Invertebrate models for hyperthermia: what we learned from *Caenorhabditis elegans* and *Hydra vulgaris*

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ABSTRACT

Animal models play critical roles in fundamental discovery and bioactivity testing to name some activities, and therefore they represent an invaluable resource for biomedical research and for predicting the efficacy and toxicity of new therapeutic and diagnostic agents. The role of animal models is crucial in many research fields, including the emerging nanomedicine, which demands not only accurate testing of novel medical nanomaterials, but also the study of the interaction between the nanomaterial and the biological target it was designed for. Model organisms provide scientists with a platform to perform biocompatibility, toxicity and pre-clinical screening. Invertebrate models also address the 3Rs principle (replacement, reduction, and refinement) by reducing ethical concerns, economic costs and the number of superior animals to be used in further studies.

In this chapter, we will show the feasibility of using two promising invertebrate model animals, *Caenorhabditis elegans* (*C. elegans*) and *Hydra vulgaris* (*H. vulgaris*), for both optical and magnetic hyperthermia studies. Following a brief introduction on the biology of both organisms presenting their advantages and limitations, we will overview their versatility to undergo multiple manipulations, hoping to provide the reader accurate criteria to select the most suitable animal model.

Although we will limit our discussion to two models, we predict a significant overlap in mechanisms and concepts with other simple model organisms not discussed in this chapter. Therefore, the final aim of this chapter is to contribute to the safe design and screen of nanomaterials for hyperthermia, boosting its translation to clinical use.

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9.1. Introduction to animal models in nanoscience

A growing interest is directed toward the fast developing field of nanotechnology due to the great potential it could offer to advance in biomedical research. The purpose of nanomedical studies is to obtain biological effects both from the intrinsic properties of nanomaterials and from their functionalization with bioactive moieties. These new experimental approaches require the development of new skills, a close collaboration between disciplines and the emergence of new knowledge paradigms; as a result, new fields of application appeared in the last decades including nanomedicine and nanotoxicology. The capability to design novel devices to diagnose, treat and restore the homeostatic equilibrium, while gaining deeper knowledge of fundamental biology, is of high importance.

Small animal models are precious resources to perform both fundamental and translational research in this growing field. Actually, small invertebrates are useful model animals for several reasons, i.e. the simple and low-cost facilities required to grow and maintain them, and the absence of ethical issues allowing high-throughput experimentation. Therefore, the use of simple non-mammalian model organisms can cut off the costs associated with in vivo experiments at the early stages of discovery yet yielding highly informative and robust biological outputs.[1] Additionally, small invertebrate models often share interesting experimental features including high reproduction rate, short lifecycle, transparency during early developmental stages and easy whole-animal visualization. Furthermore, they have been widely investigated; hence abundant bibliography and resources are available about their biology, in particular regarding the molecular conservation of signal transduction pathways and gene expression regulation compared to superior animals. Indeed, the post-genomic era has boosted the potentialities of these animals extending their use to genetic analysis as well as to toxicological screenings.

Among simple model organisms, some invertebrates have gained both scientific and industrial interest, such as the fruit fly *Drosophila melanogaster*, the zebra fish *Danio rerio* or the tiny worm *Caenorhabditis elegans*, which are especially used in biomedicine.[2] More recently, other invertebrate animals such as the freshwater polyp *Hydra vulgaris* have started to gain attention in nanomedicine. The use of simple model organisms is in high demand, particularly for scientists willing to assess the safety and efficacy of novel materials for biomedical applications within the chemical laboratories where these materials are developed.[3, 4] The pharmaceutical industry also relies on these models to validate their early biological results based on computer simulations or cell culture experiments.[2] Simple animal models contribute to bridge in vitro and in vivo experiments in mammals, offering a worthy biological platform of intermediate complexity during the initial stages of new product development.

Each animal model has specific features and culture requirements that make it more suitable for one or another purpose (Table 9.1). For instance, the fruit fly *Drosophila melanogaster* has been long used to study the effects of mutations by crossing and studying the subsequent generations. In contrast, zebra fish is widely employed to study the heart and vascular system, which are not present in *C. elegans*. Zebra fish embryos, as well as *C. elegans*, are very interesting for developmental biology due to their small size and body transparency.[5, 6] Cnidarian species such as *Nematostella vectensis* and *Hydra vulgaris* represent species of choice to study development and regeneration, due to the remarkable capacity to regenerate amputated body parts.

Based on their minimal maintenance and growth requirements, these animal models can be hosted in any laboratory after notification and authorisation by the corresponding authority. In contrast, preclinical assays in rodents result far more

demanding and require dedicated infrastructures, i.e. mice are kept in cages in a dedicated room and necessitate daily maintenance by specialised technicians. Moreover, experiments require the approval by an ethical committee, which is tedious and costly.[7, 8] Therefore, simple animals are good model systems to screen the bioactivity and biocompatibility of novel nanomedical materials within synthetic laboratories, and help to elucidate the mechanisms (biological pathways) underlying their bioactivity, before moving to mammalian models. Table 9.1 resumes and compare main biological features of invertebrate models, widely used in biology and nanoscience.

Table 9.1. Key features of different invertebrate animal models. Adapted from Gonzalez-Moragas et al.[9]

	<i>Hydra vulgaris</i>	<i>Caenorhabditis elegans</i>	<i>Drosophila melanogaster</i>
Common name	<i>Hydra</i>	Worm	Fruit fly
Habitat	Aquatic (freshwater)	Terrestrial (soil)	Terrestrial
Cultivation	Inexpensive and easy	Inexpensive and easy	Inexpensive and easy
Space	0.02L- 20L aerated aquaria	Hundreds of animals in a 10-cm Petri dishes in an incubator in the lab	Bottles in a dedicated room.
Food	Shrimp larvae	Bacteria	Fly food (water, agar, sugar, corn meal, yeast...)
Environmental conditions	18 – 21°C	16 – 25°C	18 – 29°C
Adult Size (length x width)	Column: 2-10 × 0.3-1.8 mm; Tentacles (6-9): ~ $\frac{3}{4}$ column	~1 mm x 70-90 μ m	~3 mm x ~2 mm
Adult Weight	~400 μ g	~6 μ g	200-250 μ g
Gender	Asexual reproduction; male and female	Hermaphrodite and ♂ (~0.1%)	♀ and ♂
Lifecycle	They do not undergo senescence and can regenerate.	Short (2-3 days)	Short (10 days)
Embryogenesis	Budding predominates over sexual reproduction.	18 h at 20°C	24 h at 25°C
Lifespan	'biologically immortal'	2-3 weeks; up to months as dauer larva	~30 days
Number of offspring (per animal)	7 days old adult: ~200/year, mainly clonal offspring	~300	~400
Year Genome sequenced	2010 (draft)	1998	2000
%Homology with humans	Low divergent genome	60-80%	50-80%
Automated high throughput assays	Possible at all stages	Possible at all stages	Only possible with larvae
Other features	Biological indicator of water pollution	Transparency Storage by freezing Many mutants available	Difficulty of conservation of mutants

9.1.1 The freshwater polyp *Hydra vulgaris*

Hydra anatomy can be sketched as a hollow tube with a tentacle crown and a foot at the two ends (Figure 9.1A). In the centre of the tentacles crown, there is a mouth almost hidden at rest but clearly visible when open. In contrast with its gracious and simple body plan, *Hydra* is a voracious carnivore whose limbs carry the most complex

and deadly organelle in living beings.[10] Well-fed *Hydra* reproduces by budding 3-4 young adults a week; in turn, the newborn *Hydra* will reproduce by budding within the following week. This exponential clonal growth rate makes *Hydra* a useful model for massive culturing, and the availability of large populations may enable high throughput screening. In addition to the asexual reproduction, *Hydra* reproduces sexually but the growth rate is slow and unsteady (Figure 9.1B). A particular strain, named *H. vulgaris* AEP, presents a high rate of egg production and is used to generate embryos for production of transgenic polyps.

Hydra simple body plan is composed of three independent cell lineages (Figure 9.1C). Two lineages of epithelial muscle cells form unicellular sheets (ectoderm, endoderm) and shape the body of the polyp. The third lineage is a stem cell system dispersed in the interstitial spaces between epitheliomuscular cells. The multipotent stem cells are located in the ectoderm of the gastric region. They give rise to differentiated cells essential for the polyp's behaviour (nerve cells, nematocysts, and gland cells) and sexual reproduction (gametes). The two layers of specialized epithelia are separated by an extracellular matrix (mesoglea) and filled with a mesh-like nervous system together with other specialized cells. Nematocytes are highly specialized armed cells concentrated on the tentacles and contain the stinging capsule, the nematocyst, the common feature of all cnidarians.

Figure 9.1. A) Optical microscopy image of *Hydra vulgaris* (Scale Bar=0.1 mm). B) *Hydra* sexual and asexual reproduction. C) *Hydra* anatomy consisting in three layers, ectoderm, endoderm and mesoglea. .

Although it is only a few millimetres long, *Hydra* constantly grows not only toward the budding zone but also along the head-foot direction. This balance is achieved by a fine regulation of cellular homeostasis including but not limited to: mitosis and differentiation of three stem cell lineages, autophagy/apoptotic processes, cell remodelling and cell displacements at the body extremities. The ability of *Hydra* to renew indefinitely the body tissue confer this organism the peculiar characteristics of immortality and regeneration. In fact, normal fed *Hydra* is almost identical to a young adult even after long time. Regeneration in *Hydra* consists in its ability to recover in a few days to a full functional organism starting from small tissue fragments or even cell aggregates. These peculiarities fascinated scientists from its first description by Abraham Trembley in 1744 until today.

Beside its usefulness in development and regeneration studies, *Hydra* is also an amenable system for ecotoxicology and it has been used in the past to study the toxicity of effluents and heavy metals. Polyps exposure to medium-suspended toxicants may cause alteration of morphological traits and developmental programs, adversely affect regeneration process, pattern formation, reproductive capabilities, parameters that can be all precisely and accurately estimated and quantified by reliable assays. Owing to its remarkable regenerative capacity, it has been employed to examine the teratogenic potential of several chemicals including ethinylestradiol, bisphenol, nonylphenol and

several pharmaceuticals. The molecular tools available, i.e. whole genomic sequence, gain and loss of function techniques, may enable the study of the mechanisms underlying the toxicity at a molecular level.

Starting from 2007 we challenged *Hydra* with a deal of nanoparticles (NPs) diverse in shape, dimension, chemical composition and surface coatings. Our interest was multifaceted, ranging from the evaluation of nanoparticle toxicity to the use of fluorescent nanoparticle for bioimaging, and development of new methods for ribonucleic acid (RNA) interference mediated by nanoparticles. Most of these approaches successfully revealed the great versatility of *Hydra* as an experimental model to screen new nanomaterials and understand how they interact with the biological counterpart.

To face the peculiarity of each nanoparticle studied, assays must be tailored according to the interaction of interest. For instance, the toxicity of a given nanoparticle can be assessed by evaluating the morphology of the exposed polyp either as intact specimen, or during regeneration, in case the test nanoparticles were meant to affect cell proliferation, for example. To understand the cellular distribution, optical and transmission electron microscope (TEM) microscopy can be applied on single cell and tissues; eventually, to understand the extent to which the gene expression is influenced by the modifications recorded on cell tissues and animals, single gene expression levels can be studied by quantitative real-time polymerase chain reaction (qRT-PCR) or changes in the global gene expression can be analysed by performing omics approaches such as RNA sequencing (RNA seq). Thus, an important asset of these experiments is the ability to study in the same lab and at relatively low cost, how nanoparticles behave in living organism at animal, cellular and molecular levels.

The opportunity to study in the same test animal both morphological effects and cellular/molecular events is particularly useful to characterize nanoparticles designed and synthesized for hyperthermia.

9.1.2 The roundworm *Caenorhabditis elegans*

Caenorhabditis elegans (*C. elegans*) is a 1-mm soil nematode living in the decaying organic matter. It is composed by a constant number of cells, approximately 1000, being one third of them neurons. It also exhibits a muscular, alimentary, excretory and reproductive system with perfectly studied and defined cell lineages, facilitating its use as model organism in applied sciences (Figure 9.2A). Hence, despite its small size and simplicity, it shows functional and anatomical traits that are conserved in higher animals.[11]

It is mainly hermaphrodite, while males arise spontaneously at a frequency of 0.01%. Its life cycle progresses through four larval stages (L1 to L4), separated by moulting, until it reaches adulthood in 2-3 days and is able to lay eggs (Figure 9.2B). It lives up to three weeks under normal conditions, however it can survive few months by entering an alternative larval status known as ‘dauer’ under harsh conditions (i.e. lack of food, overcrowding). In this way, the animal reduces its metabolism to the minimum to maximize survival. If favourable conditions are restored, dauer larva re-enter the normal lifecycle and become a mature adult.[11]

Figure 9.2. A) Life cycle of *C. elegans*. Adapted from Gonzalez-Moragas et al.[9] B) *C. elegans* micrograph and anatomy. Adapted from Altun et al.[11]

C. elegans was first proposed as model organism in 1967 by Sydney Brenner. Since then, many scientific breakthroughs have occurred using this round worm, leading to better understanding of how the complex nervous system works and shedding light on the unknown function of a range of genes. In particular, it has been widely employed as a model to investigate neurodegenerative diseases such as Alzheimer or Parkinson, and also in functional genetics.[12, 13]

C. elegans can be easily grown in any laboratory in a Petri dish containing nematode growth media (NGM). Due to its small size, its visualization and manipulation requires the use of a microscope. Its transparency at all developmental stages facilitates the visualization of its internal anatomical structure, and even its dissection.[14] *C. elegans* can also be manipulated genetically, i.e. by exposing them to mutagenic agents, by RNA interference (RNAi) or by microinjection, and the resulting strains can be indefinitely stored in frozen state. The easiness to create deletion mutants and/or transgenic strains has led to the availability of an extensive catalogue of mutants that can be purchased by any scientist. More recently, the possibility of genome editing via a CRISPR-Cas9 system has opened new promising avenues.[15]

In the last decades, *C. elegans* has been used as an animal model to evaluate both the efficacy and the potential toxicity of a range of chemicals, including, more recently, nanomaterials. In vivo data gathered from *C. elegans* experiments can be predictive, or at least informative, of outcomes in higher organisms.[16]

9.2. Nanoparticle fate and status in vivo

Following the description of the two selected small invertebrates, *Hydra vulgaris* and *C. elegans*, we will concentrate on some aspects related to hyperthermia that can be studied with these small organisms to gather useful information for the development of the therapy. We will evaluate different topics exposing a short introduction and then we will analyse and summarize recent works with both small animals and the results obtained.

It is widely accepted that NPs uptake and fate depend on many different parameters such as size, charge, chemical functionalities or arrangement of organic ligands on nanoparticle surface, increasing the complexity of this topic.[17, 18] Additionally, it is important to consider the potential release of ions from the surface of metal-based NPs and the unique degradation profiles of some NPs, which adds the effects of the by-products to the nano-specific effects. The high conservation of the transport mechanisms at the molecular level across the animal kingdom (i.e. endocytic pathway) makes *Hydra* and *C. elegans* as ideal tools to unravel the basic mechanisms that regulate NP uptake and secretion. Also, the greater complexity of these animals when compared to cells cultured in vitro can give original information about in vivo cell dynamics.

9.2.1. *Hydra*

Hydra has been extensively used to dissect the mechanisms of uptake of different nanomaterials, including quantum rods (QR) and gold nanospheres (AuNP). For instance, in order to study the effect of surface charge in the uptake mechanisms, core/shell CdSe/CdS quantum rods sized 35x4 nm were functionalized with different amount of charged molecules.[19] When incubated with *Hydra*, only those QR exhibiting positive net charge were uptaken into ectodermal cells, which is in line with the observations described for cells cultured in vitro.[20] Just after 30 minutes post-incubation, ectodermal cells all over the body appear intensively labelled, from tentacle tips to foot region (Figure 9.3A). After 24 h (Figure 9.3B), QRs were found in the endodermal cells lining the gastric cavity and the tentacles, revealing a migration pattern inside the animal. The possible role of the protein Annexin BXII (ANX) in the regulation of the uptake was also studied. ANX is able to form bilayer pores in the membrane when inserting into it. When the animals were pre-incubated with anti-ANX antibody, QR uptake was inhibited, meaning that this process is dependent on the presence of ANX on the membrane of ectodermal cells.

In another study, Marchesano et al. revealed by TEM that 14-nm AuNPs bearing a small interfering RNA (siRNA) targeting the *Hydra* myc 1 gene were able to directly penetrate the plasma membrane just 30 minutes after incubation with *Hydra*. [21] The direct uptake of the NPs avoiding the classical endocytic pathways has been rarely reported when using in vitro cultured cells, and it has been mainly associated with the organization of the ligands on the surface of the NPs.[22, 23] Interestingly, over time

NPs could also be observed crossing other cellular layers, which would be ideal to deliver the siRNA cargo far from the administration place. In contrast, a different dynamic of uptake was described for AuNPs functionalized with a positive molecule on the surface (AuNP-N₃) instead of siRNA. In this case, an incubation of 5 minutes led to the attachment of the NPs into the negatively charged glycocalyx, a layer mainly composed of glycoproteins that surrounds the animal (Figure 9.3C-E). After longer incubation times, NPs were trapped in large vacuoles that could even fuse/divide, and they were also observed as single NPs in the cytoplasm. This means that NPs can freely move from vesicles to cytoplasm or viceversa by membrane translocation.[24] As observed in the case of the complex AuNPs-siRNA, AuNPs could directly cross plasma membranes, suggesting multiple mechanisms governing the uptake and fate of AuNPs. After 24 and 48 h of incubation, AuNPs could be found in lysosomes inside and outside the cell membrane, this last being related to the exocytosis of the AuNPs from the animals to the environment (Figure 9.3F-H). After performing a pulse-chase experiment (24h incubation with AuNPs followed by 48-72h incubation in fresh medium), it was concluded that AuNPs could be secreted out of the animal by means of different pathways. Therefore, the complete dynamics and kinetics of the uptake and secretion of AuNPs within an animal were reported for the first time.

Figure 9.3. A) In vivo fluorescence image of *Hydra*, 30 minutes post incubation (p.i) with QR.: QR red fluorescence labels uniformly all body regions, from the tentacles (t) to the peduncle (p) located in the upper part of the image. In this picture an adult (a) with a bud (b) on the left side turns towards the camera the hypostome (h), surrounded by a ring of tentacles. B) Intact *Hydra* were treated with QRs at acidic pH for 4 h, and in vivo imaged 24 h later (left panel). On this image the orientations of sectioning planes (A, B, C and D) used for sectioning are shown as dotted lines, and on the right panels the corresponding tissue sections are reported. The green color is due to tissue autofluorescence, while the red staining indicates the QR presence. Adapted from Tortiglione et al. [19] C-E) Ultrastructural analysis of AuNP-N₃ uptake by TEM. C) At 30 min time point, AuNP-N₃ were found attached to the glycocalyx, uniformly decorating the animal outer surface (gray arrows), but also inside large vacuoles (black arrow). D) At higher magnifications the inner side of this structure appears lined by the glycocalyx layer, proving the origin from membrane invagination (gray arrows). AuNP-N₃ are also captured coming out this structure (black arrow) by direct membrane translocation toward the cytosol. E) A close view of the AuNP-N₃ entrapped into the vacuoles together with the glycocalyx components. F-H) Exocytosis of AuNP-N₃. F) Low magnification image showing the whole animal tissue section in the act of AuNP-N₃ clearance. The same region at higher magnification is shown in image G. H) Other tissue regions showing NP clearance. In this case a larger membrane area is involved, probably reflecting lysosome exocytosis. Adapted with permission from [21]. Copyright (2013)

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9.2.2. *Caernohabditis elegans*

Given the technical difficulty of studying NP fate with nanometric resolution inside living organisms at present, there is limited evidence of the internalization and translocation mechanisms of NPs in *C. elegans*. Several techniques have been applied to study NP uptake and fate in this animal model, being fluorescent microscopy,[25-27] hyperspectral microscopy,[28, 29] and transmission electron microscopy (TEM)[30, 31] the most prevalent ones. Meyer et al. reported the first evidence of intracellular uptake of 10-nm silver NPs (AgNPs) in *C. elegans*, and confirmed intergenerational transfer.[29] Scharf et al. also observed intracellular uptake of 50-nm silica NPs in the intestinal and vulval cells.[27]

As shown in *Hydra*, TEM has sufficient spatial resolution to track NPs inside multicellular organisms, allowing single NP visualization. It can be applied to investigate the integrity of the intestinal barrier in NP-treated worms, and also to study the intracellular location of internalized pristine NPs (Figure 9.4), without the need of labelling the NPs to discern their location.[30-33] The recovery of NP-treated nematodes can also be investigated by TEM. For instance, Yong et al. exposed *C.elegans* to titanium oxide nanoparticles and found that the intestinal barrier of acutely-treated worms was able to recover, in contrast to the lasting defects induced after prolonged exposure.[34] TEM can also provide further clues about NP translocation routes, i.e. endocytosis, although this should be confirmed by chemical identification or molecular mechanistic evidence.[30, 35]

Figure 9.4. Use of TEM to characterize NP kinetics and dynamics in *C. elegans*. A-C) Ultrastructural changes of intestine and uptake in nematodes exposed to TiO₂ nanoparticles after transfer to control conditions. A) Unexposed nematodes. B) Nematodes exposed to 100 mg/L TiO₂ nanoparticles immediately upon transfer to control conditions. C) Nematodes exposed to 100 µg/L TiO₂ nanoparticles after 48 h in standard conditions. Asterisks indicate positions where microvilli are absent. Arrowheads indicate the location of NPs. Mitochondria, mt. Adapted from Zhao et al.[34] D) TEM image of Fe₂O₃ NP treated young nematodes, showing individual NPs in close contact with the microvilli (arrowheads) within the glycocalyx, delimited with a dotted line. Adapted from Gonzalez-Moragas et al.[30] E-F) TEM image of the pharynx of *C. elegans* treated with E) 11-nm AuNPs and F) 150-nm AuNPs, prepared following the targeted ultramicrotomy protocol. Reprinted from Gonzalez-Moragas et al.[36]

Employing genetic analysis instead of microscopy techniques, Tsyusko et al. reported elevation of clathrin expression and significant responses of endocytosis mutants to AuNPs, suggesting that cell uptake was clathrin-dependent.[37] More recently, Maurer et al. showed that early endosome formation was necessary for NP-induced toxicity.[38] Gonzalez-Moragas et al. reported intracellular uptake by clathrin-mediated endocytosis of 6-nm iron oxide NPs and regulation of other intestinal-related genes, among them the early endosome formation gene *dyn-1*. In contrast, the authors could not detect internalization of 11-nm AuNPs either by electron microscopy or by gene expression analysis.[30, 35]

9.3. Biological effects of heat

In the last two decades, fundamental biomedical studies demonstrated the fine regulated molecular processes that bring cells to death, such as programmed cell death (apoptosis and autophagy) or passive cell death (necrosis), as result of cell/tissue exposure to high temperature (see chapter 8). Programmed cell death is an evolutionary conserved mechanism of pluricellular organisms that contributes to many biological functions during both development and adult life. It is involved, for example, in maintaining the size and plasticity of organs and in the response to reduced nutrient availability or chemical/physical perturbation. Passive cell death does not follow the apoptotic signal transduction pathway and results in the loss of cell membrane integrity, leading to an uncontrolled release of products of cell death into the extracellular space. Small animal models represent precious resources to carry-on both fundamental and translational researches aimed to dissect the overall responses to heat shock, caused either by external increase of environmental temperature or by any other compound generating heat.

Ideally, hyperthermia agents should have high heating efficiency, meaning that they should generate sufficient amount of heat when applying an external source whilst using the minimum amount of NPs. In the last years, much research has been devoted to obtain NPs with great heating capability. Also, when applying the NPs in vitro or in vivo, there are other requirements that they should be fulfilled, such as biocompatibility and stability in physiological media.[39-42] However, the generation of heat can be dramatically decreased when the NPs are placed in a cellular environment. For instance, it has been described that maghemite nanoparticles can decrease their Specific Absorption Rate (SAR) value to half of the initial one upon interaction with cells after only 90 minutes.[43] Therefore, understanding the ability of heating of the NPs in vitro and in vivo could help to boost hyperthermia treatment into clinics. The use of invertebrate models can provide worthy data before reaching experimentation in vertebrate models, which are far more complex in terms of ethical and economical

issues. Also, conservation of key functional pathways makes these simple models more similar to vertebrates than experiments in vitro based on cell cultures. Preliminary investigations in model organisms can unravel the subtle responses induced by hyperthermia treatments and provide important information about the heat production and propagation in a living multicellular organism (Table 9.2).

The first studies about thermal response in *Hydra* were carried out by Bosh et al., finding a direct correlation between the ability to synthesize heat shock proteins (HSPs) and the survival to environmental stress.[44] Exposure to elevated temperature (22-23°C) for less than 1 hour led to the synthesis of HSP, as revealed by radioactive labelling. Higher temperatures (>30°C) can result in the disintegration of the animal, although the exposure time is a critical parameter to consider (Figure 9.5).

Figure 9.5: Morphological changes of *Hydra* incubated for 30 minutes at 30, 34 or 37 °C. Scale bars: 500 μ m. Adapted with permission of Future Medicine from Moros et al. [45]

Regarding the effects of heat on invertebrates, it is known that *C. elegans* respond to high temperature by a evoking reflexive escape behaviour to avoid possible tissue damage and minimize injury.[46] Indeed, the response of *C. elegans* to high temperature depends on the intensity of the heat, whereas it is moderate heat stress or hyperthermia. Indeed, these two heat modalities have opposite effects on the cholinergic system.[47] In particular, short-term noxious heat (15 min at 31–32°C) causes activation of cholinergic synaptic transmission leading to an increase in movement speed increase for escape. In contrast, hyperthermia (36–37°C) blocks cholinergic synaptic transmission resulting in behaviour failure at extreme high temperature.

9.3.1. Hyperthermia

Hyperthermia is currently exploited as a non-invasive approach for cancer therapy, and relies in the selective ablation of abnormal cells when biological tissues are exposed to high temperatures. During the last decades, many hyperthermia methods have been developed, most of them based on the use of an external heating source such as electromagnetic radiation or high-intensity focused ultrasounds. Depending on the thermal shock intensity, cells may undergo immediate necrosis, owing to irreversible cellular damage, or they may activate programmed cell death. Beside the methodological approaches, in vivo assays on model systems are crucial to test and to validate the efficacy of the treatments and the overall cell and tissue response before using vertebrate clinical models. Below we will describe examples of both optical and magnetic hyperthermia in *Hydra* and *C. elegans*, providing clear evidence of their great translational value (Table 9.2).

Table 9.2. Main heat biological sensors in *Hydra* and *C. elegans*

Model organism	Thermal stress	Heat biological sensors	Toxicity endpoints	References
<i>Hydra vulgaris</i>	Heat shock	HSP70	Gene, protein expression; Promoter activity; Morphology; Behaviour;	[44, 48-51]
	Magnetic and optical hyperthermia, Heat Shock,			[45, 52-54]
	Heat Shock	TRPM3 NOS, SOD, HSP70	Gene expression; Morphology; Behaviour	[55]
	Heat Shock	FoxO	GFP-reporter and Gene expression	[56]
<i>C. elegans</i>	Optical hyperthermia	HSP16	Gene expression (hsp16-lacZ transgene)	[57]
	Heat Shock	HSP70	GFP expressing transgenic <i>C.elegans</i> ; Gene expression	[58, 59]
	Magnetic and optical hyperthermia, Heat Shock,	SOD, HSP70	Gene, protein expression; Lipofuscin accumulation	[25, 60] [61] [62]

9.3.1.1 Photothermal cell ablation using *Hydra vulgaris*

Photothermal hyperthermia has been studied in *Hydra* both on whole animals and on single cells, using Au nanoprisms (AuNPrs) with the LSPR (localised surface plasmon resonance) in the near infrared (NIR) range.[52] Polyps were soaked with AuNPrs of 100-150 nm edge, and after 24 h of incubation the AuNPrs were found internalized into the ectodermal layer (Figure 9.6). When applying an 830nm NIR diode focused on the tentacles, some cell detachment was observed just after few seconds of irradiation. Interestingly, no damage was found in adjacent tentacles that were non-irradiated, demonstrating that spatio-temporal control in cell ablation can be attained. The possibility of selective ablation of groups of cells can greatly help to understand their function in regards to the whole animal.

Another approach consisted in the irradiation of the whole animal using the NIR laser for 6 minutes using a 1064 nm NIR illumination (30 W/cm²). Extensive tissue damage and cell detachment was found in the irradiated animals, while those non-irradiated did not show alterations at the morphological level. Staining of the cells with propidium iodide confirmed that irradiation caused a fast necrosis, as already shown when using in vitro cultured cells using the same laser intensity.[63]

To further analyze the response to heat, molecular investigations were conducted. Genes encoding for HSPs were selected as targets, since they are early response genes to thermal stress. Among this superfamily, *hsp70* was selected, as already was cloned and functionally characterized as early marker of heat shock in *Hydra*.[44] Moreover, its implication following photothermal hyperthermia in solid tumours has been extensively described.[64]

Figure 9.6. Biodistribution and subcellular localization of AuNPrs. A) In vivo fluorescence imaging of whole treated polyps. B) TEM analysis shows an ectodermal cell containing many vacuoles and a well-defined lysosome containing electron-dense materials. C) A magnification of the squared area from B) shows monodisperse AuNPrs within the lysosome. Scale bars are A) 200 μm ; B) 1 μm ; C) 0.2 μm . D-F) Photothermal cell ablation mediated by gold nanoprisms in *Hydra*. D) Induction of necrosis in detached cells (indicated by the arrow) was shown by Propidium Iodide staining. E -F) Local irradiation was achieved by using a 100-mW laser (830-nm continuous wave (CW) diode) coupled to an upright microscope. Single cells were sequentially ablated on the tentacle tip (right side), while no side effects were monitored on the adjacent tentacle. Scale bars: F) 50 μm ; D-E) 100 μm . Adapted with permission from Future Medicine from Ambrosone et al. [52]

After irradiation and recovery, RNA was extracted and quantitative real-time PCR (qPCR-RT) analysis performed. *hsp70* transcripts were significantly overexpressed compared to control animals, suggesting that photothermal hyperthermia in *Hydra* can lead to both cell death and heat tolerance mechanisms. Probably, when the amount of heat is above a characteristic threshold level, cells become necrotic, but if this threshold is not reached, defence mechanisms as the synthesis of HSPs can be activated to increase protein refolding.

9.3.1.2 Photothermal approach using *C. elegans*

In *C. elegans*, a method using a laser beam focused through the objective of a microscope was developed in the 1980s, followed by several technical refinements.[65] In biology, this tool is of great value, allowing investigating the role and function played by a specific cell, in vivo, within its physiological context. Photoinducible cell ablation has also been achieved through genetically encoded photosensitizers, that is, newly engineered proteins that generate toxic compounds (such as singlet oxygen) upon light illumination.[66] This method is time and cost consuming, and involves molecular engineering and genetic transformation, thus novel straightforward methods would be very attractive to study cell function.

As reported in *Hydra*, the application of a laser at the excitation wavelength of the LSPR of AuNPs may result in heat generation in *C. elegans*. However, it has not been yet exploited experimentally. Indeed, it has been found that near-infrared continuous-wave laser light can produce stress in *C. elegans*.[67] In that study, the

authors used a transgenic strain carrying an integrated heat-shock-responsive reporter gene (*hsp16-lacZ* transgene) and found that gene expression was most often induced by light of 760 nm, and less by 810 nm. The stress response increased with laser power and irradiation time. The expected temperature increase under the exposure conditions (between 0.7°C and 4.1°C) suggests that it is unlikely that photothermal effects accounted for the gene expression observed in animals exposed to light below 800 nm. Since the increase in temperature is significantly lower at 760 nm than at 850 nm, but the reporter gene expression showed a pronounced maximum at 760 nm, this indicates that the gene expression at 760 nm was not predominantly caused by photothermal effects. Instead, the authors hypothesized that in the 700–760 nm range, stress response was generated by photochemical reactions, while the stress caused at 810 nm mainly had a photothermal origin. However, the authors did not discuss the exact nature of such photochemical induced processes. Hence, according to this study, 810 nm at normal laser powers does not cause stress at the cellular level while the 700–760-nm wavelength region is less suitable for biological application.

It is an interesting application, which we foresee *C. elegans* could bring in the evaluation of novel materials and photothermal therapies.

9.3.2. Magnetic hyperthermia

Hyperthermia methods based on the use of light to irradiate target tissues on one side allow spatial control of the thermal shock, but on the other are limited by the narrow penetration of the irradiation source into the tissue, restricting heat production to defined regions. Magnetic hyperthermia (MHT) is the use of magnetic heat developers, such as magnetic nanoparticles (MNPs), producing heat under an alternating magnetic field (AMF). MHT may allow heat delivery to any tissues or organs, independently of their location. Any tissue containing MNPs may remotely respond to the application of the AMF and act as nanoheaters. Below, we summarise the most relevant data achieved in our model organisms, either to test MNP heating performance, or more oriented to check biocompatibility and toxicity.

9.3.2.1 Hyperthermia mediated by iron oxide nanoparticles in *Hydra*

Hydra has been also used to decipher the intracellular responses to heat after applying MHT.[45] In this case, a mild – rather than a lethal – hyperthermal effect was investigated. The interest in mild hyperthermia relies on the fact that it can increase the efficiency of radiation therapy and/or chemotherapy without posing cytotoxic effects *per se*. [68] To this end, superparamagnetic iron oxide nanoparticles (hereinafter, SPIONS) were incubated with polyps for 24 h, accumulating into intracellular compartments in the ectoderm. An AMF ($f=835$ kHz; $H=20.05$ kA/m) was applied for 30 minutes, after which no tissue or cell damage could be found. Molecular responses to heat were assessed by analysing the expression of *Hydra hsp70* (*hyhsp70*) gene. A two-fold overexpression of *hsp70* transcripts in animals loaded with MNPs and exposed to AMF was found, but not in any other control condition. This was in accordance with a mild treatment, where the final goal is intracellular heating without generating cellular damage in the surroundings. Furthermore, soaking the animals in hot baths at different temperatures served to assess the response of *Hydra* to external heating. As suggested by other works, a temperature equal to 34°C was identified as a critical threshold above which high macroscopic damage occurred.[69] Below this temperature, animals did not show extensive damage by microscopic observation, and no increase in apoptotic nuclei

could be found. Molecular analysis revealed that *hyhsp70* gene expression was activated at 26°C (~1.5-fold increase), reaching a two-fold increase at 30°C, which corresponds to the maximum expression level (Figure 9.5, 9.7). By comparing *hyhsp70* gene expression of animals exposed to an external heat stress (30°C) with the level of those treated with MNPs and exposed to MHT, a similar response was reported, indicating that a heat response was induced following an increase of 12°C (*Hydra* polyp culture temperature is 18°C). Impressively, a similar induction of *hsp70* expression levels was found in melanoma B16 cells exposed to MNP and AMF, or exposed to a 12°C thermal increase, suggesting a general mechanism underlying the response of eukaryotic cells to the delivery of sublethal dose of heat.

Figure 9.7. A) AMF exposure of MNP-treated polyps induces a twofold change of *hyhsp70* expression, while MNPs and AMF exposure alone did not modulate *hyhsp70* expression. Asterisks denote statistically significant expression change relative to untreated animals (one-way ANOVA followed by Tukey's multiple comparison post-test, $p < 0.001$). B) Molecular response to macroscopic heat shock in *Hydra*. qRT-PCR analysis revealed that *hsp70* is significantly upregulated at 30°C. Asterisks indicate statistical significance ($p < 0.001$; two-way ANOVA with Bonferroni's multiple comparison post-test). In *Hydra*, MNP treatment alone does not induce toxicity effects. Exposure to AMF neither affects viability, but induces a doubling of *hyhsp70* transcripts. C) This enhancement mirrors that induced by exposing polyps at 30°C (in a thermal bath), namely by a shift of 12°C above the initial temperature (18°C). Altogether the data show same cell and animal response to MNP-mediated hyperthermia, suggesting their combined use to assess the thermal dose delivered by MNPs in vivo. Green arrow heads represent local heat production. Adapted with permission of Future Medicine from Moros et al.[45]

9.3.2.2 Hyperthermia mediated by iron oxide nanoparticles in *C. elegans*

The use of SPIONs to manipulate *C. elegans* by applying a magnetic field has been reported by Huang et al.[70], confirming that it is definitely possible to excite SPIONs inside the *C. elegans*. In their study, they used heat induction to alter the motor behaviour of *C. elegans*, and calculated a local temperature of 34°C in the targeted neurons after application of the magnetic field (Figure 9.8). By switching on and off the magnetic field, they could manipulate the locomotion of the worms magnetically labelled with fluorescein-PEG-coated iron oxide nanoparticles.

Figure 9.8. Plot of the time course of the fluorescence intensity and temperature for the head region. During application of the magnetic field between 11 and 28 s, the fluorescein fluorescence intensity decreased due to a temperature increase from 20 to 34°C, at which point the worm retracted. Adapted from Huang et al.[70]

This approach, based on nanoparticle-based magnetothermal control of neuromodulation, involves the delivery of heat-emitting nanoparticles into the targeted area. In contrast, the approach of Long et al. was based on magnetogenetics, which although not related to cell ablation, show the possibility to control cell function by remote application of AMF (Figure 9.9). Muscle contraction and withdrawal behaviour was observed in a transgenic of *C. elegans* strain expressing an exogenous magnetoreceptor in *myo-3*-specific muscle cells or *mec-4*-specific neurons, after the application of an external magnetic field. These observations revealed a magnet-dependent activation of muscle cells and touch receptor neurons, respectively.[71] The work of Long et al. avoids the safety issues of MNPs, and combines the genetic activation of neuronal activity via a magnet-dependent magnetoreceptor with an external magnetic field. Hence, it enables non-invasive and wireless perturbation of neuronal activities with long-term continuous dosing, which would be almost impossible in the case of the photothermal strategy.

Figure 9.9. Magnetogenetic control of behavioral responses in *C. elegans*. A) Simultaneous contraction of body muscle when magnetic field was applied under white field illumination. Arrow heads indicate the head and tail of *C. elegans*. Left, body relaxation just before magnetic

field was on; right, body contraction after the magnetic field was switched on. B) Body length was measured with 1 s interval at 10 s before and 50 s after the magnetic field was turned on and also at 20 s after the magnetic field was turned off. Relative body length was calculated by dividing the length measured to the average body length before stimulus onset. Orange trace showing reduction of body length to 94 % of the initial length, while N2 wild type showed no obvious change of body length by magnetic stimulation (*myo-3*, n = 24; N2, n = 20). Adapted from Long et al.[71]

Hyperthermia experiments require a biological marker/reporter able to reveal local heat generation, as illustrated in the case of *Hydra*. In such small invertebrates, the increase in temperature is so little that the temperature of the media is not increased; hence its measurement is not informative. In collaboration with the group of Prof. Jesús M. de la Fuente (CSIC-UNIZAR, Zaragoza), we performed a preliminary assay using SPIONs-treated worms to induce a local increase of the temperature using a dedicated hyperthermia device (Nanoscale Biomagnetics). Citrate-coated MNPs of 6 and 12 nm diameter were used, but no observable difference was found between the animals which underwent AMF treatment and control animals, either in the animal phenotype, NP distribution, life cycle or gut autofluorescence (Personal communication). In *C. elegans*, gut autofluorescence is often used as a stress indicator; however it is feasible that 6-nm and 12-nm SPION act as weak nanoheaters, not efficient enough to modulate the intrinsic autofluorescence of the worm in such a short time period. In this direction, the use of mutants with *hsp* fused to green fluorescent protein (GFP) (i.e. *hsp-70:gfp* mutants) could bring promising results in read out magnetic hyperthermia treatments (Figure 9.10).

Figure 9.10 *C. elegans* express *hsp* genes when exposed to high temperatures (as visualized by transgenic animals carrying a GFP reporter for a heat shock protein gene). Left panel appears black since no fluorescence is displayed at 20°C, while fluorescent animals can be observed in the right panel due to exposure to 34°C.[72]

Recently, Wang et al. investigated the interference of lipid metabolism by phosphatidylcholine-coated magnetic nanoparticles (P-MNPs) under 0.5 T static magnetic fields (SMFs) in *C. elegans*. [73] Phosphatidylcholine is the major constituent of cell membranes. P-MNPs have been shown to have low toxicity and good dispersion, and are widely used in biomedical applications including drug delivery, magnetic resource imaging and hyperthermia.[74] In *C. elegans*, P-MNPs accumulated in the intestine. In *C. elegans* exposed to either P-MNPs or SMF, the content of lipofuscin decreased, while there was no further decrease after simultaneous exposure to both P-MNPs and SMF. Lipofuscin can be used as a "marker" to determine the amount of long-term oxidative stress cells were subjected to, hence these results indicate that the

potential oxidative effect of P-MNPs and SMF are neither additive or synergic.[75] In the presence of SMF, the lipid content was greatly decreased in P-MNPs treated worms under 0.5 T SMF, which was consistent with the mRNA expression of fatty acid metabolism genes including *elo-2*, *let-767* and *fat-5*, indicating that the lipid metabolism interfered by P-MNPs via the accelerated beta oxidation and changed gene expression in *C. elegans* under 0.5 T SMF. Hence, this work confirms the potential of *C. elegans* as a platform to further investigate the effects of MNPs and magnetic field application in vivo.

9.4. Biological effects of nanoparticles

To date, one of the main problems of nanotoxicological studies is that no standard procedures exist, despite the existing effort to create guidelines common to all.[76] The lack of standard protocols may be due to the wide variety of nanomaterials that currently exist, which results in a range of different physicochemical properties. Also, the inorganic core, organic shell and/or the molecules attached to the surface of the NPs can be responsible of different toxic effects. It has been suggested that different types of cells along with different assays should be used to test the cytotoxicity of NPs in vitro, as variation in toxicity has been observed using different cell lines.[77] In this context, the use of in vivo invertebrate models can boost the nanotoxicological research in an economical way before reaching vertebrate ones.

9.4.1 *Hydra vulgaris*

Hydra has been successfully used as a model to assess the environmental impact of different compounds such as metals or industrial effluents.[78] Therefore, *Hydra* can be used to study the toxicological impact of nanomaterials on morphology, reproduction and regeneration capabilities. Also, molecular mechanisms underlying the toxicity can be elucidated by identifying genes that are deregulated upon exposure to nanomaterials.

Gold and iron oxide nanoparticles are frequently used in optical and magnetic hyperthermia treatments, respectively. Both types of nanoparticles are generally considered biocompatible and are currently used in clinical trials in humans.[79] In *Hydra*, both types of materials have been used revealing a lack of significant adverse effects, even when used at high concentrations. For instance, 16nm MNPs functionalized with a poly(ethylene glycol) (PEG) were incubated with living polyps at a concentration of 2.5 mg/mL for 24h and adverse effect of the MNPs tested at different levels, namely impact on morphological alterations, reproductive capability and induction of cell death, either apoptosis or necrosis. [45] For morphological tests, a score system ranging from 10 (indicative of healthy condition) to 0 (animal fully disintegrated), was employed.[80] Upon microscopic inspections, treated animals did not show signs of morphological alterations (tentacle clubbing or disintegration, body contraction or swelling). Similarly, reproductive capability was not altered in treated polyps and no increase of apoptosis, neither significant necrosis was found compared with control animals. Likewise, polyps treated with 5mg/mL of spherical or triangular AuNPs for 24h did not show any signs of toxicity when evaluated at the same levels.[21, 52]

Using other nanoparticles, however, toxic effects have been described in *Hydra*. For instance, Ambrosone et al.[81] tested for the first time the toxicity of CdSe QDs in *Hydra vulgaris* using an array of diverse methods. After soaking the animals with the QDs, a clear dose-dependent noxious effect on the animal was found. Nuclear damage

revealed by 4',6-diamidino-2-phenylindole (DAPI) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining was also time- and dose-dependent. Interestingly, the exposure to animals to QDs caused more severe damage than the same concentration of Cd²⁺ ions, impairing both reproductive and regeneration capabilities. In order to gain further knowledge of the toxicology origin, changes in the transcript levels of different genes were assessed by qRT-PCR. The selection of genes involved in oxidative stress detoxification, apoptosis and cell proliferation was based on the conservation of key molecular pathways (Figure 9.11). Early activation in the expression of genes related to general stress and apoptotic routes was identified. The effect on gene expression was found for all tested concentrations, pointing out the effect on gene expression even when morphological changes were not apparent. This pioneering work served as the starting point to test the toxicity of nanoparticles in *Hydra*.

More recently, others have used similar techniques to test other materials. For instance, Murugadas et al. assessed the ecotoxicological impact of copper oxide (CuO) NR in vivo using *Hydra magnipapillata*. [82] Although growth rate and reproductive capabilities were not affected, impairment of regeneration capabilities could be observed. Exposure of animals to CuO NRs caused a cellular cycle arrest in phase S, followed by an increase in the number of apoptotic cells, suggesting DNA damage. Results suggested that the NRs had the ability to inhibit the antioxidant defense system of *Hydra*, leading to an accumulation of Reactive Oxygen Species (ROS) and therefore to oxidative stress. The increase of apoptosis was suggested to occur via the mitochondria-mediated intrinsic pathway, as proteins of these pathways were deregulated upon the treatment with CuO NRs. Further, Tino et al. revealed that toxicity of CdSe QD was related to the capping agent, being more toxic those QD functionalized with thioglycolic acid than those functionalized with glutathione. [83]

Figure 9.11. Genotoxic effect of CdTe QDs on *Hydra* A) Schematic representation of molecular pathways involved in the cell response to environmental stimuli. Cell exposure to CdTe QDs may elicit a number of reactions, which may lead to death or stress adaptation. These processes are mediated by signaling pathways, which induce deregulation of various stress-inducing or protective molecules. Key components of the apoptotic pathways are shown on the left panel and depend on the activation of the death receptor. Genes selected in this study, namely the *c-myc*, *Bcl2* and *caspase 3*, are indicated in violet. Selected stress responsive genes are depicted in orange (*hsp70*, *FoxO*), together with their relationship with components of other signaling pathways (plain arrowed red line indicates activation, dotted line indicates inhibition). On the right panel key reactions of the oxidative pathways are presented, from the membrane lipid peroxidation to the conversion of oxygen radicals into non-toxic products by the SOD gene (colored in light blue). B) Temporal expression patterns of selected stress responsive genes in QD treated polyps by qRT-PCR analysis using Elongation factor 1-alpha (*Ef-1a*) as reference gene. Animals were treated with 10 nM and 25 nM QDs for the indicated periods, and processed for RNA extraction and qRT-PCR analysis using specific primers. The general trend

is an early (8 h) upregulation of apoptotic genes induced by the 10 nM QD dose, and a late response (from 24 h) of the protective *hsp70* gene, suggesting a possible animal recovery. Data represent mean+Standard error (SE) of three technical repeats from three biological replicates. Reprinted from [81] Copyright (2012), with permission from Elsevier

All these results are in line with a plethora of works that describe that QD produce cytotoxic, inflammatory and oxidative stress responses in a variety of systems.[84]

However, the possible ecotoxicological effects of QDs have been scarcely investigated up to date. Therefore, to evaluate the potential risks of QDs to the environment, the impact of CdSe/ZnS QDs was also investigate in other ecologically important cnidarian species, such as the starlet anemone *Nematostella vectensis* and the coral *S. pistillata*. [85, 86] Transcriptome analysis by RNA-seq indicated that, in *Hydra*, 493 genes were deregulated upon 8 h of exposure, increasing to 1562 deregulated genes when the incubation was performed for 24 h. On the other hand, in corals the number of deregulated genes was higher at 6 h than at 12 h post incubation, suggesting a faster mechanism of response to the stimulus and a certain recovery over time. Interestingly, the identified function of the deregulated genes was different between *Hydra* and *Stylophora*, and therefore the authors concluded that *Hydra* and corals present different adaptive responses to environmental contamination.

Other types of nanoparticles have been also investigated using *Hydra*. Yeo et al. studied the toxicity of TiO₂ and Zn-doped TiO₂ nanoparticles using *Hydra magnipapillata* as model system.[87] The rationale of this study was the fact that TiO₂ and ZnO nanoparticles are widely used in sunscreen products and as photocatalysts. None of the nanoparticles caused toxicity in *Hydra* when light was turned off. However, animals treated simultaneously with nanoparticles and UV light exhibited great morphological changes, although an increase in the number of apoptotic/necrotic cells was not found.

9.4.2 *C. elegans*

In the evaluation of metal-based NPs, it is important to consider that some metals have an intrinsic biological role in animals and plants; these are called essential metals. They are required in small amount, however above a threshold value they become toxic. Zinc, copper, manganese, iron and selenium are essential metals for both humans and *C. elegans*, and they play an important role in diverse biological processes (Table 9.3).[88, 89] Organisms have sophisticated mechanisms of metal homeostasis and transport to regulate uptake and distribution of these elements within their body, since an imbalance can cause severe dysfunctions.[88, 90] Additionally, in some cases non-essential metals can be taken up and transported throughout the body by mimicry of essential metals; e.g., Ag can mimic Cu, Cd can mimic Zn and Pb can mimic Ca.[36]

Table 9.3. Functions of essential metals. Adapted from Gonzalez-Moragas et al.[36]

Copper	Iron	Manganese	Zinc	Selenium
- Iron homeostasis - Neurotransmitter biosynthesis - Oxidative phosphorylation, - Oxidative stress protection	- DNA synthesis - Mitochondrial respiration - Oxygen transport - Neurotransmitter synthesis.	- Fat and carbohydrate metabolism - Oxidative stress protection (SOD) - Neurotransmitter synthesis and metabolism.	- Cofactor in several cellular processes and cellular signaling pathways.	- Development - Reproduction - Antioxidant activity - Neuroprotection

Different strategies have been employed to investigate the molecular mechanisms of nanotoxicity in *C. elegans*, among them assays to evaluate the production of ROS; studies to assess altered gene or protein expression, including transcriptomics; or pharmacological experiments (e.g., by NP co-exposure with an antioxidant treatment).[28, 91-93] Genetic studies offer an alternative approach to evaluate how a genetic alteration can affect the biological response to a stressor. By combining biochemical and genetic studies, different putative mechanisms have been proposed to account for the effects of metal-based NPs in *C. elegans*, including general stress, oxidative stress and endoplasmic reticulum (ER) stress (Figure 9.12). These responses could, then, activate signaling cascades (i.e. unfolded protein response, UPR; calcium; Mitogen-activated protein kinase MAPK) leading to organellar effects (e.g. mitochondrial dysfunction, lysosomal impairment, DNA damage) and finally, to premature cell death by apoptosis.

Figure 9.12. Effects and mechanisms observed in the screening of NPs in *C. elegans* at the molecular level. Adapted from Gonzalez-Moragas et al.[36]

Recent works have also provided evidence of metal toxicity when *C. elegans* is challenged with metal-based NPs, as nanotoxicity was significantly amplified in metal sensitive mutants while protection against metals reduced NP-derived cytotoxic damage.[92] Hence NP dissolution could also drive nanotoxicity, at least partially.

The specific biological mechanisms reported in response to NP treatment are further detailed in Table 9.4, illustrating the lack of agreement between different studies regarding the prevalent mechanism of nanotoxicity. To date, it is still to be elucidated whether nanotoxicity can be explained by a general mechanism, or if it depends on the physico-chemical profile of the NP under study.

Table 9.4. Biological mechanisms triggered by metal-based NPs in *C. elegans*. Adapted from Gonzalez-Moragas et al.[36]

MECHANISM	CONTRIBUTION TO NANOTOXICITY
Metal NPs (Ag, Au, Pt)	
Oxidative Stress	Controversy (●[25, 28, 94-96] ○[97] x[29, 98])
Metal stress	Major role [95, 99]
Dissolution	Major role [97, 100]
NP-specific effects	Major role[100]
Other mechanisms	- Alteration of metabolic processes.[100] - Dermal effects.[95, 100] - Early endosome formation is necessary for AgNP-induced toxicity in vivo.[38] - NP-induced cellular damage after intracellular uptake of AgNPs.[28] - Oxidative stress-related mitochondrial and DNA damage.[28] - MAPK-based integrated stress signaling network as a defense against AgNP exposure.[62, 94] - Pre-exposed nematodes suffered cumulative damage.[101] - Cell uptake by clathrin-mediated endocytosis causes ER stress, and activates UPR pathways that can lead to cell death. Cell uptake also activates Calcium signaling and amyloid processing pathways, which can lead to intracellular Ca²⁺ increase and trigger calpain–cathepsin-mediated events causing cell necrosis and ultimately mortality.[37] - Nano-Pt scavenges endogenous ROS, attenuating intracellular damage.[25, 96]
Metal Oxide NPs (ZnO, TiO₂, SiO₂, CeO₂, FeO_x, Al₂O₃)	
Oxidative Stress	Controversy (●[31, 91, 92, 102, 103], ○[104-107], x[108])
Metal stress	Major role[92, 109]
Dissolution	Controversy (●[109, 110], ○[104], ?[106, 110])
NP-specific effects	Major role[108, 110]
Other mechanisms	- Deficit in development of intestinal barrier and neurons controlling defecation; no recovery after chronic exposure.[34] - Aging phenotype.[26, 27] - Growth defects by inhibition of feeding caused by NP aggregates.[107] - Defense and/or compensatory mechanism mediated by <i>cyp35a2</i>. [108]

● major role, ○ minor role, x lack of evidence, ? hypothesis

9.5. Methodological approaches for tracking NPs used for optical and magnetic hyperthermia in *Hydra* and *C. elegans*

Understanding NP behavior and status in real complex environments is critical for both environmental and biomedical scenarios. Due to the nano-sized scale of the materials under study, techniques with very high spatial resolution are required to identify and characterize NPs in a biological matrix. The study at multiple biological levels, from whole-organism to intracellular scale, can shed light on the translocation routes and NP status in vivo with single-particle precision.

Advanced imaging techniques including TEM, Scanning Electron Microscopy (SEM), Magnetic Resonance Imaging (MRI), hyperspectral dark field microscopy (HDFM) or Synchrotron Radiation X-ray Fluorescence Microanalysis (μ -SRXRF) can help to identify, locate and characterize individual NPs in specific regions of the body of small animals. These techniques can also contribute to unravel the mechanisms by which NPs cross biological barriers in pluricellular organisms. Combined with spectroscopy, it is possible to investigate the in vivo fate, aggregation and degradation of NPs, as well as bio-accumulation and bio-persistence patterns. Quantitative techniques such as inductively coupled plasma mass spectrometry (ICP-MS) allow the quantification of NPs inside small animals to investigate dose- and time- dependent accumulation, and the study of NP metabolism. Interestingly, some techniques permit simultaneous characterization and quantitation of NPs inside treated animals. However, protocols for sample preparation might require specific adaptations for the specific biological specimens, e.g. *C. elegans*, *Hydra*.^[111, 112]

In this section, we present several techniques that have been used to investigate the entrance route, uptake, biodistribution and fate of nanoparticles in small animals, trying to focus on materials used for optical and magnetic hyperthermia.

9.5.1 Hyperspectral microscopy

Hyperspectral microscopy is a visualization tool that combines hyperspectral imaging with advanced optics and computer software enabling a rapid screening, identification and characterization of micro and nanomaterials. Meyer et al. studied the biodistribution of citrate and PVP-coated AgNPs (10-75 nm) using hyperspectral microscopy and observed that all the NPs tested were internalized by the intestinal cells, but only the citrate-coated AgNPs detectably transferred to the germ line of *C. elegans* (Figure 9.13).^[29] Yang et al. reported that the majority of AgNPs (8-38 nm) were located in the digestive tract, and detected limited tissue uptake by hyperspectral microscopy but not by TEM.^[33] Arnold et al. also applied hyperspectral imaging to study the localization of 50-nm CeO₂ nanoparticles and detected NPs both in the intestinal tract and on the surface of the worm, but not inside the intestinal cells.^[107]

Even though hyperspectral microscopy has not been used to the study of AuNPs and iron oxide nanoparticles, we foresee that could bring light to their biodistribution.

Figure 9.13. Use of fluorescence and hyperspectral imaging to characterize NP pharmacokinetics. Worms fed on a bacteria together with PS NPs and AgNPs. Upper panels show epifluorescence images of carboxy 50-nm PS NPs in the intestine (left) and cytoplasm of early embryos (right). Lower panels present hyperspectral images showing 10-nm citrate AgNPs in the intestine (left) and transference to the offspring (right). AgNP identity was confirmed by hyperspectral analysis. Reprinted from Gonzalez-Moragas et al.[36]

9.5.2 Transmission Electron Microscopy

TEM has sufficient spatial resolution to allow single NP detection. However, applying protocols for optimal sample preparation is crucial to minimize technical difficulties, for instance body orientation in the case of *C. elegans*. Sampling at random locations along the body of the worm can limit the information obtained by TEM visualizations; moreover, the analysis of a large number of sections is costly and laborious. To maximize the control of the anatomical area investigated in the cross-sections, targeted ultramicrotomy protocols can be applied using correlated light and electron microscopy (CLEM), with the aim of establishing a statistically significant and biologically meaningful link between the location in the body and the NP status in vivo.[111]

TEM has been frequently used to detect the localization of different types of NPs in *Hydra*, including MNPs and AuNPs.[45, 52] Animal tentacles and body region below the head level can be selected to perform the cuts in order to analyze different locations along the whole animal. To enable an ultrastructural analysis closer to the living state, cryo-TEM can be also performed.[21] Ultrarapid freezing of the animals using high-pressure freezing avoids ice-crystal formation and damage of the tissues.

9.5.3 Scanning Electron Microscopy

SEM allows investigation of the morphology of the objects up to the nanoscale, hence it can reveal the binding of NPs to the external surface of small model organisms. The visual information obtained by SEM, coupled to elemental analysis techniques (Energy-Dispersive X-ray spectroscopy (EDX)) within the same device, provides information about the chemical composition of the visualized areas and allows, for instance, the acquisition of chemical composition maps.

In *C. elegans*, Kim et al. explored the dermal effects of NP exposure by SEM after a 1-day contact with 10 nm AgNPs in NGM agar, observing severe epidemic edema and bursting of the cuticle of *C. elegans* (Figure 9.14A-C).[36] Hence, these results suggest that AgNPs can induce adverse physical effects via the dermal route.

More recently, we evaluated the external surface of *C. elegans* treated with iron oxide and gold NPs for 24 h in liquid by SEM coupled to EDX, and none of the techniques revealed presence of NPs or the chemical elements (Figure 9.14D-F).[36] These findings are in good agreement with the well-accepted notion that NP uptake mainly occurs through the intestine, while in specific cases such as that of NP with high dissolution rate, the released ions could also cause adverse effects on the cuticle.[29, 113, 114]

Figure 9.14. Investigation of the interaction between NPs and the external surface of *C. elegans* by SEM. A-C) SEM of *C. elegans* exposed to citrate AgNPs: A) control, B) 10 mg/L, and C) 100 mg/L. The white arrows indicate epidermal divisions and necrosis. D-F) SEM-EDX analysis of E) Fe₂O₃ NP treated *C. elegans* and F) AuNP treated *C. elegans*. Different locations of the body of treated animals were analyzed by EDX, as schematized in panel D. Reprinted from Gonzalez-Moragas et al.[36]

9.5.4 Synchrotron and microprobe techniques

Among the synchrotron techniques, synchrotron radiation X-ray fluorescence (μ -SRXRF) has been used to map the metal distribution in small animals, while synchrotron X-ray absorption near-edge spectroscopy (μ -XANES) has provided information regarding the oxidation state and coordination environment of metals.[115, 116] The combination of μ -SRXRF and μ -XANES is a powerful tool to study the subcellular distribution and chemical species of noble metal and metal NPs of interest, and might provide valuable information for both gold and iron oxide NP.

Using μ -SRXRF in *C. elegans* specimens, Gao et al. showed that 24-nm copper nanoparticle exposure resulted in elevation of Cu and K levels in the *C. elegans* body, and also in changes in elemental biodistribution of Cu, Fe and Zn within the animal (Figure 9.15).[115] However, Cu²⁺ exposure resulted in a much higher absorption and accumulation. Regarding the use of nuclear microprobe techniques, Le Trequesser et al. combined scanning transmission ion microscopy (STIM) and micro-proton-induced X-ray emission (μ -PIXE) to detect and quantify 30-nm TiO₂ nanoparticles in *C. elegans*. After 4 h exposure, NPs were visible only in the lumen of the alimentary system extending from the pharynx to the anal region, and were retained there even 24 h after feeding.[116] Given that alterations in the distribution of trace metal such as Fe, Cu, Zn

or Mn are sometimes related to certain pathological states, the use of these techniques is of value in the study of alterations in metal homeostasis.

Figure 9.15. Use of μ -PIXE to characterize NP toxicokinetics in *C. elegans*. Above, synchronized worm observed by conventional light microscopy, indicating the different anatomical structures. Below, μ -PIXE maps of titanium in *C. elegans* body. Scale bar, 150 μ m. Adapted from Le Trequesser et al.[116]

In *Hydra*, to the best of our knowledge, synchrotron techniques have not been applied yet to study the distribution of NPs. We have used μ -XANES and μ -SRXRF to evaluate the biodistribution of different types of InP QDs and the integrity of the NPs after the uptake (Mattera et al., manuscript in preparation). Elemental distribution of the QDs in tissue sections was retrieved through μ -SRXRF in cryogenic conditions, which allows measuring the tissues in their frozen hydrated state minimizing radiation damage. On selected spots, μ -XANES spectra can assess the local speciation of the metals.

9.5.5 Analytical chemistry techniques

Among the analytical techniques with potential to investigate NPs in biological environments, quantitation of NP uptake has been mainly addressed by chemical elemental analysis (ICP-MS), while different micro-spectroscopy techniques have been used to characterize NP status. The following paragraphs will exemplify how these techniques have been applied.

Höss et al. investigated the accumulation of soil-derived iron oxide NPs in *C. elegans* using the ferrozine assay for iron determination. They detected relatively high Fe concentrations after a 6 h exposure, however they confirmed NP ejection during defecation (disposal of 50% Fe), and further disposal of the surface-attached Fe during molting (additional 80% reduction) (Figure 9.16). It is worth to note that the ferrozine test did not provide information of the form of iron, hence it is not possible to determine if Fe uptake corresponded to intact NPs or to iron ions.[106] More recently, Johnson et al. applied ICP-MS to quantify AuNP uptake by *C. elegans* and, operating in single particle ICP-MS mode, to characterize AuNP status inside the animals.[117] However, the requirement of NP extraction from treated *C. elegans* using aggressive solvents may have an impact on NP status, which might not be consistent with NP status *in vivo*. Conversely, TEM allows the *in situ* characterization of NP size and aggregation status inside the intestine of NP-treated *C. elegans*. [118]

Figure 9.16. Evaluation of NP uptake by analytical chemistry techniques. Fe concentrations measured in *C. elegans* after 6 h exposure to K-medium (control) and ferrihydrite colloids associated with citrate (Fh_citrate) (28 mg Fe/L); 0 h post exposure: comprises bioaccumulated, attached and ingested Fe; 2 h post exposure: comprises bioaccumulated and attached Fe; 8 h post exposure: comprises bioaccumulated Fe; bars: arithmetic mean, error bars: standard deviation (n = 3). Adapted from Höss *et al.*[106]

9.5.6 Other techniques

The characterization of NP status in biological systems including size, shape or aggregation status *in vivo* is still scarce. Indeed, few studies have investigated how the contact between NPs and a biological matrix affects the latter, for instance, via the formation of a protein corona or the digestion of pH-sensitive NPs in acidic environments, among other potential effects.[29, 109, 119] The combination of materials science techniques and biological assays can lead to a more solid evaluation of nanomaterials in simple animals. We propose below some possibilities that could help to advance in this direction.

9.5.6.1 Advanced microscopy coupled to micro-spectroscopy

Spectroscopy coupled to microscopy can provide a comprehensive understanding of nano/biological interactions. For instance, the identity of the NP-like content of endosomes spotted by TEM in animals treated with SPIONs, can be then confirmed by high angle annular dark field (HAADF) scanning transmission electron microscopy (STEM) coupled to EDX (Figure 9.17). Under HAADF STEM imaging modality, the intensity of the material is proportional to the square of the atomic number, Z^2 . Hence, SPIONs appear with a higher intensity (that is, brighter) than the cellular background due to the higher atomic number of Fe ($Z = 26$) compared to C ($Z = 6$). EDX spectroscopy allows elemental identification by measuring the number and energy of X-rays emitted from a specimen after excitation with an electron beam. Electron energy loss spectroscopy (EELS) can also reveal cell uptake of SPIONs by endocytosis, by measuring the energy loss when the sample is irradiated with an electron beam to determine the elemental components of the material.

Figure 9.17. Investigation of endocytosis of 6-nm iron oxide nanoparticles by the intestinal cells of *C. elegans*, combining the imaging and analytical capabilities of A) TEM, B) HAADF STEM coupled with EDX, and C) EELS. The combination of these techniques allows the researcher to locate and identify NPs intracellularly in endosomes unambiguously. Adapted from Yu *et al.*[30]

Following a similar strategy, the spectral properties of AuNPs can be assessed inside *C. elegans* by absorbance μ -spectroscopy with high precision. Using this approach, Gonzalez-Moragas *et al.* detected a reversible aggregation pattern of AuNPs depending on the physiological features of the anatomical of the animal.[118] Combining the spectral data with the Two-Photon Luminescent Micrographs (TPLM), both biodistribution and aggregation status in *C. elegans* could be depicted in a single image, and the effect of NP size could be investigated (Figure 18).[120-124] Compared to bright-field or dark-field microscopy, TPLM shows superior contrast, is intrinsically confocal and has better spatial resolution, resulting in a more accurate NP identification.

Figure 9.18. Combination of two-photon luminescence microscopy and absorbance micro-spectroscopy to characterize nematodes treated with gold nanoparticles of A) 11 nm AuNPs and B) 150 nm. The two-photon luminescent signal from AuNPs is merged with a dark-field micrograph of the treated animals, and colored according to the peak shift of the absorption maxima (by absorbance micro-spectroscopy) compared to the respective AuNP in dispersion. C) Color legend of the peak shift (expressed in nanometers). Adapted from Gonzalez-Moragas *et al.*[118]

9.5.6.2 Magnetometry to characterize ingested MNPs

Magnetometry techniques have been applied to determine the uptake of MNPs in cells *in vitro* due to its high sensitivity. This approach was extended recently to

quantify the ingestion of MNPs in *C. elegans*. [119, 125] NP quantification by magnetometry shows good agreement with ICP-MS results, confirming the value of this technique in the study of the uptake of MNPs in biological systems. [30, 119, 125] Magnetometry can be complemented with light micrographs of Prussian blue-stained specimens, which reveal the patterns of NP biodistribution throughout the biological specimen.

Magnetometry can also be applied to evaluate the magnetic properties of NPs inside the animal and to determine NP aggregation status and degradation profile. These parameters can be easily obtained by magnetometry and are in good agreement with the results obtained in more laborious analysis methodologies, e.g. cross-sections of *C. elegans* by TEM. [30, 119]

Table 9.5 describes the main features of a range of NP characterization techniques with potential to characterize inside simple animal models such as *C. elegans* or *Hydra*. It presents the NP parameters that each technique can assess, the biological information gathered, and the main advantages and drawbacks of each technique. Being able to relate the physicochemical properties of the NPs with their in vivo performance would provide a vital information to maximize the quality, efficiency and safety of novel nanomaterial at the early stages of discovery without wasting time and money. [126]

9.5.6.3 Molecular Biology Techniques

Molecular biology methodologies deal with the study of genes and their products in terms of gene expression and proteins, respectively. One powerful approach is the study of gene expression modulation by qRT-PCR (quantitative real time polymerase chain reaction), a robust technique that allows acknowledging the level of gene expression in a fast and reliable way. Total RNA from *Hydra* or *C. elegans* treated with nanoparticles and incubated in specific conditions can be purified and used as template to synthesize cDNA by a reverse transcription reaction. Pairs of DNA primers specific for the candidate genes and reference gene respectively, are used as probes to quantify the amount of RNA of interest into the sample. The quantification can be obtained by the delta-delta Ct ($2^{-\Delta\Delta CT}$) method. [127]

A powerful approach to study the effects of nanoparticles on whole gene expression is the transcriptomics profile by RNA seq. To this end, samples of RNA purified from treated animals versus control animals are reverse transcribed to obtain the relative cDNA libraries. Deep sequencing automated reactions give the complete transcriptome with relative amount of specific transcripts. In order to extract functional information from the raw data, bioinformatics tools play an essential role. Functional annotation can be achieved by Blast2GO, a powerful platform to investigate the biological meaning of RNA seq data with different graphical and statistical functions. This platform interoperates the classical platforms for gene and protein-domains homology search including BlastX, toward the gene repository of interests. In order to understand the gene targeted by NPs treatments in *Hydra*, the following can be used 1) the National Center for Biotechnology Information (NCBI) GenBank non-redundant (nr) database (<http://www.ncbi.nlm.nih.gov>), 2) the full Swissprot database, and 3) the cnidaria-UniProt database. Moreover, the distribution of GO (gene ontology) terms can be calculated when possible using the combined graphs function in Blast2GO. In order to decipher further biological function of differentially expressed sequences, KEGG pathways can be assigned to sequences using the online Kyoto Encyclopedia of Genes

and Genomes (KEGG) Automatic Annotation Server (KAAS), <http://www.genome.jp/kegg/kaas/>. [86, 128]

This strategy can be applied in *C. elegans* in an exact manner based on the abundant resources on the biology of this animal model such as WormBase or Functional annotation tool such as DAVID or PANTHER.[129-132]

Table 9.5. Advanced techniques to investigate nano/bio interactions in small animals: state-of-the-art techniques and proposed novel uses. Adapted from Gonzalez-Moragas et al.[36]

	Parameter evaluated	Information derived	Advantages	Limitations
Microscopy				
HDFM	NP Spectra	NP biodistribution NP status	Easy sample preparation.	Lacks spatial resolution.
TEM	NP Size NP Aggregation	NP biodistribution	High resolution (up to 1 nm)	Complex sample preparation of TEM cross-sections. Not quantitative. Contrast between the cellular structures and the NPs is required.
HAADF	NP Size NP Aggregation	NP biodistribution (cellular level)	Higher contrast than TEM. High resolution (up to 1 nm).	Complex sample preparation of <i>C. elegans</i> cross-sections.
SEM	NP Aggregation	NP biodistribution	Allows investigation of the external surface of treated <i>C. elegans</i>	Low spatial resolution – single particle detection is not possible.
TPLM	NP Luminescence	NP biodistribution NP uptake	Enhanced contrast compared to LM. No fluorescence required. Confocal in nature; offers tomography capabilities	Limited to particles with UV-Vis-NIR absorption. Lacks spatial resolution. Not quantitative.
μ-SRXRF	Chemical composition	NP uptake	High sensitivity.	Limited access to synchrotron-based techniques. Limited to elemental identification (phase identification is not possible).
Spectroscopy				
EDX	Chemical composition	NP biodistribution	Multi-element detection. High sensitivity.	Not quantitative. Not possible to discern the form of the element (NP / ionic)
EELS	Chemical composition	NP biodistribution	Multi-element detection. High sensitivity.	Not quantitative. Not possible to discern the form of the element (NP / ionic)
ICP-MS	Chemical composition	NP uptake	Multi-element detection. Quantitative.	High cost. Not possible to discern the form of the element (NP / ionic)
μ-FT-IR	--	Degree of tissue oxidation	Highly informative about lipid and protein status.	Limited access to synchrotron-based techniques.
Absorbance μ-spectroscopy	NP aggregation	NP uptake	Quantitative.	Limited to particles with UV-Vis-NIR absorption.
Raman μ-spectroscopy	--	Biomolecular phenotype	Quantitative.	Difficult interpretation.
μ-XANES	Redox status of chemical elements	Ionic homeostasis	Informative of oxidation state. High sensitivity.	Limited access to synchrotron-based techniques.
μ-PIXE	Chemical composition	NP uptake Ionic homeostasis	Multi-element detection (also in 2D). High sensitivity.	Limited access to microbeam line facilities.
Other techniques				
Magnetometry	NP uptake and magnetic properties	NP composition	Informative of NP size and magnetic properties. High sensitivity. Quantitative.	Limited to magnetic particles.
MRI	In vivo T ₁ / T ₂	NP biodistribution	Safe imaging modality.	High sensitivity is demanded. Difficult to make it quantitative. Limited to magnetic NPs.

Legend: TPLM: Two-Photon Luminescence Microscopy, MRI: Magnetic Resonance Imaging, SQUID: Superconducting Quantum Interference Devices; HAADF: High Angle Annular Dark Field, HDFM: Hyperspectral Dark Field Microscopy, SEM: Scanning Electron Microscopy, μ-SRXRF: Synchrotron Radiation X-ray Fluorescence; STORM: Stochastic optical reconstruction microscopy; XANES: X-ray Absorption Near Edge Spectroscopy; EDX: Energy-dispersive X-ray spectroscopy; EELS: Electron energy loss spectroscopy; ICP-MS: Inductively coupled plasma mass spectrometry; FT-IR: Fourier-

Transform

Infrared

Spectroscopy;

μ -PIXE:

micro-proton-induced

X-ray

emission

9.6. References

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