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PAPER

Biosafety assessment of $Gd@C_{82}(OH)_{22}$ nanoparticles on *Caenorhabditis elegans*

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$Gd@C_{82}(OH)_{22}$, a water-soluble endohedral metallofullerene derivative, has been proven to possess significant antineoplastic activity in mice. Toxicity studies of the nanoparticle have shown some evidence of low or non toxicity in mice and cell models. Here we employed *Caenorhabditis elegans* (*C. elegans*) as a model organism to further evaluate the short- and long-term toxicity of $Gd@C_{82}(OH)_{22}$ and possible behavior changes under normal and stress culture conditions. With treatment of $Gd@C_{82}(OH)_{22}$ at 0.01, 0.1, 1.0 and 10 μg ml^{-1} within one generation (short-term), *C. elegans* showed no significant decrease in longevity or thermotolerance compared to the controls. Furthermore, when $Gd@C_{82}(OH)_{22}$ treatment was extended up to six generations (long-term), non-toxic effects to the nematodes were found. In addition, data from body length measurement, feeding rate and egg-laying assays with short-term treatment demonstrated that the nanoparticles have no significant impact on the individual growth, feeding behavior and reproductive ability, respectively. In summary, this work has shown that $Gd@C_{82}(OH)_{22}$ is tolerated well by worms and it has no apparent toxic effects on longevity, stress resistance, growth and behaviors that were observed in both adult and young worms. Our work lays the foundations for further developments of this anti-neoplastic agent for clinical applications.

Introduction

Currently, the antitumor drugs prevailing in clinical tumor therapy are in most cases accompanied by inevitable high side effects. For instance, the commonly used antineoplastic agent, cisplatin, is highly toxic and drug resistant in many types of tumors. Hence, its applications in chemotherapeutics have been restricted.[1,2] However, $Gd@C_{82}(OH)_{22}$, a new metallofullerenol with an average size of 22 nm in saline solution,[3] has recently been found to be an antitumor candidate with distinctive low or no cytotoxicity. Chen *et al.*[3] discovered that $Gd@C_{82}(OH)_{22}$ exhibited high inhibitory activity on hepatoma cell (H22) implanted in mice with high efficacy but low toxicity. Interestingly, the nanoparticles do not kill the tumor cells directly. The exact mechanism has not yet been fully understood, but previous studies have suggested involvements of improved immunity,[4,5] the inhibition of angiogenesis and antioxidant activity[6–8] and its size-based selective targeting to tumor sites.[3,9]

Meanwhile, in the past few years, researchers have also explored the toxicity of $Gd@C_{82}(OH)_{22}$ *in vivo* and *in vitro*. It has been shown that the nanoparticles could efficiently prohibit hapatocellular and renal damage by tumor implantation in tumor-bearing mice.[3,7] Besides, $Gd@C_{82}(OH)_{22}$ caused no abnormal behavior or deaths or other systemic toxicity in tumor-bearing mice.[4,7] In addition, $Gd@C_{82}(OH)_{22}$ was found to be nearly non-cytotoxic to immune system cells.[4] These results initially proved the low toxicity of the nanoparticles.

In the above studies, the tumor-bearing mice were typically chosen as models for the *in vivo* toxicity assessment of $Gd@C_{82}(OH)_{22}$ for weeks accompanied by studies on potent antineoplastic effects of the nanoparticles.[3,7] Although rodent animals have been the traditional model in the toxicity studies, the long experimental period and relatively high cost restrict their applications in long-term toxicity studies. To the best of our knowledge, the *in vivo* effects of the nanoparticles under tumor-free and long-term administrative conditions have not been investigated. It is known to us that the lack of *in vivo* toxicological studies have hampered clinical use of a large number of candidate agents. Therefore, to fully study the potential adverse influence of the nanoparticles on organisms, we carried out further exploration using a new model with a shorter life cycle and simpler internal environment, thus facilitating chronic

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experiments and reducing the interference of complicated mechanisms existing in higher animals. In our work, we chose nematode *Caenorhabditis elegans* (*C. elegans*) as a system to further test the potential toxicological effects of $Gd@C_{82}(OH)_{22}$. As a simple, invertebrate animal model, *C. elegans* has features that differ from both rodent animal and cell models in toxicological research. The ease of maintenance, short life cycle, and small body size of nematodes facilitate their use in long-term toxicity investigations as well as studies of drug effects on early age toxicity and reproductive capacity. Furthermore, the whole organism allow researchers to observe the phenotypes that are well-characterized and more biologically relevant than cell models. More importantly, many of the basic physiological processes and stress responses that are observed in higher organisms (*e.g.*, humans) are conserved in *C. elegans*, making the results obtained from them predictive for vertebrates.[10] Due to these virtues, *C. elegans* has become an excellent test invertebrate for the rapid toxicological assessment of a variety of chemicals on agar plates.[11–13]

In this paper, our objectives are to explore whether $Gd@C_{82}(OH)_{22}$ can influence the aging process, stress responses and some other physiological behaviors. For these purposes, the uptake of $Gd@C_{82}(OH)_{22}$ by *C. elegans* is firstly determined. Then, both the lifespan and heat shock assays are carried out within one-generation treatment of $Gd@C_{82}(OH)_{22}$. To study the long-term effects of the nanoparticles on *C. elegans*, we extend the period of the treatment from one to successively six generations, and both the lifespan and heat shock assays are also performed. Besides, body length, pharyngeal pumping rate and the reproductive capacity of worms are also investigated.

Results and discussion

$Gd@C_{82}(OH)_{22}$ nanoparticle can be taken up by *C. elegans*

Stability and the inability of Gd^{3+} liberating from the fullerene cage have been confirmed.[4,7] Therefore, we can determine that the status of Gd *in vivo* representing exact the distribution as well as the content of $Gd@C_{82}(OH)_{22}$ nanoparticles in organisms.

The total content of $Gd@C_{82}(OH)_{22}$ in the body was measured *via* inductively coupled plasma-mass spectrometry (ICP-MS) (Fig. 1). The content of Gd in a control group is here taken as the baseline value in *C. elegans*. The content of Gd in $Gd@C_{82}(OH)_{22}$ group at 2 μg ml^{-1} and 10 μg ml^{-1} were 1.21 and 1.68 folds of that in control, respectively. These results suggested that $Gd@C_{82}(OH)_{22}$ nanoparticles has been ingested by *C. elegans*.

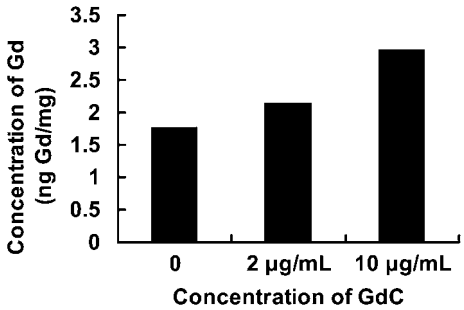

Fig. 1 The content of Gd in *C. elegans* (expressed as concentrations of gadolinium element, ng Gd mg^{-1} total protein in a pool of about 1,000 adults for each group). $Gd@C_{82}(OH)_{22}$ treatment (0, 2.0 μg ml^{-1} and 10 μg ml^{-1}) started at L1 stage and onward for 72 h.

Neither does short- or long-term administration of $Gd@C_{82}(OH)_{22}$ significantly decrease the lifespan and thermotolerance of *C. elegans*

The lifespan assay indicates the intervention of toxicants on the the aging rate of the nematodes and is a simple signal for toxicity in *C. elegans*. Hence, it has been widely used in environmental and pharmacological toxicity studies.[14–16] Besides, there are many correlations between stress resistance and longevity.[17] Previous studies have shown that lifespan extension is associated with improved thermotolerance,[18,19] and genetic manipulations that increase life span in many species also enhance resistance stress.[14,20–27] In this study, the two signals, the lifespan of worms under normal conditions and thermotolerance at 35 °C are both performed. The dosages for *C. elegans* in our work were designated as 0.01, 0.1, 1.0 and 10 μg ml^{-1}, respectively, which were generally in the concentration range in the cytotoxicity experiment on HepG2 and RH35 by Chen *et al.*[3] Herein, we name the treatment of $Gd@C_{82}(OH)_{22}$ for one generation as short-term treatment, and that for successively six generations as long-term treatment.

Lifespan assays with short-term treatment manifested that $Gd@C_{82}(OH)_{22}$ did not significantly affect the worm lifespan at all tested dosages at 20 °C (the optimal culture temperature for *C. elegans*). (Fig. 2a and Table 1). To test the short-term effect of $Gd@C_{82}(OH)_{22}$ on the thermotolerance of *C. elegans*, we exposed synchronous worms to heat shock at 35 °C after

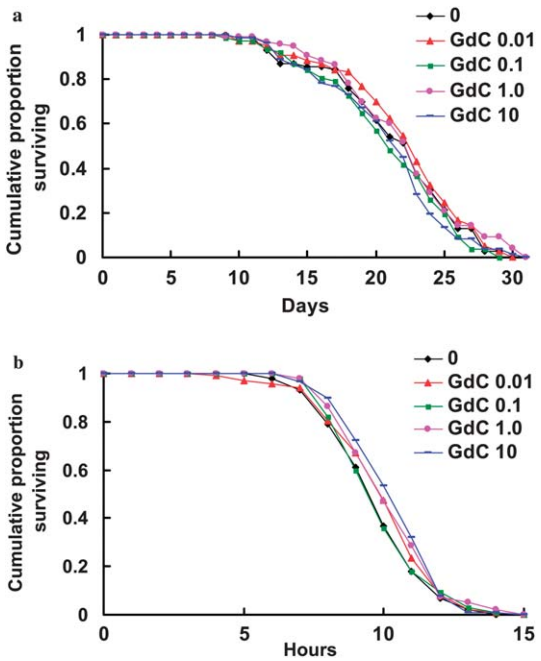

Fig. 2 Short-term effects of $Gd@C_{82}(OH)_{22}$ on the lifespan (a) and thermotolerance (b) of N2 nematodes with treatment for one generation. For lifespan assays, the worms were grown at 20 °C and the nanoparticles treatment began shortly after adulthood until the end; For thermotolerance assays, L1 larva were fed on $Gd@C_{82}(OH)_{22}$ plates onward for 72 h before exposed to 35 °C.

Table 1 The effects of $Gd@C_{82}(OH)_{22}$ on the lifespan and thermotolerance of *C. elegans*[a]

Treatment of GdC	Assays	Concentration (ug ml^{-1})	MLS	S.E.	%	*P*	n
Short-term	Lifespan assays at 20 °C	0	21.5	0.584			70
		GdC 0.01	22.2	0.542	3.0	0.541	77
		GdC 0.1	20.9	0.541	−2.8	0.305	77
		GdC 1.0	22.1	0.480	2.8	0.437	96
		GdC 10	20.9	0.520	−2.7	0.371	82
Short-term	Thermotolerance assays at 35 °C	0	9.9	0.180			90
		GdC 0.01	10.1	0.197	2.1	0.242	97
		GdC 0.1	10.1	0.170	1.3	0.704	95
		GdC 1.0	10.4	0.178	4.7	0.068	94
		GdC 10	10.5	0.147	5.9	0.022	116
Long-term	Lifespan assays at 20 °C	0	19.4	0.568			74
		GdC 0.01	20.8	0.621	7.3	0.047	72
		GdC 0.1	18.6	0.539	−3.7	0.454	82
		GdC 1.0	19.8	0.624	2.0	0.572	62
		GdC 10	19.9	0.677	2.5	0.522	54
Long-term	Thermotolerance assays at 35 °C	0	9.9	0.184			95
		GdC 0.01	10.3	0.180	4.4	0.043	126
		GdC 0.1	10.1	0.167	2.2	0.481	107
		GdC 1.0	9.9	0.182	0.1	0.949	96
		GdC 10	9.6	0.174	−3.7	0.229	109

[a] Short- and long-term treatment refer to treatment of $Gd@C_{82}(OH)_{22}$ for one and six generations to worms, respectively. MLS, Mean lifespan; S.E., Standard error; %, change in mean lifespan compared with control; *P*, comparison with untreated animals by log-rank test; n, numbers of death observed.

pretreatment with $Gd@C_{82}(OH)_{22}$ for 72 h. We found that $Gd@C_{82}(OH)_{22}$ at 0.01, 0.1, and 1.0 μg ml^{-1} enhanced the mean longevity of *C.elegans* by 2.1%, 1.3% and 4.7%, respectively, no significant differences observed ($P > 0.05$). Interestingly, $Gd@C_{82}(OH)_{22}$ at 10 μg ml^{-1} increased the mean lifespan by 5.9% ($P = 0.022$), but the extension on lifespan was not obvious (Fig. 2b and Table 1).

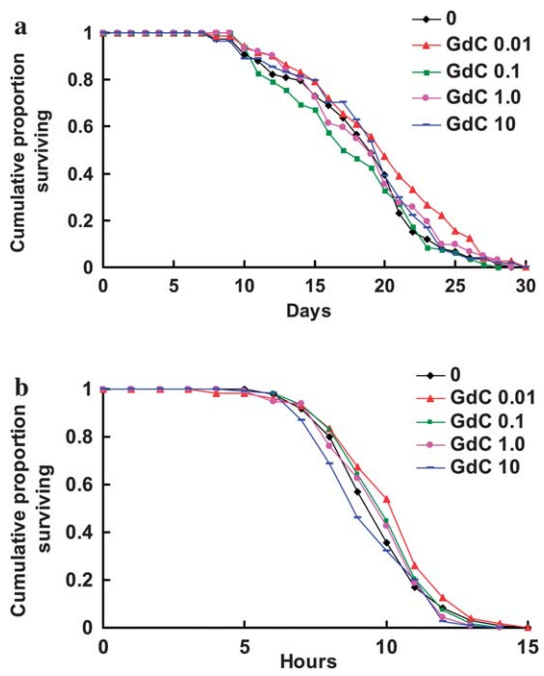

Fig. 3 Long-term effects of $Gd@C_{82}(OH)_{22}$ on the lifespan (a) and thermotolerance (b) of N2 nematodes with treatment for six generations. The worms were cultured at 20 °C in the presence of the nanoparticles for six generations. The 6th worms were assayed for the lifespan. For thermotolerance assays, the 6th worms at L1 stage were continuously fed with particles for 72 h before subjected to heat shock.

To test whether the potential toxic effect of $Gd@C_{82}(OH)_{22}$ could be highlighted after long-term treatment to *C. elegans*, we extended the treatment to worms from one to successively six generations at 20 °C. Lifespan and thermotolerance were investigated on the 6th generation. Again, we found that $Gd@C_{82}(OH)_{22}$ at all tested concentrations demonstrated no obvious adverse effects on the mean longevity ($P > 0.05$) compared with the control in either assays (Fig. 3 and Table 1). Interestingly, $Gd@C_{82}(OH)_{22}$ at 0.01 μg ml^{-1} enhanced the mean longevity of worms by 7.3% ($P = 0.047$) and 4.4% ($P = 0.043$) in lifespan and thermotolerance assays, respectively. These observations may suggest that the nanoparticles might possess anti-aging and stress-resistance enhancing activities, though the effect is not significant in the current experimental systems.

In the results above, no significant decrease was observed in the mean lifetime of *C. elegans* upon short- or long-term treatment of $Gd@C_{82}(OH)_{22}$ in both lifespan and heat shock assays. On the contrary, $Gd@C_{82}(OH)_{22}$ seemed to possess some activities of promoting the longevity and thermotolerance. $Gd@C_{82}(OH)_{22}$ proved to have high antioxidative activity both *in vitro* and *in vivo*.[7,8] Based on the free radical hypothesis of ageing advanced by Harman[28] we assumed that the effects on the lifespan and thermotolerance may be contributed to the antioxidant capabilities of $Gd@C_{82}(OH)_{22}$ nanoparticles.

The growth, feeding behavior and reproductivity of *C. elegans* remain unchanged upon the administration of $Gd@C_{82}(OH)_{22}$

Apart from the survival rate assays above, the short-term toxicity of $Gd@C_{82}(OH)_{22}$ was also investigated in *C. elegans* using growth, feeding rate and reproductive capacity as toxicological endpoints. The index of body length reveals the rate of development and physiological status of *C. elegans*. We conducted

Table 2 The short-term effects of $Gd@C_{82}(OH)_{22}$ on body length, pumping rate and total progeny.[a]

Treatment	Body length/μm	*P* value	Pumping rate/min	*P* value	Total progeny	*P* value
0	1464 ± 53	—	339 ± 19	—	236 ± 7	—
GdC 0.01	1490 ± 34	0.210	328 ± 25	0.477	246 ± 17	0.424
GdC 0.1	1453 ± 61	0.685	332 ± 37	0.688	246 ± 12	0.267
GdC 1.0	1473 ± 62	0.470	318 ± 23	0.181	262 ± 15	0.077
GdC 10	1482 ± 57	0.460	335 ± 34	0.816	236 ± 34	0.989

[a] Body length and pumping rate were measured when worms reached adulthood. The total progeny was counted within the designated duration of 5 days.

body length measurement at the endpoint of treatment (from L1 stage for 72 h). Body length results showed no significant differences between the control and $Gd@C_{82}(OH)_{22}$ treatment groups (0.01, 0.1, 1.0 and 10 μg ml^{-1}), suggesting that no growth delay was caused by the putative toxicity of $Gd@C_{82}(OH)_{22}$ (Table 2). Besides, abnormalities were not detected in all groups during the lifespan assays mentioned above as well as this endpoint assay (data not shown).

Pharyngeal pumping rate was assessed to study the possible toxicity of $Gd@C_{82}(OH)_{22}$ to the feeding behavior of the nematodes. Compared with the control, $Gd@C_{82}(OH)_{22}$ at all designated concentrations (0.01, 0.1, 1.0 and 10 μg ml^{-1}) did not significantly affect the pumping rate (Table 2).

Self-fertile hermaphrodites display an age-related decline in progeny production that has been measured by determining the number of progeny produced on each day of adulthood.[29–31] This method provides accurate and precise measurements of daily progeny production. We found that the egg-laying behavior of nematodes, including the startpoint and the declining tendency of progeny number *versus* time, displayed no obvious differences between the control and treatment groups (Fig. 4). Although egg-laying curves exhibited progeny at 1.0 μg ml^{-1} on day 2 were slightly enhanced, there were no significant differences compared with the control otherwise ($P = 0.074$). Furthermore, the total progeny per worm during the designated period had no significant variation due to the exposure to $Gd@C_{82}(OH)_{22}$ (Table 2). Taken together, we can conclude that $Gd@C_{82}(OH)_{22}$ at all designated concentrations represented nearly no impact on the reproductive capacity of the nematodes.

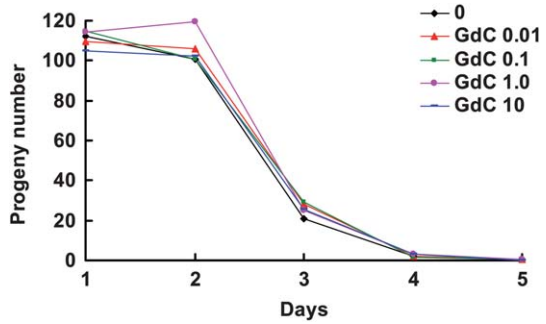

Fig. 4 Short-term effect of $Gd@C_{82}(OH)_{22}$ on the reproductive activity of N2 nematodes. This figure shows average daily progeny production of live hermaphrodites. The worms were fed with or without $Gd@C_{82}(OH)_{22}$ from egg stage to the end of egg-laying assays. Day 1 of this assay was designated as the duration from the startpoint (0 h) of egg-depositing to 24 h of the control. The animals were transferred to a fresh dish daily, and the numbers of live progeny produced daily were scored for a total of 5 days.

Wild-type hermaphrodites were reported to have a mean self-fertile reproductive span of 5.8 (±2.0) days[32] previously. In this study, the self-fertile reproductive span is referred to the time from L4 stage to the last day of self-progeny production. In contrast, in our work the startpoint of the measurement was defined as the beginning of egg-laying. It is known that wild-type hermaphrodites cultured at 20 °C begin to deposit fertilized eggs about 12 h after the L4 stage.[33] On the day 5 in our assays, each worm deposited no more than one egg on average. Taken both of these observations, our results of the mean reproductive span was generally consistent with the reports.

Conclusion

In this paper we employed *C. elegans* as a model to investigate the potential toxicity of the promising antitumor drug candidate, $Gd@C_{82}(OH)_{22}$ nanoparticles. Our results suggest that the $Gd@C_{82}(OH)_{22}$ nanoparticle exhibits nearly no toxic effects on the well-established toxicological testing model, *C. elegans.* With either short- or long-term administration of $Gd@C_{82}(OH)_{22}$ at a concentration range of 1000 times (from 0.01 to 10 μg ml^{-1}) to nematodes, the lifespan and thermotolerance were not significantly influenced. Besides, the developmental process, pharyngeal pumping behaviour and reproductive capabilities also remained nearly unchanged due to exposure to the nanoparticles within one generation (short-term treatment). Thus, the features of low or no toxicity $Gd@C_{82}(OH)_{22}$ nanoparticles may accelerate their development as a potent potential antitumor agent in the future.

Experimental section

Reagents and worms maintenance

$Gd@C_{82}(OH)_{22}$ were synthesized by the Krätschmer–Huffman method[34] and extracted by a high-temperature and high-pressure method.[35] The nanoparticle characterizations have been described by Chen *et al.*[3] FUDR (5-fluoro-2′-deoxyuridine), 98%, was bought from Sigma. Bristol N2 (Caenorhaditis Genetics Center; CGC) was used as the wild-type strain. Worms were maintained on standard nematode growth medium (NGM) plates at 20 °C.[36] Unless stated otherwise, plates were seeded with live *Escherichia coli* (*E. coli*) OP50 bacteria[36] (CGC). All of the assays in this study started with synchronized worms in the L1 stage to avoid the influence of developmental stage on the toxicity test. To obtain the synchronized L1 worms, gravid adults

were washed with 4 ml M9 buffer into 15-ml centrifuge tubes and 0.5 ml of 5 M NaOH and 0.5 ml of 10% household bleach were subsequently added. Then shake well the tube and repeat shaking every a few seconds for a total of 7 min before centrifuging for 3 min at 1, 300 g M9 buffer was then added to the pellet and centrifuged again to remove the remaining NaOH and bleach. The released eggs were allowed to hatch and L1 worms were then gathered for assays.

Determination of the uptake of $Gd@C_{82}(OH)_{22}$ by *C. elegans*

The content of Gd in *C. elegans* was analyzed *via* inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500a). We began administration of $Gd@C_{82}(OH)_{22}$ (0, 2.0 μg ml^{-1} and 10 μg ml^{-1}) to worms from L1 stage onward for 72 h. At the endpoint of treatment, the worms were washed with ice-cold ultrapure water for four times as above. Then worm samples were quantified, digested and analyzed for Gd content. For quantification of worms, the worm pellet was lysed (lysis buffer: 50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100 and protease inhibitors), sonicated and centrifugated for 10 min at 12,000 g to precipitate insoluble components. Supernatants were used for protein quantification as described in the protocol of BCA assay kit (Applygen technologies Inc., China). Prior to elemental analysis, the samples were digested in nitric acid and heated at 150 °C. Hydrogen peroxide solution was used to drive off the vapor of nitrogen oxides until the solution was colorless and clear. After the solution volume was adjusted to 2 ml using 2% nitric acid, the Gd content was analyzed using ICP-MS. Indium (20 ng ml^{-1}) was used as an internal standard.

Lifespan assays

Lifespan assays were performed on *C. elegans* wild-type N2 at 20 °C. $Gd@C_{82}(OH)_{22}$ treatment started when worms reached adulthood and worms were transferred to fresh $Gd@C_{82}(OH)_{22}$ treatment plates (treatment plates) every 2 days for the first 10 days of the treatment and every 4 days for the days onward. The death numbers of *C. elegans* were counted and recorded every day. To prevent the accumulation of progeny on $Gd@C_{82}(OH)_{22}$ treatment plates, the reproductive suppressant FUDR was supplemented over the entire brood period. $Gd@C_{82}(OH)_{22}$ at 0.01, 0.1, 1.0 or 10 μg ml^{-1} and FUDR (Sigma; 100 mg L^{-1}) was diluted into *E. coli* OP50 suspension and added to the surface of the dry NGM plates to the indicated final concentrations.[37,38] The day when L1 worms were recovered and exposed to *E. coli* OP50 was designated as day 0.

Except for the lifespan assays with one-generation treatment of $Gd@C_{82}(OH)_{22}$ mentioned above, successively treatment for 6 generations were also conducted for lifespan assays so as to magnify the potential effects of $Gd@C_{82}(OH)_{22}$ on *C. elegans*. Synchronous L1 hermaphrodites were transferred to $Gd@C_{82}(OH)_{22}$ treatment plates at concentrations of 0, 0.01, 0.1, 1.0 or 10 μg ml^{-1}, which were designated as the first generation (G1). When G1 reached young adult stage, several G1 worms of each group were picked up to fresh $Gd@C_{82}(OH)_{22}$ treatment plates correspondingly, allowed to lay eggs (G2) for some hours and then picked out of the plates. Subsequently, G2 worms were treated following this method till G5. We used synchronous G6 worms for the lifespan assays. G6 worms were exposed to $Gd@C_{82}(OH)_{22}$ from L1 till the end of the assays.

Thermal stress assays

To investigate whether $Gd@C_{82}(OH)_{22}$ could influence the stress-resistance of *C. elegans* under stress, we conducted thermal stress assays on *C. elegans* wild-type N2 at 35 °C. For this, synchronous L1 worms were exposed to plates with $Gd@C_{82}(OH)_{22}$ at concentrations of 0, 0.01, 0.1, 1.0 or 10 μg ml^{-1} for 72 h before being exposed to heat shock at 35 °C. During thermal stress treatment, dead worms were counted every hour.[18,38] Consistent with that in lifespan assays, treatment of $Gd@C_{82}(OH)_{22}$ successively for 6 generations at 20 °C were also performed before thermal stress assays. Synchronous L1 nematodes of G6 were exposed to $Gd@C_{82}(OH)_{22}$ at 20 °C for 72 h and then followed by heat shock.

Body length measurement

L1 nematodes were cultured in the presence of $Gd@C_{82}(OH)_{22}$ for 72 h. At the end of the exposure period, about 10 living worms per time were pipetted into a drop of water on a glass slide, covered with a coverglass and heated to approximately 50 °C. This treatment resulted in straight and easily measurable worms. The body images were snapped in light field by an inverted fluorescent microscope (Olympus X71) and the length of the body was measured using the distance measurement of the software.

Pharyngeal pumping rate

Pumping assays were performed on agar at room temperature (approximately 22 °C). Pumping rate was defined as the number of contractions in the terminal bulb in a 1-min period. For pumping rates assessment, worms were raised with or without $Gd@C_{82}(OH)_{22}$ from day 0 for 72 h. At the endpoint of treatment, pumping was recorded as videos in light field by an inverted fluorescent microscope (Leica DMI 3000B) equipped with a camera. We counted the number of pumping in each video for three times, the average of which was used for statistics.[32,39]

Egg laying assays

The reproduction was examined by the method by Huang *et al.*[32] with some modifications. To obtain synchronized worms for reproduction assessment, we put several gravid worms on NGM plates with or without $Gd@C_{82}(OH)_{22}$, allowing them to lay eggs for one hour. Day 1 of this assay was designated as the duration from the startpoint (0 h) of egg-depositing to 24 h of the control. The animals were subsequently transferred to a fresh dish daily, and the numbers of live progeny produced daily were scored for a total of 5 days. Reproductive capacity was evaluated by determining the egg-laying curve and the total offspring produced in the designated period.

Statistical tools

The data of lifespan assays and stress resistance assays were processed with Kalpan-Meir survival analysis of SPSS 13.0.

P values were calculated by Kaplan–Meier log-rank pairwise comparison between the control and the treated groups.

Acknowledgements

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