a concentration of 100 mM was added to suspensions to increase the ionic strength to a value of ~150 mM.

pH	**Na_2HPO_4 (mM)**	**NaH_2PO_4 (mM)**
5	0.11	8.22
6.5	2.512	6.29
8	7.76	0.57

ONPs suspensions for the thermal stress study were prepared without the addition of KCl, but adding Na_2HPO_4 and NaH_2PO_4, as reported in Table S2, to understand the role played by the different ions on the colloidal stability. Finally, the *pH* was adjusted by adding 2 N HCl or NaOH aqueous solutions until the desired *pH* value was obtained.

Table S2. Amounts of mono- and bi-basic sodium phosphate salts used for the preparation of 25 mM solution (ionic strength corresponding to 150 mM KCl), used for the thermal stress analysis.

pH	**Na_2HPO_4 (mM)**	**NaH_2PO_4 (mM)**
5	0.33	24.67
6.5	7.535	17.465
8	23.29	1.71

5. Effect of temperature changes on ONPs emissions

The luminescence intensity of ONPs suspensions was recorded with a Cary Eclipse Fluorescence Spectrophotometer coupled with a temperature controller. The multi-holder of the fluorimeter has four holders for cuvettes. Two cuvettes were filled with water, used as a reference, and the other two with the ONP suspensions. We introduced a thermo-couple in the cuvettes in order to effectively measure the temperature and inside all of them (references and sample) were stirred by a

little magnetic stirrer achieving a uniform temperature control. The emission spectra of ONPs suspensions were tested in the range of temperature 278 – 328 K with a temperature ramp of 1 K min^{-1}. The analyzed samples were excited with a wavelength of 377 nm (at the absorption maximum of ONPs) and it was recorded the intensity at the maximum of monomer (I_M) and excimer (I_E) bands each 0.5 K. In all measurements it was not recorded the entire emission spectrum was not recorded to avoid possible photobleaching effects.

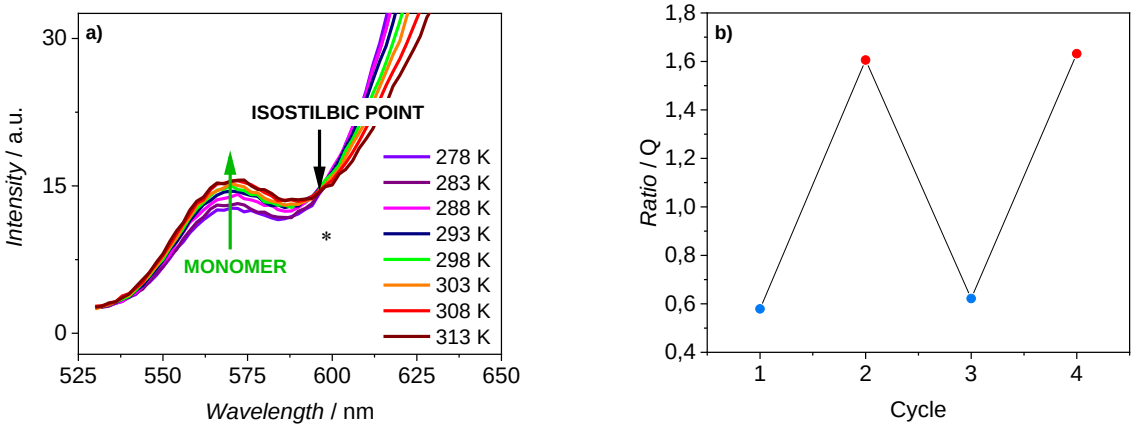


Figure S1. a) Zoom of the temperature effect on the monomer emission showing the isostilbic point; b) Reversibility of the fluorescence emission in water, where Q is the ratiometric output of the sensor, given by the fluorescent intensity ratio, I_M/I_E, where I_M is the intensity at the maximum of the monomer band, and I_E is the intensity at the maximum of the excimer one.

6. Optimization of the ratiometric sensitivity and accuracy of ONPs with different radical doping levels

Temperature dependence of fluorescent intensity ratios, I_M/I_E, of ONPs suspensions were fitted with the empirical equation LIR=a+b·exp(c·T), using Origin 2015® software, and the resulting functions were derived with respect to the temperature ($\partial Q(T)/\partial T$) and the corresponding sensitivity values, S (given in % K^{-1}), were estimated following the Equation S1 for the absolute sensitivity and Equation 2, for the relative sensitivity.

$$S_a = \left|\frac{\partial Q}{\partial T}\right| \times 100\% \ , \ [\% \cdot K^{-1}] \qquad \text{(S1)}$$

where $Q(T)$ is the ratiometric output or LIR of the sensor, given by the fluorescence intensity ratio, I_M/I_E, where I_M is the intensity at the maximum of the monomer band, and I_E is the intensity at the excimer maximum (**Figure S2d**).

The temperature dependence of suspensions of TTMd-ONPs with different TTM radical doping of 15, 20, and 25 % are given in Figure S2a-c and the resulting sensitivities, absolute and relative, in the temperature range of 278-328 K in Figure S2d and e, respectively.

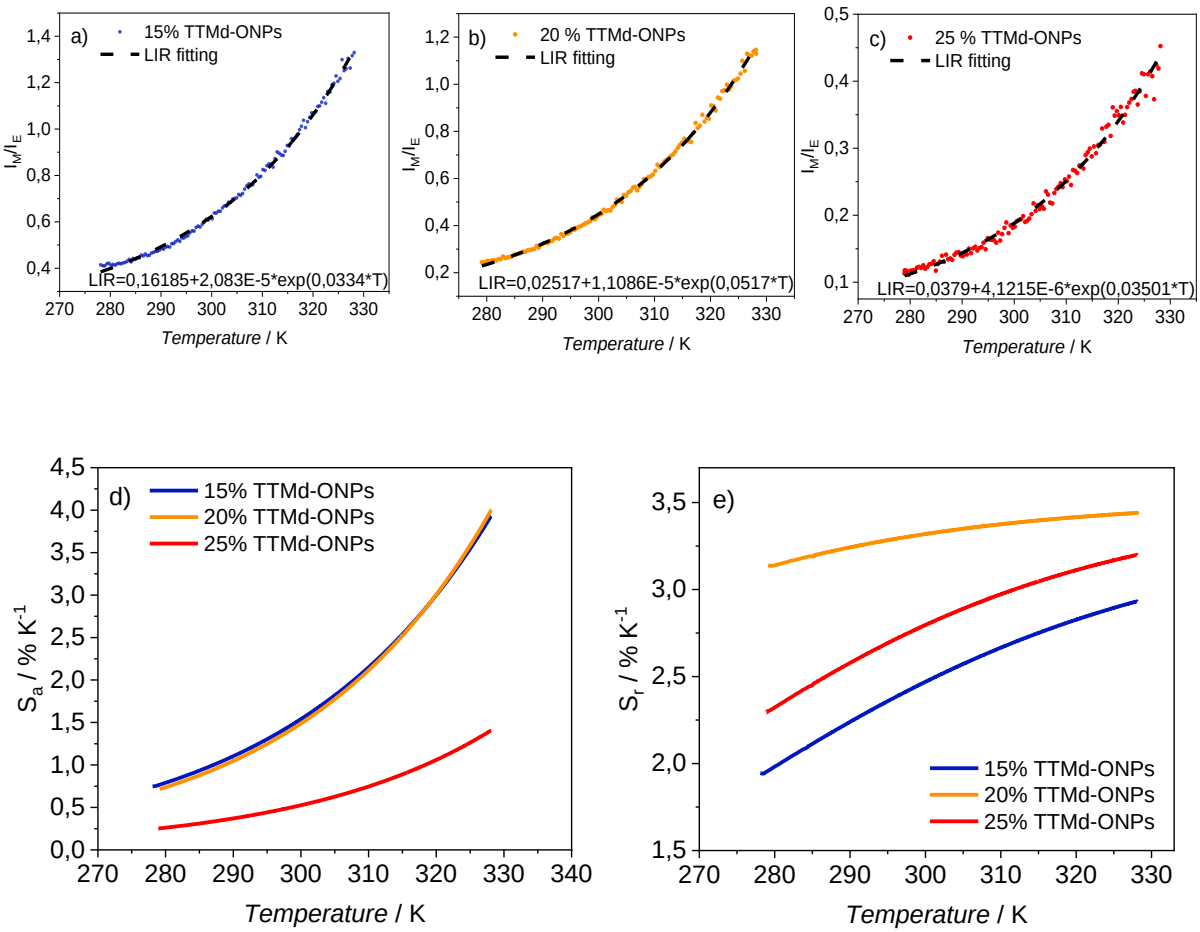


Figure S2. Temperature-dependent ratiometric outputs of TTMd-ONPs suspensions with radical doping of a) 15 %, b) 20 % and c) 25 %. d) Absolute and e) relative sensitivities of the 15, 20, and 25 % TTMd-ONPs suspensions.

The smallest detectable temperature changes, or minimum temperature resolution, ΔT_{min}, is another crucial parameter to characterize the accuracy of a nanothermometer. This parameter can be estimated by the following expression, [4]

$$\Delta T_{min} = \frac{\sigma Q}{S_a} \quad \text{(S2)}$$

where σQ is the uncertainty of the measured ratio (I_M/I_E), which apparently is similar at all the studied temperature range, and S_a is the absolute sensitivity.

7. Effect on the ratiometric sensitivity of dialyzed ONPs suspensions

Finally, as obtained, the aqueous suspension of 20 % TTMd-ONPs was dialyzed in order to remove all content of the organic THF solvent used for their preparation by the re-precipitation method. After the dialysis, the maxima fluorescence intensity ratio, I_M/I_E, was recorded and the sensitivity for the dialyzed suspension was calculated as before. In Figure S3 the temperature dependence of I_M/I_E and the variation of the sensitivities of a 20 % TTMd-ONPs suspension before and after the dialysis are compared with the variation of the sensitivities of a 20 % TTMd-ONPs suspension before and after the dialysis is compared.

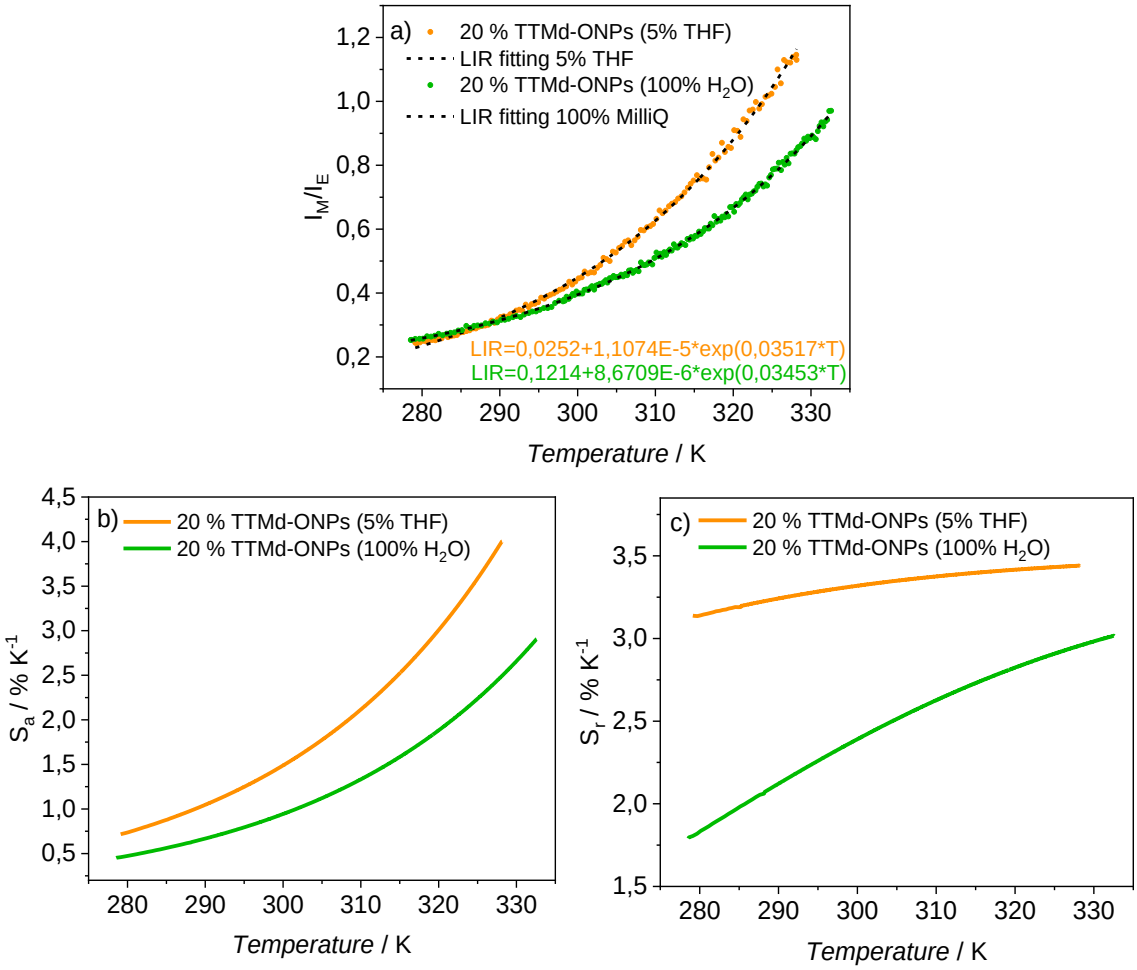


Figure S3. a) Temperature-dependent ratiometric output of 20 % TTMd-ONPs suspension before and after the dialysis. b) Absolute and c) relative temperature sensitivities of 20 % TTMd-ONPs suspensions before and after the dialysis.

8. Characterization of colloidal properties of TTMd-ONPs

Sizes of 20 % TTMd-ONPs were determined by Dynamic Light Scattering (DLS) which was performed with a Malvern Nano ZS using a laser at 633 nm at RT. Each measure consisted of 13

scans and the reported values are given as the averaged value of three measures of 100-150 nm with a low polydispersity index > 0.2, expressed in nm, implying a high monodispersity (Figure S4 right). Due to the high ionic strength of suspensions containing KCl and buffer salts, it was not possible to estimate their Z-potentials. 20 % TTMd-ONPs were also characterized using transmission electron microscopy (TEM) with the aid of negative staining. For that, one drop of the suspension was applied to glow-discharged carbon-coated copper grids (SPI) for 3 min. Subsequently, one drop of 2 % uranyl acetate was placed on the grid for 1-2 min before being drained off. The grid was then placed in a transmission electron microscope (Jeol JEM 1400) operating at an accelerating voltage of 120 kV. Images were acquired using an Orius SC200 (Gatan) showing that ONPs have a spherical morphology (Figure S4 left).[2]

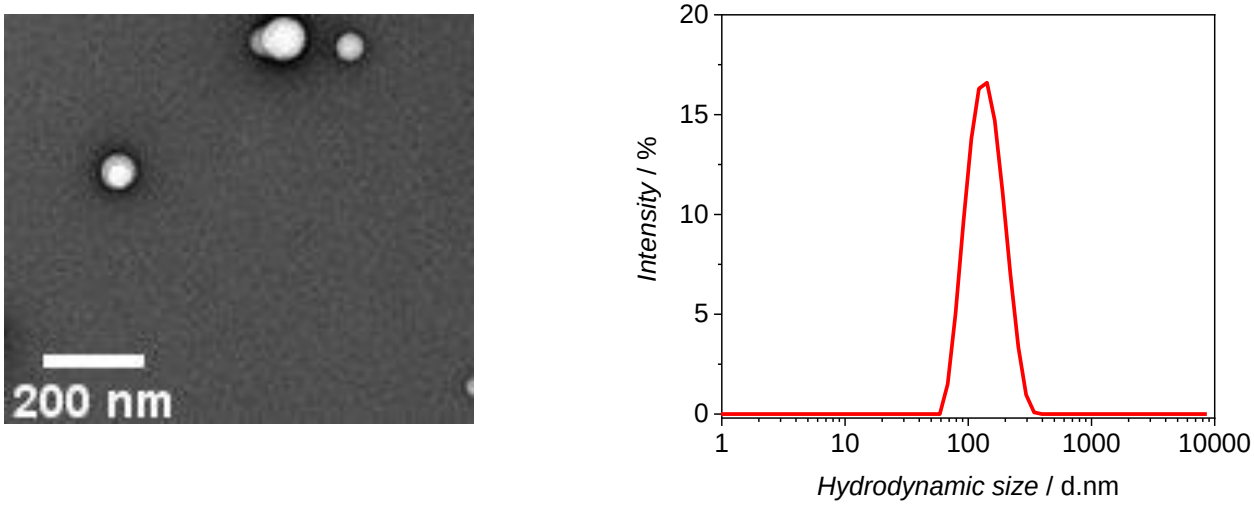


Figure S4. Left) TEM images of 20 % TTMd-ONPs after the dialysis. The size and the morphology of the ONPs are unaffected by the dialysis process showing a spherical morphology. Right) Size distribution of 20 % TTMd-ONPs after the dialysis obtained by DLS.

9. Effect of concentration changes on ratiometric sensitivity of ONPs suspensions

To use the ONPs as ratiometric nanothermometer, it is important to verify that the thermometric parameter (I_M/I_E) is independent of possible concentration fluctuations of ONPs since they may accumulate differently in specific areas of the analyzed cells or tissues. The thermometric parameter of 20 % TTMd-ONPs in pure MilliQ water was compared with the one derived by a 1 to 3 volumetric dilution of the original suspension (20 % TTMd-ONPs dil). Results are presented in Figure S5 where it is shown that the outputs of the two suspensions are not exactly the same. In particular, the signal of the diluted sample seems to be slightly shifted compared to the concentrated one. This difference is ascribed to the different scattering in the two suspensions. In order to verify

this assumption, the two signals were normalized with respect to the maximum and the resulting normalized graphs were identical, demonstrating that both suspensions exhibit the same ratiometric outputs as well as the same sensitivities.

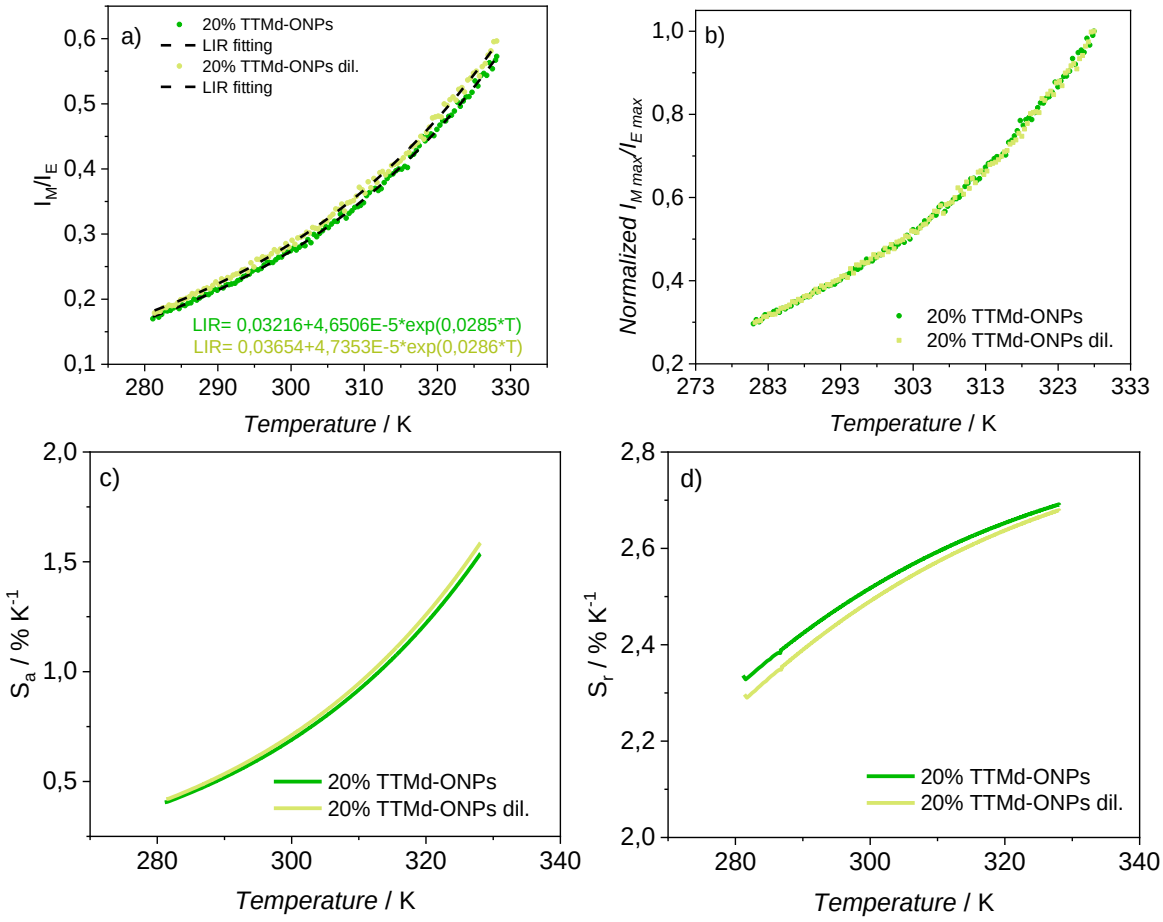


Figure S5. a) Temperature-dependent ratiometric outputs of 20 % TTMd-ONPs suspension in pure MilliQ water and after its 1 to 3 volumetric dilution. b) Normalized temperature-dependent ratiometric output of 20 % TTMd-ONPs suspensions in pure MilliQ water and after its 1 to 3 volumetric dilution 1 to 3. c) Absolute and d) relative temperature-dependent sensitivities of 20 % TTMd-ONPs suspensions in pure MilliQ water and after its 1 to 3 dilution.

10. Effect of *pH* changes over time and temperature of ONPs suspension

To evaluate the stability of the ONPs at different *pH* the size of the ONPs was followed by DLS with time and the fluorescence emission at increasing temperature, and both result in stable nanoparticles at a different *pH*.

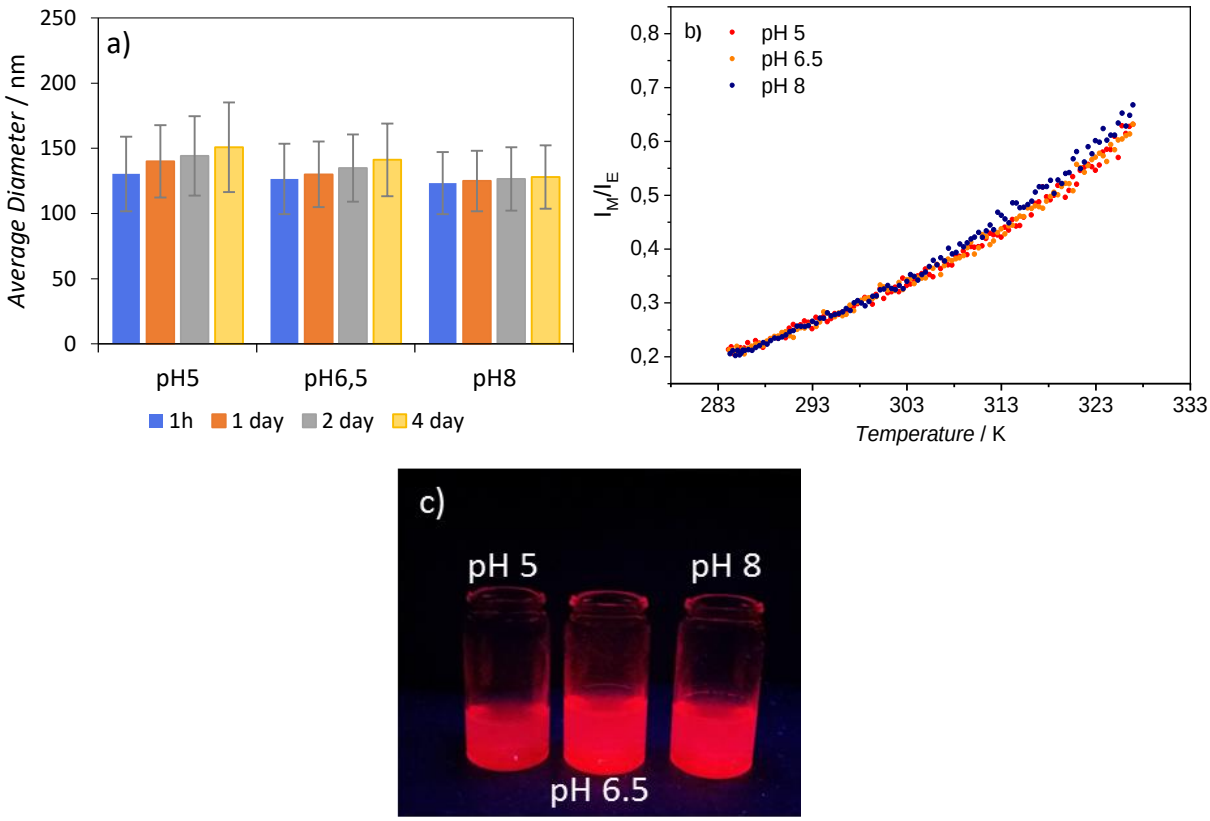


Figure S6. Effect of *pH* on the optical properties and colloidal stability of 20 % TTMd-ONPs suspensions at three different *pH* in the range 5-8 using PBS as buffer media. a) DLS measurement showing a slight increase of ONPs particle size in acidic *pH* during 4-day storing for n=3. b) Evolution of the I_M/I_E fluorescence intensity ratios with temperature changes in the range of 283-328 K. c) Appearance of 20 % TTMd-ONPs suspensions in PBS solution at *pH* of 5, 6.5, and 8 with an λ_{exc} of 365 nm at 298 K.

11. Effect of thermal stress of the colloidal properties of ONPs suspensions at different conditions

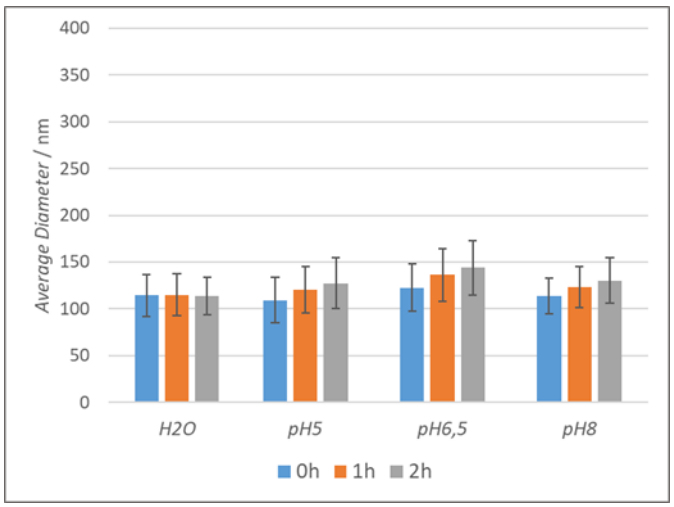


Figure S7. Effect of prolonged thermal stress for two hours at 50 °C on the colloidal stability of 20 % TTMd-ONPs in different conditions, as observed by DLS: pure MilliQ water (H_2O) and PBS solutions with an ionic strength of 150 mM at different *pH* (*pH* 5, *pH* 6.5 and *pH* 8) for n=3.

12. Cell viability, biocompatibility, and fluorescence imaging of ONPs

A (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) colorimetric assay was used to evaluate the metabolic activity of cells in the presence of TTMd-ONPs suspensions. For such assay, 5000 COS-7 cells (Monkey African green kidney, SV40 transformed fibroblast-like cells, ATCC) were seeded in a 96-well plate. Different concentrations of ONPs were added, ranging from a 0.7 µM of TTMd-ONPs to 1.4 nM. As the ONPs are conserved in water, the maximum concentration that could be tested in the MTT assay was 175 nM (vehicle 25% water in media). As it is shown in Figure S8, treating cells with a higher proportion of water (vehicle 50% water in media) decreased the viability of the cell culture to 70 %. According to the MTT data shown in Figure S8, concentrations below 35 nM (25% water-media) do not compromise the viability of the treated cells.

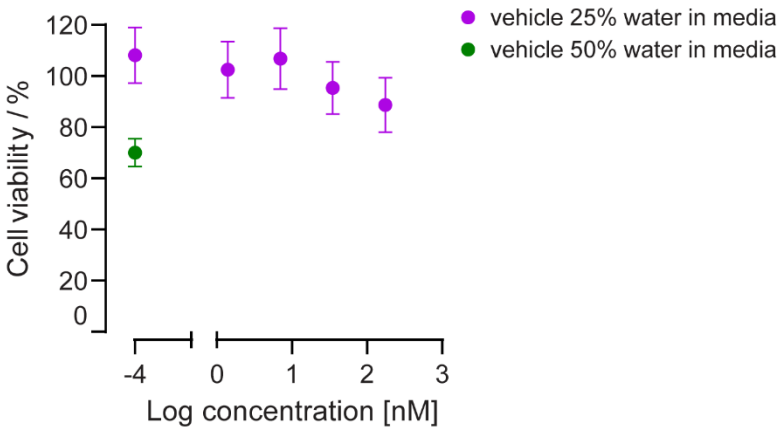


Figure S8. Cell viability assay with COS-7 cells at different ONPs concentrations. The absorbance measurements were performed at 570 nm after 48 h. It can be seen that the 50-50 % water-medium condition induces a negative effect on the culture viability. On the other hand, the concentrations that contain a 25 % proportion of water show no negative impact on the culture.

To further check the biocompatibility of TTMd-ONPs, an in vitro assay on tsa201 cells, a transformed human kidney (293; Sigma Nr. 85120602) cell line stable expressing an SV40 temperature-sensitive T antigen, was also performed using a fluorescence optical microscope. Tsa201 cells were grown on glass supports treated with poly-lysine one day before the measurements. Cells were incubated with a 1:10 dilution of 20 % TTMd-ONPS in MilliQ water suspension for 4 h at 37 °C. Before being analyzed, samples were washed with a buffer solution at room temperature (140 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 10 mM Hepes, 10 mM Glucose, and 2 mM $CaCl_2$, *pH* 7.4) to remove the excess of ONPs. Acquisition of the images was done with an inverted fully-motorized digital microscope (iMIC 2000, Till Photonics) using an exposure time of 200 ms. ONPs were excited at 380 nm with a polychrome V light source (Till Photonics) using a 505 nm dichroic beam splitter (Chroma Technology). Emission from the excimer was filtered by an ET630/75 nm emission filter (Chroma Technology) and finally collected by a cooled CCD camera (Interline Transfer IMAGO QE, Till Photonics). In Figure S9 are reported the bright field, the fluorescence images, and the merged images of tsa201 cells irradiated at 380 nm after the incubation (4 hours at 37 °C) with 20 % TTMd-ONPs. In such images the luminescence of the ONPs is observed, which confirms their stability in the cell environment, as well as the cells, with ONPs on their cellular membrane, present a healthy aspect (no apoptotic cell morphology) supporting a good biocompatibility of the ONPs.

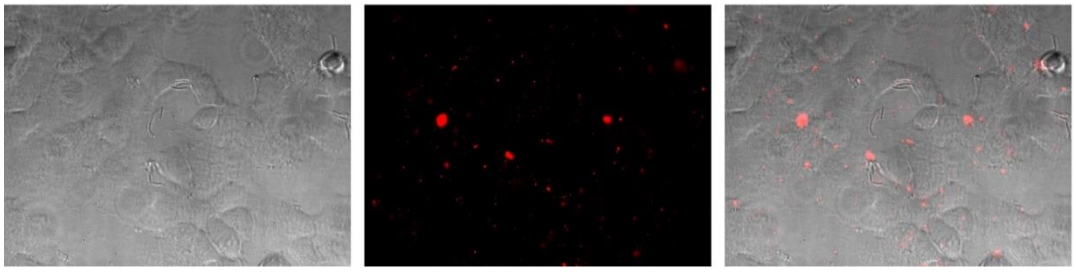


Figure S9. From left to right: bright field image, fluorescence image and merged image of Tsa 201 cells incubated 4 hours at 37 °C with 20 % TTMd-ONPs. Red spots represent the fluorescence of the TTMd-ONPs.

13. Effect of the temperature in the emission of a collection of 20 % TTMd-ONPs deposited on a treated glass surface.

TTMd-ONPs (20 % in MilliQ water (H2O) were incubated on poly-L-Lys (Sigma-Aldrich) pre-treated glass coverslips (16-mm diameter, Fisher Scientific) mounted on a recording chamber (Open Diamond Bath Imaging Chamber for Round Coverslips from Warner Instruments) for 1 hour. After that, the coverslips were washed with an imaging buffer solution at room temperature (140 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 10 mM Hepes, 10 mM Glucose, and 2 mM CaCl2, *pH* 7.4) to remove nanoparticles that remained in suspension. Imaging was performed on an IX71 inverted microscope (Olympus) with a UApo/340, 40x/1.35 oil immersion objective (Olympus). Nanoparticles were excited during 25 ms every 1 s at 377 nm using a Polychrome V monochromic light source (Till Photonics) equipped with a Xenon Short Arc lamp (Ushio) and a 505 nm dichroic beam splitter (Chroma Technology) and a 585 nm dichroic beam splitter (Chroma Technology). Emission from the monomer was filtered by a D535/40 nm emission filter (Chroma Technology) and for the excimer by an ET630/75 nm emission filter (Chroma Technology) and finally collected by a C9100-13 EM-CCD camera (Hamamatsu).

Temperature variation in the recording chamber was carried out by perfusing the imaging buffer solution that was previously heated or kept cold. Fast switching of both solutions was done with a VC-6 six-channel valve controller (Warner Instruments Corp.). The temperature in the recording chamber was monitored using a K-type digital thermometer (Kosmon) at the accessory pool of the recording chamber.

Imaging analysis was done with FIJI software (ImageJ) and analysis of the obtained data was done with Excel and Prism Graphpad software.

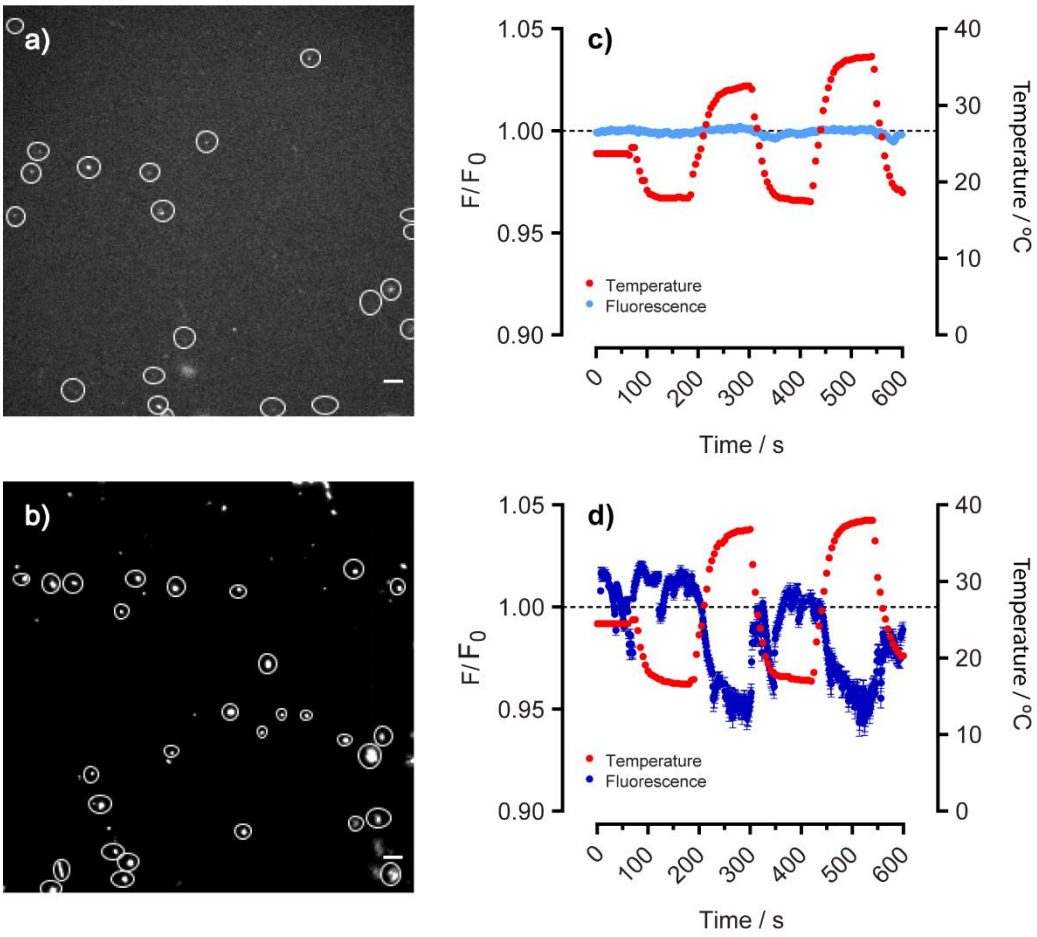


Figure S10. Fluorescence microscopy images corresponding to TTMd-ONPs with different aggregation levels excited at λ_{exc}= 377 nm and detected with a 510-560 nm filter, corresponding to the emission of monomers (a), and with a 595-670 nm filter for the excimers (b). Real-time variation of relative fluorescence intensity signals (F/F_0) with temperature changes up to 20 degrees emitted by the monomers (c) and by the excimers (d), in all nanoparticles inside ROIs shown in a) and b) respectively (N=21 for the monomer and N=29 for the excimer). ROIs shown in b) and plotted in d) correspond to 29 of the 35 total selected ROIs, that displayed temperature-dependent changes in relative fluorescence intensity (83 % of the selected ROIs). Scale bar, 10 µm.

14. Stability of ONPs in M9 buffer

15. M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 1 mL of 1 M of $MgSO_4$ and 5 g of NaCl per liter of miliQ H_2O)

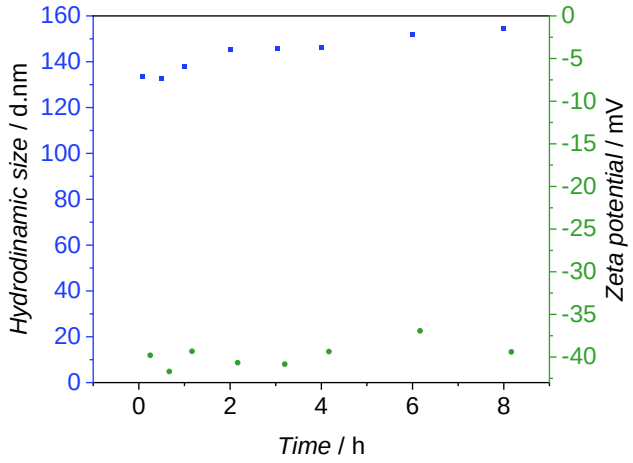


Figure S11. Effect of M9 buffer on the colloidal stability of 20 % TTMd-ONPs during 8 hours.

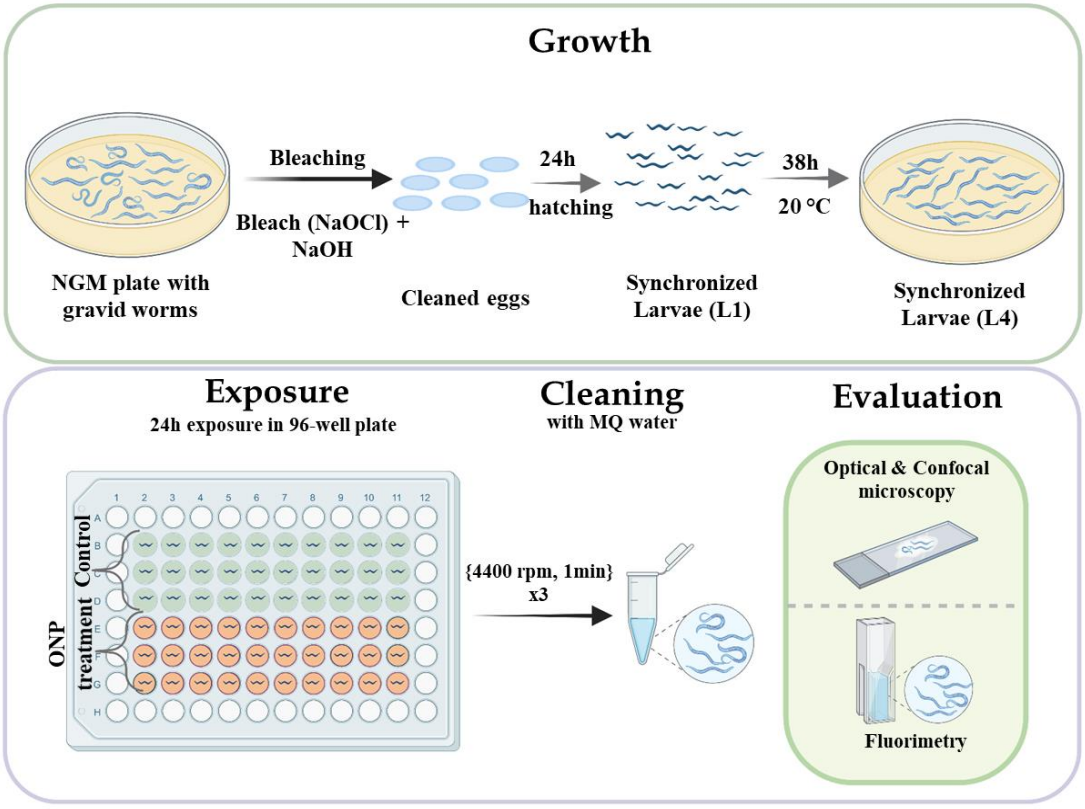
16. Growth, treatment of *C. elegans* with ONPs and imaging.

The *C. elegans* used in this work are wild-type strain (N2), purchased from Caenorhabditis Genetics Center, Minnesota (USA). Nematode growth media plates (NGM) containing agar, peptone and NaCl were used for maintaining the worms at 293 K in the laboratory. M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 1 mL of 1 M of $MgSO_4$ and 5 g of NaCl per liter of miliQ H_2O), is used to maintain the worms alive and healthy in liquid environment such as during hatching from eggs to larva and during exposure of the worms to the nanoparticles. The *Escherichia coli* strain OP50 is used as a bacterial food source in the laboratory for *C. elegans*. Usually, 550 µL of OP50 is spread on top of the solidified NGM plates and incubated at 303 K overnight prior to further use for *C. elegans* cultivation.

For liquid exposure of ONPs to *C. elegans*, heat-killed OP50 is used. The liquid culture with alive OP50 is heat-killed at 353 K for 30 min and washed to remove debris and toxins from heat treatment. The warms are re-suspended in equal volume of M9 buffer. It is important to use heat-killed OP50 so that they do not interfere with the ingestion of ONPs during co-administration to *C. elegans*.

C. elegans are exposed to the nanoparticles when all of them have attained a synchronized L4 larval stage. First, eggs from gravid worms are collected through a process called bleaching (Figure S12).

When the NGM plates reach confluency with mostly gravid worms, the worms are treated with household bleach (sodium hypochlorite) and sodium hydroxide under vortex or vigorous shaking. This process dissolves the mother worms and releases the eggs into the solution, which is washed several times with M9 buffer by centrifugation to clean the excess bleach mixture and debris. The eggs were placed in M9 solution and kept for 24 hours to hatch and become Larvae (L1). Bleaching and collecting eggs is crucial for experiments where age synchronized population is needed, such as in the case of studying the effect of treatment of any substance. The synchronized L1 population is then transferred to NGM plates containing OP50 and the worms are grown at 293 K for ≈38 hours, or until the population is at a synchronized L4 larval stage. L4 worms were collected from NGM plates in M9 buffer and exposed to ONPs in a 96-well plate. The experiment consists of two treatment groups, untreated (control) and ONP-treated *C. elegans.* The exposure in liquid system is carried out for 24 hours so that at the end of exposure during imaging and analysis, we have a population of a single generation of fully-grown adult worms with eggs inside. Each well consists of ≈20-25 worms (in 3 µL M9), 5 µL of OP50, 42 µL of M9, and lastly, 50 µL of TTM ONPs aqueous suspension (110 µg mL^{-1}) for ONP treated group or 50 µL MilliQ water for the control



group. In total, we will have 50 µL of M9 buffer and 50 µL MilliQ water per well to provide an optimal system for both ONPs dispersion as well as *C. elegans* growth condition.

Figure S12. Growth of synchronized population of *C. elegans*, exposure to ONPs & evaluation of ONP inside the worms.

After 24 hours, the worms were washed several times with MilliQ water by centrifugation remove all debris and excess non-ingested nanoparticles in order to obtain clean control and treated worms. For microscopic imaging, *C. elegans* were fixed using 8 % PFA in equal volume to the worms' suspension. When the worms are fixed, and no swimming movement is observed (~2 min), the slide is sealed using a cover glass and nail polish. The imaging was carried out with a fluorescence microscopy Olympus BX51 microscope with digital camera Olympus DP20 y reflected fluorescence system Olympus U-RFL-T at magnifications 5X and 20X, with UV and GFP filters. The intensity of fluorescence under both the filters war quantified using ImageJ and statistical analysis was performed using Mann Whitney test.

17. Survival rates of *C elegans* to ingested ONPs and temperature changes

After 24 hours of exposure, both the untreated control and the ONP treated worms are counted by observing directly under the microscope. Worms are considered dead if they are not moving even after a gentle push with a filament. The survival rate is calculated as,

$$\{Number\ of\ alive\ worms \div total\ number\ of\ worms\} \times 100$$

Body length of the worms in the control and treated group was measured from the microscopic images using ImageJ. In the second control experiment, the survival rate of *C. elegans* at different temperatures was investigated. A healthy synchronized adult population of *C. elegans* was collected in an Eppendorf tube in M9 buffer. Then, the Eppendorf tube was placed in a thermo-shaker (BioSan TS-100), equipped with a plate (BioSan SC-24 block) for Eppendorf tubes. The sensitivity of worms to different temperatures was calculated, in the temperature range from 293-328 K with steps of 5 K. Worms were incubated at each temperature was 2 min to disperse the heating and stabilize the solution to the temperature. A known volume of the suspension was added to the glass slide to count number of dead and alive worms at each temperature. Sometimes, under extreme conditions, the *C. elegans* enter a stage called Dauer state (dormant state), which is adopted to protect themselves from stressful conditions. In this stage, the worms remain motionless, so they can be confused with dead ones. Therefore, after heating up to 328 K, worms were placed on a plate with food until the next day, to check if they can recover into their normal life cycle. However, none of the worms recovered after heating to 328 K and were declared as dead. Table S3 shows the observed survival rates at the assayed temperatures showing that *C. elegans* can be used as an in

vivo model system for validating ONPs as nanothermometers only at temperatures between 293 and 308 K.

Table S3. Survival rates of *C. elegans* at different temperatures

Temperature (K)	Total worms	Dead worms	Survival rate (%)
293	124	3	98
298	70	10	86
303	55	14	75
308	65	18	72
313	62	51	17
318	60	60	0
323	56	56	0
328	51	51	0

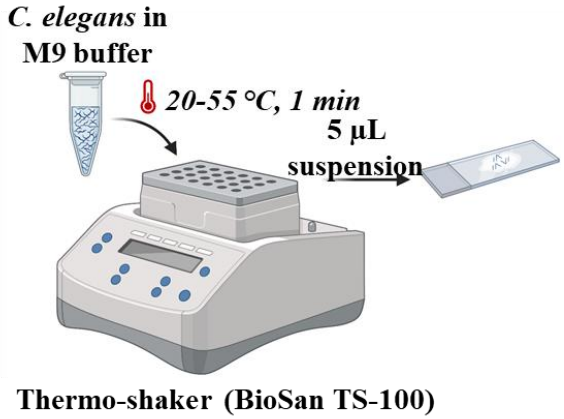


Figure S13. Experimental setup for measuring temperature sensitivity of *C. elegans*

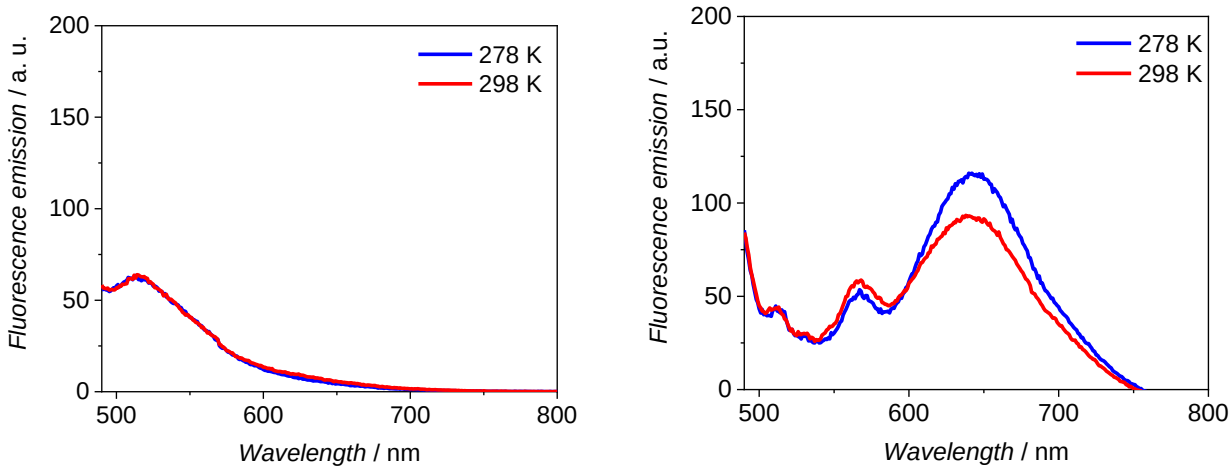


Figure S14. Left) Emission spectra of *C. elegans* control group without TTMd-ONPs. Right) Emission spectra at two different temperatures *C. elegans* exposed to 20 % TTMd-ONPs. Spectra recorded at two different temperatures with an excitation wavelength λ_{exc} =337 nm.

18. Preparation of samples for confocal microscopy

To record a local temperature, change confocal images were taken at different temperatures, focusing on a single *C. elegans*. To ensure a good acquisition of the images and avoid movement of live *C. elegans*, NemaGel (InVivo Biosystems) was used to immobilize them. Briefly, a drop of the sample (*C. elegans* suspension in M9) was placed in the middle of a 35 mm diameter plate (ibiTreat; Ibidi GmbH, Germany) and a drop of NemaGel was placed on the cover glass. The cover glass was then inverted and gently placed over the liquid drop of worms so that NemaGel is able to trap and immobilize the worms. After 2 mins, the worms are immobilized and visualized under the microscope.

19. Confocal Microscopy and Image Processing

Confocal microscopy analysis was performed on a Leica TCS SP8 microscope equipped with a white light laser and Hybrid spectral detectors (Leica Microsystems GmbH, Mannheim, Germany). The confocal images were acquired using an HC PL APO 63×/1.4 oil immersion objective with a 1 AU pinhole. Both types of nanoparticles were excited with a blue diode laser (405 nm) and detected in the 420–570 nm and the 620–720 nm range, respectively. Fluorescence and transmitted light imaging were combined to study the nanoparticles' distribution inside *C. elegans*. An image acquisition sequence xyz for both channels was used to acquire the 3D stacks. The image format chosen was 12-bit and 1024 × 1024 pixels resolution. Ten sections (step size= 1.5 µm) were acquired to get an image volume stack along the organism thickness. Appropriate negative controls were used to adjust confocal settings and avoid non-specific fluorescence artifacts. Image quantification of the integrated density (sum of the intensity of all the pixels in the area delimited by the mask) was performed using ImageJ/Fiji software (NIH, Bethesda, MD, USA). The ratio of red versus green fluorescence was calculated within a representative region of each image. Finally, 3D representation images were deconvoluted using the software Huygens Professional Software v17.10.0p8 (Scientific Volume Imaging B.V., Leiden, The Netherlands) in order to improve image quality and resolution. 3D models were generated with Imaris x64 v7.2.1 software (Bitplane, Zurich Switzerland) with Surpass Mode.

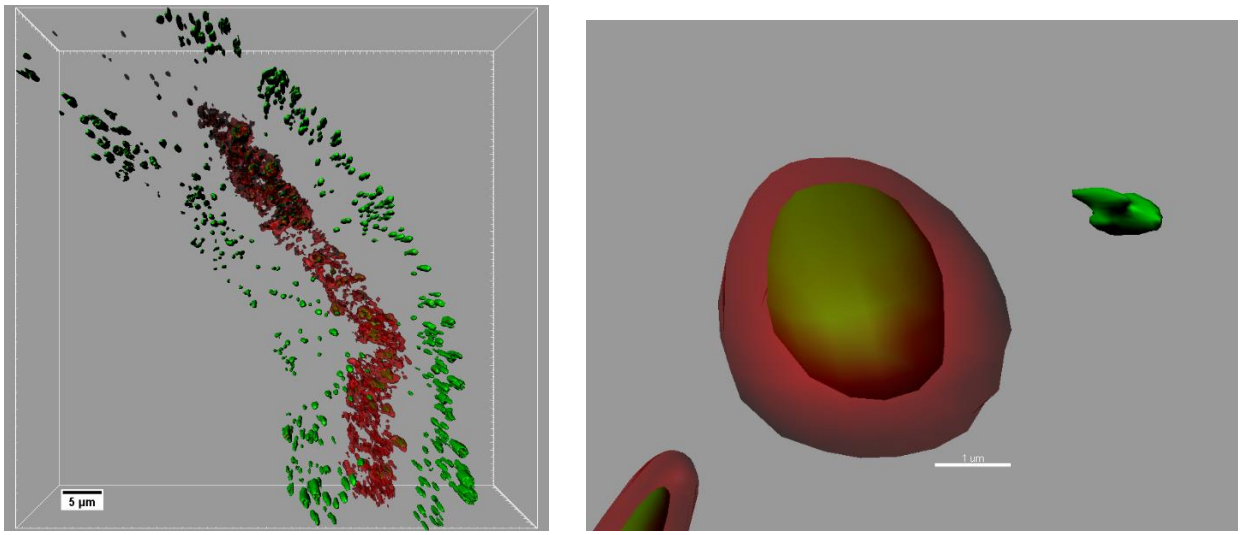


Figure S15. Representative 3D model reconstruction from the confocal stack images of a *C. elegans*. Left) Part of the body, showing the TTMd-ONPs (red) inside the intestine and the lipids (green); Right) Zoom of a small area showing emission of monomer (green) and excimer (red) of a single TTMd-ONPs particle/aggregate. The shell structure of the two different emissions is due to the treatment of the images with two emission channels for which the most intense luminescence stays always outside.

20. Ratiometic output (Q) from confocal analysis of the top part of *C. elegans*

Confocal images were analyzed to see if the ratio (Q) with temperature was following the same trend as the suspension of the ONPs. Figure S16 represents the data of the top part of the *C. elegans* body. Around the pharynx of *C. elegans* a higher amount of lipids is concentrated, which are autofluorescence and influence the overall fluorescence emission.

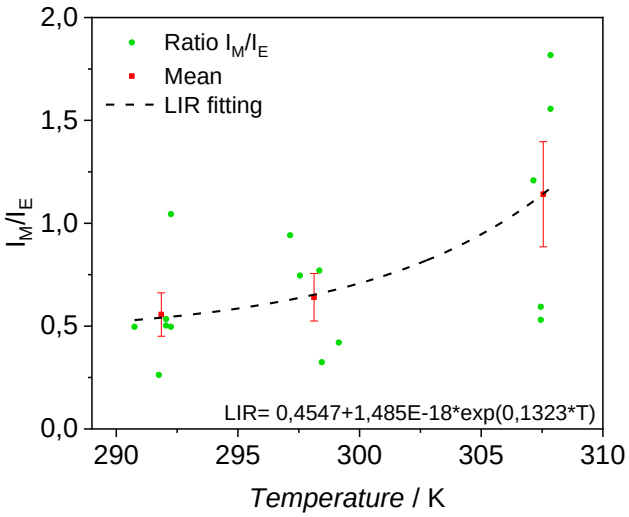


Figure S16. Ratio (Q) between monomer and excimer maxima fluorescence emission at an increasing temperature of the TTMd-ONPs inside *C elegans*, from a region of the top of the body. Red points represent the average of the ratios and the error bars are the deviation caused by different worms for the same temperature range, for n= 6 and p= 0.8419, 0.7675 and 0.7390, respectively.

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