Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Electronic supplementary information:

Toxicity of halloysite clay nanotubes *in vivo*: a *Caenorhabditis elegans* study

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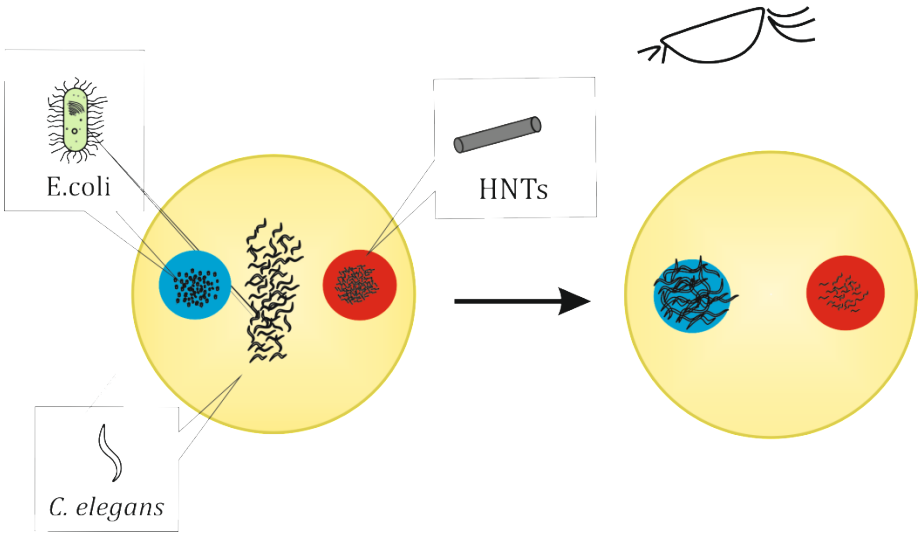
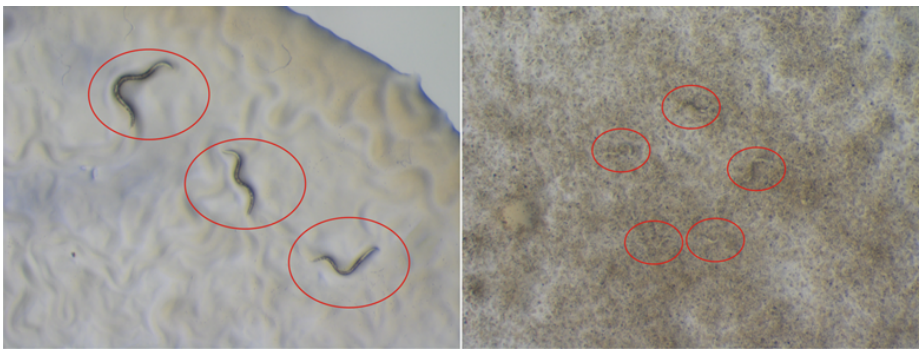


Figure S1. A scheme demonstrating the behavioural test to investigate the chemotaxis of nematodes for pure and halloysite-mixed food.



a b

Figure S2. Typical stereomicroscopy images (taken at the same zoom) of nematodes feeding on pure *E. coli* (a) and *E. coli* mixed with HNTs (b)

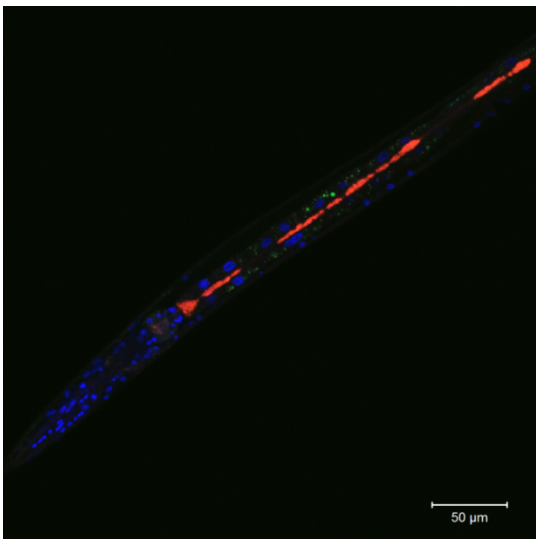


Figure S3. A typical confocal microscopy image of rhodamine-labelled HNTs localisation inside the intestines of a *C. elegans* nematode.

Table S1. The effects of HNTs on the lifespan of *C. elegans*

Concentration of HNTs (mg ml^{-1})	Mean lifespan ± Standard error	% control	P value against control (log rank test)	Total number of animals
0	11,37 ± 0,180			114
0,05	11,23±0,173	-1,23	0,047	118
0,1	11,14±0.1963	-2,02	0,068	162
0,5	10,08±0,221	-11,3	0,29	126
1	9,51±0,29	-16,4	0,34	117

Real-time footages:

Video 1: Brownian motion of a single isolated halloysite nanotube recorded using enhanced dark-field microscopy

Video 2: Localisation of halloysite nanotubes in intestines of the nematode – a whole body imaging of a fixed animal

Video 3: Movement of dispersed halloysite nanotubes in the head region of a live nematode

Video 4: Movement of dispersed halloysite nanotubes in the midgut region of a live nematode

Experimental section

Materials and reagents

Halloysite nanotubes (HNTs) have been obtained from Applied Minerals Inc (USA). HNTs prior to use were washed trice with ethanol and then with sterile water and dispersed on an ultrasonic bath. Fluorodeoxyuridine (FUDR), 4',6-diamidino-2-phenylindole (DAPI), Poly(allylamine

hydrochloride) (PAH, molecular weight ca. 58 kDa) and poly(styrene sulfonate sodium salt) (PSS molecular weight ca. 70 kDa) were purchased from Sigma-Aldrich, were of analytical grade and used as received. MilliQ water purified by reverse osmosis system (Millipore) was used throughout the study.

C. elegans worms maintenance

C. elegans wild type strain (N2 Bristol) was maintained at 20°C in darkness on agar-based nematode growth medium (NGM) (US Biological, USA) 15 cm plates with *Escherichia coli* OP50 bacteria as a food source.[S1] To obtain the synchronized worms, the gravid adults were washed from the Petri dishes with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85.5 mM NaCl, 1 mM $MgSO_4$), centrifuged and then the pellet was mixed with aqueous 2% NaOCl and 0.45 M NaOH and incubated for 10 min while shaking. After the complete destruction of all worms the preserved eggs were washed with M9 buffer several times and then transferred into sterile NGM plates. After 24 hours of incubation at 20 °C, the plates were inoculated either with intact or with HNTs-coated microbial cells, as described below. Observation of nematodes was performed using a Nikon SMZ 745 T stereomicroscope. For taxis and toxicity tests, the nematodes were cultivated on 5 cm plastic Petri dishes with NGM.

Coating of E. coli cells with polyelectrolytes and HNTs

E. coli were cultivated overnight at 37 °C, harvested and washed prior the deposition of HNTs. The concentration of the cells was determined using optical density measurements at 600 nm using a Perkin Elmer Lambda 35 spectrophotometer. Cells were harvested from the nutrient media prior to deposition and washed with water. HNTs were tailored to *E. coli* cell walls using an LbL approach.[S2] Briefly, *E. coli* cells were coated with 1 mL PAH (5 mg mL^{-1}). Then the PAH-coated cells were introduced into 1 mL of aqueous HNTs dispersion (0.05 – 1 mg mL^{-1}). After washing, the cells were similarly coated with another PAH/PSS layer, to immobilise HNTs and render the cells with the resulting negative charge. All incubations were performed for 15 min, followed by washing. The resulting architecture of the coating was *E.coli*@PAH/HNTs/PAH/PSS. All coating procedures were performed under sterile conditions. In several experiments, HNTs were labelled with rhodamine B, to visualise nanotubes inside the worms using confocal microscopy.

Taxis test

NGM dishes were prepared as shown in Figure S1 – on the opposite sides of the dishes 50 µL of either pure E. coli cells or E. coli cells mixed with HNTs (1 mg mL^{-1}) were dropped, then L1 nematodes (which received no food after hatching), were placed in the middle of the dish. After feeding on the bacteria for 8 hours, the dishes were inspected under a microscope, and the number of worms near either spot was counted.

Delivery of nanoparticles into C. elegans nematodes

Adult synchronised nematodes were starved for 24 hours and then supplemented with 100 µL of food (HNTs (1 mg mL^{-1})-coated bacteria at 10^{10} cells mL^{-1}) and were allowed to feed freely for 1 hour. After feeding, the worms were washed from the plates, immobilised with 40 mM NaN_3, and then further characterized. Alternatively, for HNTs distribution study, the worms were fixed with 2.5% glutar aldehyde in PBS.

Body size assay

The body length of the HNTs-treated worms was measured using optical microscopy images and the ZEN microscopy software. The measurements of the bodies length was performed after 72 h of feeding. The worms were isolated from the NGM plates and fixated by heating. The measurements were performed in 6 replicates (>100 individual worms each).

Egg number assay

The effects of nano-coated bacteria (PAH-MNPs or AgNPs) on the reproduction of *C. elegans* were investigated by counting the number of eggs from in adult hermaphrodites 72 h after feeding. The number of eggs inside gravid worms was counted using optical microscopy. The measurements were performed in 6 replicates (>100 individual worms each).[S3]

Nematode lifespan assay

Synchronised nematodes were kept in 96 well microplates (~ 60 animals per well) and treated with 0.6 mM FUDR during L4 stage. Then, on the next day, the adult nematodes were fed with 10 μL per well HNTs-coated E. coli cells (0.05 – 1 mg mL^{-1}). The microplates were sealed with a breathable film. Next, the live and dead worms were monitored during 16 days (each two days), the animals which failed to respond for a touch were considered dead. The measurements were performed in 3 replicates.

Characterisation techniques

Optical microscopy images were obtained using a Carl Zeiss Imager Z2 microscope (Germany) equipped with an AxioCam HRC CCD camera operated using ZEN software. Enhanced dark field (EDF) microscopy images were obtained using a CytoViva® enhanced dark-field condenser attached to an Olympus BX51 upright microscope equipped with fluorite 100x objectives and DAGE CCD camera. Occasionally, CytoViva® Dual Mode Fluorescence system (UV excitation) was used to visualise HNTs in nematodes along with DAPI nuclei stain. Extra clean dust-free Nexterion® glass slides and coverslips (Schott, Germany) were used for HRDF microscopy imaging to minimise dust interference. Laser scanning confocal microscopy images were obtained using a Carl Zeiss LSM 780 instrument. Hydrodynamic diameters and zeta-potentials of HNTs and *E. coli* cells as a function of the layer deposited were measured using a Malvern Zetasizer Nano ZS instrument and standard U-shaped plastic cells.

ESI references

S1. S. Brenner, *Genetics*, 1974, **77**, 71-94.

S2. M. Kahraman, A. I. Zamaleeva, R. F. Fakhrullin and M. Culha, *Analytical and bioanalytical chemistry*, 2009, **395**, 2559-2567.

S3. R. Dhawan, D. B. Dusenbery and P. L. Williams, *Journal of Toxicology and Environmental Health Part A*, 1999, **58**, 451-462.