Supplementary information

Study of shape-tunable bimodal $GdPO_4:Eu^{3+}$ nanoparticles and their impact on *Daphnia magna*

Augustas Morkvenas[1,4], Egle Ezerskyte[1,2], Vaidas Klimkevicius[1,2], Zivile Jurgelene[3], Jonas Venius[1], Marius Burkanas[1], Arturas Katelnikovas[2], and Vitalijus Karabanovas[1,4]

[1]Biomedical Physics Laboratory, National Cancer Institute, Baublio 3b, LT-08406, Vilnius, Lithuania

[2]Institute of Chemistry, Faculty of Chemistry and Geosciences, Vilnius University, Naugarduko 24, LT-03225 Vilnius, Lithuania

[3] Nature Research Centre, Akademijos 2, LT-08412 Vilnius, Lithuania

[4]Department of Chemistry and Bioengineering, Vilnius Gediminas Technical University, Sauletekio 11, LT-10223 Vilnius, Lithuania

1. Synthesis of $GdPO_4:Eu^{3+}$ nanoparticles with different morphology

1.1 *Materials*

Gadolinium(III) oxide (Gd_2O_3, 99.99%, Tailorlux, CAS 12064-62-9), europium(III) oxide (Eu_2O_3, 99.99%, Tailorlux, CAS 1308-96-9), ammonium dihydrogen phosphate ($NH_4H_2PO_4$, ≥ 99%, Carl Roth, CAS 7722-76-1), tartaric acid ($C_4H_6O_6$, 99.99%, Eurochemicals, CAS 133-37-9), nitric acid (HNO_3, 70%, Eurochemicals, CAS 7697-37-2), and ammonia solution (NH_3(aq), 30%, Chempur, CAS 1336-21-6). $RE(NO_3)_3$ were prepared by dissolving RE_2O_3 in nitric acid.

1.2 Synthesis procedure

The $GdPO_4$:15%Eu^{3+} samples were obtained via hydrothermal synthesis in an alkaline aqueous medium (pH = 10). The variation in the initial <Gd>:<P> molar ratio resulted in the formation of $GdPO_4$:15%Eu^{3+} particles with different morphologies. The <Gd>:<P> molar ratios were 1:10,

1:50, and 1:100, leading to the formation of nanorods, nanoprisms, and sub-microspheres, respectively.

The synthesis procedure started with the formation of tartaric acid – RE^{3+} complex, which was induced by mixing aqueous solutions of $Gd(NO_3)_3$ (0.85 mL 0.4 M), $Eu(NO_3)_3$ (0.15 mL 0.4 M) and tartaric acid (30 mL 0.3 M). The resulting mixture was magnetically stirred for 30 minutes at room temperature. Afterwards, the pH of the produced solution was adjusted to 10 by adding a concentrated ammonia solution. Subsequently, 20 mL of freshly prepared aqueous $NH_4H_2PO_4$ solution was poured at once and the reaction mixture became turbid. Considering the desired morphology of the particles to be prepared, the concentration of $NH_4H_2PO_4$ solution used was equal to 0.2, 1, and 2 M when the <Gd>:<P> molar ratio was equal to 1:10, 1:50, and 1:100, respectively. Furthermore, the pH of the obtained reaction mixture was again adjusted to 10 using a concentrated ammonia solution and then diluted to 80 mL by adding DI water, followed by adjusting the pH value once again, if required. The resulting solution was magnetically stirred at room temperature for 30 minutes. Finally, the reaction mixture was poured into a Teflon liner and placed in a stainless steel autoclave. The hydrothermal reaction was performed at 160 °C temperature for 12 hours using a hydrothermal reactor by Berghof and BTC-3000 Temperature Controller, as well as a Data Logger by Berghof.

The synthesized particles were centrifuged four times at 10000 rpm for 10 minutes each time. Between centrifugation cycles, the particles were washed with DI water. The obtained powders were dried at 70 °C for 24 hours or stored in aqueous medium.

2. Analysis methods of synthesized $GdPO_4:Eu^{3+}$ NPs

2.1 *X-ray diffraction (XRD) analysis*

The phase purity of the prepared $GdPO_4$:15%Eu^{3+} samples was examined by XRD. XRD patterns were recorded using a Rigaku MiniFlexII diffractometer operating in Bragg-Brentano geometry in

the 5° ≤ 2θ ≤ 80° range under Cu Kα radiation (Ni filter). Scanning step width: 0.02°; speed: 5°/min.

2.2 Scanning electron microscopy (SEM)

Field-emission Hitachi SU-70 SEM images were taken in order to determine the morphology and size distribution of the synthesized phosphate particles. The electron acceleration voltage was 5 kV. The particle size was measured manually (100 particles per sample) using the ImageJ software (v. 1.53m).

2.3 Determination of surface area via N_2 absorption using Brunauer–Emmet–Teller (BET) method

The surface areas of the produced $GdPO_4$:15%Eu^{3+} nanorods, nanoprisms, and sub-microspheres were examined by conducting nitrogen gas adsorption via the BET method using a surface area and porosity analyzer TriStar II 3020.

2.4 *Photoluminescence measurements*

The excitation and emission spectra and photoluminescence (PL) decay curves were recorded using an Edinburgh Instruments FLS980 spectrometer (double grating Czerny-Turner excitation and emission monochromators, 450 W Xe arc lamp, single photon counting photomultiplier Hamamatsu R928P). Excitation spectra were recorded at λ_{em} set to 586 nm, with the excitation and emission slits being 0.5 nm and 1.5 nm, respectively. At the same time, emission spectra were recorded at λ_{ex} set to 393 nm, with the excitation and emission slits being 1.5 nm and 0.5 nm, respectively. In both cases, the measurement step was equal to 0.5 nm and dwell time – 0.2 s. Emission spectra were corrected using an instrument response correction file provided by Edinburgh Instruments. The PL decay curves at λ_{em} = 586 nm were recorded using a μF2 μ-flash lamp as an excitation source (λ_{ex} = 393 nm) with a pulse repetition rate of 25 Hz.

2.5 *MRI measurements and calculations*

The MRI contrast properties of $GdPO_4$:15%Eu^{3+} NPs with different morphology were evaluated as a function of concentration using a clinical 1.5 T MRI scanner (Philips Achieva).

2.5.1 T_1 calculation

To estimate the longitudinal relaxation time T_1, a T_1-weighted turbo spin-echo (TSE) inversion recovery sequence (T_1 WI) was employed. The chosen registration parameters were as follows: time to echo (TE), 10 ms; integration time (IT), ranged between 30 and 2000 ms; repetition time (TR), 4000+IT ms; number of averages, 2; slice thickness, 6 mm; flip angle, 90; number of echoes, 1; TSE factor, 8; matrix, 224 × 224; and field of view, 120 × 120 mm.

The SI values under different inversion times were plotted graphically, and T_1 was calculated by approximating the experimental data by exponential decay using Equation 1.

$$SI(TI) = SI_0\left(1 - 2\left(exp^{\frac{-TI}{T_1}}\right)\right) \quad (1)$$

2.5.2 *T_2 calculation.*

Transversal relaxation time T_2 images were obtained using a turbo spin-echo sequence that was T_2-weighted (T_2 WI). The chosen registration parameters included a range of echo durations (TE) from 10 to 200 ms at various intervals: repetition time (TR), 2500 ms; number of averages, 10; slice thickness, 5 mm; flip angle, 90; echoes, 20; TSE factor, 20; matrix, 224 × 224; FOV, 120 × 120 mm.

The SI values under various TE were visually shown, and T_2 was estimated by approximating the experimental data with exponential decay using Equation 2.

$$SI(TI) = SI_0 exp^{\frac{-TE}{T2}} \quad (2)$$

2.5.3 Relaxivity r_1 & r_2 calculations.

T_1 and T_2 were calculated for r_1 and r_2, respectively, using previously established methods for various Gd molar concentrations. The values of $1/T_1$ and $1/T_2$ at multiple concentrations were displayed and linearly approximated. The slope of this line determines the molar relaxivity r.

3. *Confocal fluorescence microscopy and imaging*

After 24-hour exposure to NPs with different morphologies, alive *D. magna* were placed on glass bottom dishes (NuncTM, Thermo Fisher Scientific, Tokyo, Japan) in small water droplets using a Pasteur pipette (Biosigma S.r.l.). This step ensures that the *D. magna* remains alive and prevents excessive movement, allowing for accurate imaging with a confocal microscope. *D. magna* was imaged using a confocal Nikon Eclipse Te2000-U microscope (Nikon, Yokohama, Japan) with a C1si laser scanning confocal system. The microscope was equipped with a Fianium WhiteLase Micro supercontinuum laser (NKT Photonics, Birkerød, Denmark) with a pulse repetition rate of 30 MHz and filtered using a 400 ± 10 nm band-pass filter (Thorlabs Inc., Newton, NJ, USA) for NPs and autofluorescence detection in *D. magna*. Imaging was performed using a 10/0.25 NA (Plan Apo VC, Nikon, Yokohama, Japan). The bright-field mode of Nikon Eclipse Te2000-U and detectors with band-pass filters of 515/30 nm and 605/75 nm for the green and red channels, respectively, were used. Image processing was performed using the EZ-C1 Bronze software (v.3.80, Nikon, Tokyo, Japan) and ImageJ software (v.1.53e, U.S. National Institutes of Health, Bethesda, MD, USA).

4. Bioassay

D. magna was obtained from the Laboratory of Evolutionary Ecology of Hydrobionts (Nature Research Centre, Vilnius, Lithuania) and has been kept in the laboratory for several hundred generations. The offspring from the fourth brood were used for inhibition assays (duration 24-hours) performed according to the ISO 6341:2012 standard (ISO 6341:2012, 2012) and OECD recommendations for toxicity tests of *Daphnia sp*. (OECD, 2004) with some modifications. *D. magna* were cultivated in deep-well water at 20 °C under a 16/8 hours light/dark cycle and no

feeding regiments were applied. Six test concentrations of NPs with different morphologies (3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) and a control (0 μg/mL) were used. The NPs solutions were sonicated (15 min) prior to concentration preparation. In all experiments, deep-well water was used as the dilution water. The main physicochemical parameters of the deep-well water are presented in Table S1. The mean pH of dilution water was 8.2.

Table S1. Chemical characteristics (mg/L) of the deep-well water.

Metals		**Cations**		**Anions**	
Zn	0.0128	Na^+	3.2	Cl^-	3.7
Cu	<0.001	K^+	1.2	SO_4^{2-}	18.4
Cr	<0.001	Ca^{2+}	70.1	HCO_3^-	258
Ni	<0.002	Mg^{2+}	16.5	CO_3^{2-}	0.18
Pb	<0.001	Fe^{2+}	0.1	NO_2^-	<0.010
Cd	<0.0003	Fe^{3+}	<0.01	NO_3^-	<0.050
		Fe_{total}	0.1		

For each NPs concentration, 10 neonates (< 24 hours old) from a designated brood were placed in 50 mL glass beakers containing 20 mL test solution for 24 hours. The daphnids were maintained at a constant temperature with a 16:8 h light: dark cycle in a climate cabinet (PGC-660, Bronson, Zaltbommel, the Netherlands). After 24 hours of exposure, the mortality of the individuals in each glass beaker was assessed. Animals were considered dead when there was no movement and no heartbeat . All experiments were conducted in triplicates.

4.1 Heart rates counting

Alive *D. magna* was placed on an objective slide in a 50-µL droplet of solution from the respective NPs concentration after 24 hours of exposure. The heart rate (HR) of 15 *D. magna* individuals was measured during a 10 s periods using an optical microscope (Optika B-600TiFL (Italy) equipped with a digital camera (Leica, Germany)) at x4 magnification, recorded at low speed. The mean HR value for 1 minute of the 15 daphnids was calculated.

4.2 *Behavior alterations*

Behavioral parameters were investigated through video tracking using a DanioVision Observation Chamber (Noldus, Wageningen, the Netherlands). It was equipped with a GigE digital IR-sensitive camera (Noldus, Wageningen, the Netherlands), which recorded 30 frames/second at 1080p resolution, and a temperature control unit. The temperature was set to 21 °C, and after 5 min of acclimation, the recording proceeded for 30 min. To analyze the video recordings, we used EthoVision XT version 17 software (Noldus), which can distinguish daphnids from background media and track their whereabouts in the well plate, with each well defined as an arena. Video tracking analyses were performed in 24-well plates and tracked individually by placing one individual in one well containing 500 µL of the exposure medium. After the 24 hours test, the behavioral effects (distance moved (cm), which is the distance traveled continuously for a 30 min period and swimming velocity (cm/s), which is determined by movement portrayed by accelerations followed by slowdowns) were estimated.

5. Statistical analysis

The 24 hours LC_{50} (i.e., the lethal concentration that kills 50% of a *D. magna* population) values, as well as their associated 95% confidence intervals (95% CI), were calculated using the trimmed Spearman-Karber method (Hamilton et al., 1977). A log (dose)-normalized response curve (Y = 100/ (1+10(logEC50−x) × hillslope) was plotted using *D. magna* mortality rates (%) and GraphPad Prism (version 8.1.2, GraphPad software, San Diego, CA, USA). Toxicity data were first checked for normal distribution with Kolmogorov-Smirnov and Shapiro-Wilk tests and homogeneity of

variances with Bartlett's test. ANOVA (F-statistics, two-way) followed by a Post-hoc Tukey's HSD test was used. Toxicity data analyses were performed using the STATISTICA software (10.0 Software, Inc. PA, USA).

Results

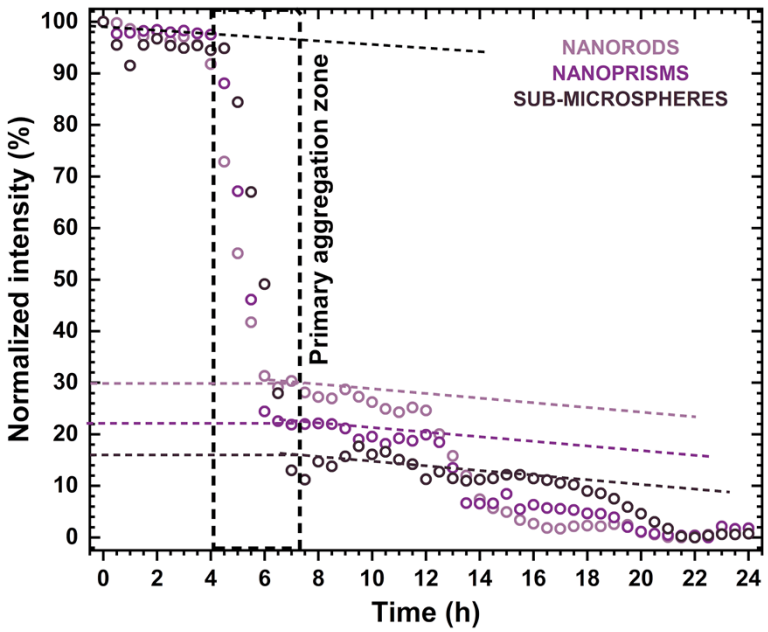

Fig. S1. Changes in emission intensity of NPs in deep-well water over 24 hours.

Table S2. Comparison of 24 h LC_{50} values of *D. magna* exposed to nanoprisms, nanorods, and sub-microspheres.

NPs	LC_{50} (µg/mL)	0.95 confidence interval (µg/mL)
Nanorods	52.36	44.75 – 61.28

Nanoprisms	60.15	53.7 – 67.38
Sub-microspheres	64.47	59.15 – 70.26

References

ISO 6341:2012(En), Water Quality — Determination of the Inhibition of the Mobility of *Daphnia magna* Straus (Cladocera, Crustacea) — Acute Toxicity Test. 29, 2023. https://www.iso.org/obp/ui/#iso:std:iso:6341:ed-4:v1:en.

Hamilton, Martin A., Rosemarie C. Russo, and Robert V. Thurston. 1977. Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. Environmental Science & Technology 11 (7): 714–19. https://doi.org/10.1021/es60130a004.

OECD, 2004. Test No. 202: Daphnia sp. Acute Immobilisation Test. https://doi.org/10.1787/9789264069947-en.