Ingestion of gallium phosphide nanowires has no adverse effect on \textit{Drosophila} tissue function

Karl Adolfsson$^{1,4}$, Martina Schneider$^{2,4}$, Greger Hammarin$^{1}$, Udo Häcker$^{2}$ and Christelle N Prinz$^{1,3}$

$^1$ Division of Solid State Physics/The Nanometer Structure Consortium, Lund University, Box 118, SE-22100 Lund, Sweden
$^2$ Department of Experimental Medical Science, Lund University, Box 117, SE-22100 Lund, Sweden
$^3$ Neuronano Research Center, Lund University, Box 117, SE-22100 Lund, Sweden

E-mail: udo.hacker@med.lu.se and christelle.prinz@ftf.lth.se

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Abstract

Engineered nanoparticles have been under increasing scrutiny in recent years. High aspect ratio nanoparticles such as carbon nanotubes and nanowires have raised safety concerns due to their geometrical similarity to asbestos fibers. III–V epitaxial semiconductor nanowires are expected to be utilized in devices such as LEDs and solar cells and will thus be available to the public. In addition, clean-room staff fabricating and characterizing the nanowires are at risk of exposure, emphasizing the importance of investigating their possible toxicity. Here we investigated the effects of gallium phosphide nanowires on the fruit fly \textit{Drosophila melanogaster}. \textit{Drosophila} larvae and/or adults were exposed to gallium phosphide nanowires by ingestion with food. The toxicity and tissue interaction of the nanowires was evaluated by investigating tissue distribution, activation of immune response, genome-wide gene expression, life span, fecundity and somatic mutation rates. Our results show that gallium phosphide nanowires applied through the diet are not taken up into \textit{Drosophila} tissues, do not elicit a measurable immune response or changes in genome-wide gene expression and do not significantly affect life span or somatic mutation rate.

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(Some figures may appear in colour only in the online journal)

1. Introduction

A variety of engineered nanoparticles are used in products available to the public, emphasizing the urgency to investigate their effects on living species and on the environment. Carbon nanotubes are high aspect ratio nanoparticles that can be produced in high quantities at relatively low cost and have great potential to be incorporated into consumer electronics or sports products such as bicycle frames because of their exceptional mechanical properties. Carbon nanotubes are under scrutiny because of their geometrical similarities to asbestos fibers and have been the subject of many toxicity studies, with contradictory results [1–6]. Another type of high aspect ratio nanoparticle is semiconductor nanowires from the III–V group, which have not yet been introduced into commercially available products. These nanowires are produced using epitaxy [7], which is a costly process and therefore mass production of III–V nanowires cannot currently be considered. However, III–V nanowires are expected to be part of electronic devices, biosensors and solar cells in the near future [8–12]. Moreover, many research
groups are working with III–V nanowires and are at risk of exposure during production and handling, which stresses the need to investigate the effect of systemic exposure to III–V nanowires. Since this nanomaterial is expensive and in limited supply, suitable model systems must allow in vivo investigations while minimizing the quantity of material required for the study. We chose the fruit fly Drosophila melanogaster, which is an established model system in developmental biology and has recently been shown to be a valuable system for investigating the effects of nanoparticles [13–18]. Its small size, short generation time, genetic amenability and extensive homology to vertebrate systems make Drosophila an excellent system to investigate the properties of nanowires at the molecular level.

Here, we used Drosophila melanogaster to investigate the effect of exposure to gallium phosphide (GaP) nanowires, which are part of the III–V material family. Flies or larvae were exposed to nanowires contained in the food and effects on immune response, gene expression, life span, fertility and gene mutation rate were subsequently investigated. In summary, we did not detect a significant adverse effect of nanowire ingestion on Drosophila flies.

2. Materials and methods

2.1. Nanowires

Gallium phosphide nanowires were grown using metal organic vapor phase epitaxy (MOVPE) from catalytic gold nanoparticles [7]. Pure single crystalline gold nanoparticles were deposited randomly on a GaP (111) B substrate by aerosol deposition [19]. The average particle density on the surface was chosen to be 1 or 10 $\mu$m$^{-2}$. The substrates were subsequently transferred to a commercial MOVPE reactor (Aixtron 200/4) for nanowire growth. In order to remove the surface oxide and to alloy the Au particles with the substrate, the samples were annealed at 550°C for 10 min in an atmosphere of hydrogen and phosphine. The nanowire growth was initiated by supplying trimethylgallium in addition to the phosphine at 470°C. The precursor gas molar fractions were $10^{-5}$ for trimethylgallium and $10^{-2}$ for phosphine, in a hydrogen carrier gas flow of 6 l min$^{-1}$. The growth was conducted under low pressure (10 kPa). The resulting nanowires were perpendicular to the surface with very low tapering and with exceptional homogeneity in length and diameter. The size distribution of the nanowires used in this study can be seen in figure 1. In order to make nanowires with the same dimensions as the GaP nanowires mentioned above but with higher material Young’s modulus and higher chemical stability than GaP, some 60 nm diameter GaP nanowires were coated with a 10 nm layer of hafnium oxide (HfO$_x$) using atomic layer deposition (Savannah-100 system, Cambridge NanoTech, USA). The nanowires were broken off their substrate and suspended in DI water using ultrasonication. The sonication time was minimized in order to avoid breaking the nanowires. The length of the nanowires after sonication was quantified using the ‘particle analyzer’ function in ImageJ (see figure 1).
2.2. Fly strains

All experiments were performed with wild type Drosophila melanogaster (Oregon R) unless stated otherwise. The mutant allele of warts (wts) was wts3−17.

2.3. Administration of nanowires

Larvae. An aqueous suspension of nanowires was mixed with fresh yeast to a final concentration of 10 nanowires ml−1. Larvae were presented with food on top of an agarose gel (1%). Nanowire ingestion was verified using confocal microscopy (see supplementary file 1 available at stacks.iop.org/Nano/24/285101/mmedia).

Adults. In the longevity experiments, adult flies were kept in small vials, each of them prepared by pipetting 50 µl of a 6 × 107 nanowires ml−1 water solution on top of standard fly food and letting it dry. The surface concentration was 3 × 106 nanowires cm−2 (see supplementary file 2 available at stacks.iop.org/Nano/24/285101/mmedia). Ingestion was verified by confocal microscopy (see supplementary file 3 available at stacks.iop.org/Nano/24/285101/mmedia).

2.4. Sample preparation of confocal imaging of Drosophila guts

Third instar larvae or CO2 sedated flies were dissected in phosphate buffered saline (PBS) under a stereomicroscope. Fixation and staining were done at 4 ºC. The entire gastrointestinal tract was isolated and placed in PBS. The dissected guts were fixed for 10 min in 3.7% paraformaldehyde (PF), rinsed for 30 min in PBS. The guts were stained for cell nuclei, actin filaments and chitin by incubating them in bisbenzimide (1:125), Alexa Fluor 488 phalloidin (1:60) (Invitrogen) and wheat germ agglutinin CF640R conjugate (1:100) (Biotium) for 4 h, followed by a 2 h rinsing step.

The stained guts were placed in glycerol and mounted on a microscope slide. Approximately ten intestines were investigated using confocal microscopy (Zeiss LSM 510) for all experiments examining equal numbers of males and females. Nanowires inside the intestine were visualized by the reflected laser light detection (488 nm).

2.5. Crystal cell assay

All samples were prepared by collecting approximately 100 eggs in vials containing 1% agarose and sample specific mixtures (see feeding methods) of yeast on top. At the third instar stage (L3), the larvae were collected and incubated at 70 ºC for 10 min, inducing the rupture of crystal cells, followed by release of enzymes leading to melanin production. Melanized dots (see supplementary file 4 available at stacks.iop.org/Nano/24/285101/mmedia) were counted in abdominal segments A6, A7 and A8. As a positive control, larvae were exposed to E. coli-containing food that was prepared as follows: one E. coli MG1655 colony was picked from a culture plate, transferred to LB broth and amplified at 37 ºC overnight in a shaker incubator. The bacterium-containing solution was mixed with dry yeast to produce a semi-fluid yeast mixture.

2.6. Longevity and fecundity investigation

**Longevity assay after larval exposure to nanowires.** The procedure was the same as in the crystal cell experiments during the larval stages with the exception that larvae were allowed to develop into pupae. After hatching, the flies were transferred to small vials with standard food, which were changed every third day.

**Longevity and fecundity assay of adult flies during long-term exposure to nanowires.** Newly hatched flies were collected during a 24 h period and distributed in the following way: 15 males and 15 females in a small vial with standard food and dried nanowire solution. Living flies were counted and transferred to new vials every third day. Every tenth day, a fecundity test was performed, during which the flies were allowed to lay eggs on a fresh vial under a 24 h period. The experiment continued until at least 50% of the flies had died. The assay was performed with three replicates for each group.

2.7. RNA microarrays

**Larval feeding scheme.** Wild type flies (four cages, 30 females and 30 males per cage) were placed on apple juice agar plates and allowed to lay eggs for 7–8 h at 25 ºC. Ten pieces of agar containing 60–80 eggs each were cut out and transferred to ten agarose plates (1% agarose in water). Fresh yeast paste with nanowires was added to half of the plates. For the other half plain yeast paste was used as control (n = 5 for each group). Once the larvae had hatched and migrated into the yeast, they were transferred together with the yeast to new agarose plates. Plates were kept at 25 ºC and new yeast paste with/without nanowires was added daily until larvae had reached the third instar and were aged 88–96 h (after egg laying). From each plate 30–50 larvae were collected, homogenized on ice in 1 ml TRIzol (Invitrogen) and stored at −20 ºC until RNA extraction.

**RNA preparation and microarray analysis.** RNA was isolated using the TRIzol method and purified with the clean-up column of an RNeasy kit (Qiagen). Subsequent RNA quality control and microarray analysis using a Drosophila Genome 2.0 GeneChip (Affymetrix) was performed by SCI BLU Genomics (Lund, Sweden). Basic Affymetrix chip and experimental quality analysis were performed using the Expression Console software. The probe summarization and data normalization method was robust multi-array analysis (RMA) [20]. A significance analysis of microarrays (SAM) was performed to identify significantly differentially expressed genes between groups [21].
2.8. Somatic mutation and recombination test

Females carrying one copy of the mutant warts [3–17] tumor suppressor allele and a balancer chromosome (TM3, Sb) were crossed to wild type males. The resulting progeny was fed on either standard or nanowire-containing food until the third instar larval stage. As a positive control, third instar larvae were irradiated with 200 kV x-rays using a Gulmay RT6 200 kV setup working at 1 Gy min$^{-1}$. The total irradiation dose was 8 Gy. The irradiated larvae were placed in a petri dish with apple juice agar and a wet filter paper to avoid dehydration. Among the emerging adults, heterozygous wts flies (wts/+ ) were identified by the absence of the balancer chromosome and scored for tumors. The total numbers of flies scored in each group were the following: nanowire-fed group, 300 adult flies; control group, 384 flies; positive control group, 118 flies.

3. Results

3.1. Nanowires

Gallium phosphide nanowires with a length distribution centered around 4 µm and an average diameter of 80 nm (see figure 1) were mixed into the Drosophila diet and progression through the intestinal tract was monitored using confocal microscopy (see supplementary files 1 and 3 available at stacks.iop.org/Nano/24/285101/mmedia).

3.2. Investigation of immune response

Ingestion of nanowires may lead to their uptake in the larval intestinal tract and transfer to the hemolymph, where the nanowires could become a target for encapsulation by effector cells of the immune system. Challenge of the Drosophila immune system results in the proliferation of crystal cells, a type of blood cell that can be visualized and quantified by heat exposure of the larvae. Heat-mediated rupturing of tissues can be detected, which is in accordance with our direct analysis of the immune response and confirms that nanowires do not elicit an immune response in Drosophila melanogaster third instar larvae.

3.3. Gene expression—RNA microarrays

Interaction of nanowires with Drosophila tissues can be expected to result in changes in gene expression. We therefore fed third instar Drosophila larvae with GaP nanowire-containing food and determined changes in gene expression by RNA microarray analysis. A SAM was performed in order to identify significantly differentially expressed genes between groups (see supplementary file 5 available at stacks.iop.org/Nano/24/285101/mmedia). No significant ($q < 5\%$) difference in gene expression was observed between the nanowire-treated group and the control group (see table 1). Notably, an up-regulation of immune genes could not be detected, which is in accordance with our direct analysis of the immune response and confirms that nanowires do not elicit an immune response in Drosophila melanogaster third instar larvae.

3.4. Longevity and fecundity study

In order to investigate whether ingestion of nanowires can alter the life span of Drosophila, we performed a longevity assay on flies that were exposed to GaP and HfO$_x$-coated nanowires either throughout adult life or during the larval stages only. Efficient nanowire ingestion was verified using confocal microscopy (see supplementary files 1 and 3 available at stacks.iop.org/Nano/24/285101/mmedia). The results show that nanowire ingestion does not adversely affect the Drosophila life span (see figure 3 and supplementary file 6 available at stacks.iop.org/Nano/24/285101/mmedia). We observed a difference in longevity between male and female flies that has been previously reported [22].

In parallel to the chronic exposure longevity assay, we examined fecundity for all groups. Our results show that fecundity decreases with time in all groups, which is consistent with previously published observations [23] (see figure 4). We did not see any significant difference in the number of laid eggs between any of the groups except at day 29, when we measured a slightly smaller number of laid eggs for flies exposed to HfO$_x$ coated nanowires ($p < 0.05$). However, the graph shows that the significance arises from low variability in the number of collected eggs within each
Table 1. Fold change in expression level (fold change) and significance ($q$-value) of selected genes comparing nanowire-fed larvae and control larvae. No significant difference ($q < 5\%$) in gene expression could be measured between the two groups. NaN (not a number) means that the analysis could not find any significance for the fold change.

| Function        | Gene symbol | Gene title       | Fold change | $q$-value (%) |
|-----------------|-------------|------------------|-------------|---------------|
| Humoral response| AttC        | Attacin-C        | 0.953 935 15 | 84.777 65     |
|                 | CerC        | Cecropin-C       | 0.971 6165  | 84.777 65     |
|                 | Def         | Defensin         | 1.012 5477  | NaN           |
|                 | Drs         | Drosomycin       | 0.975 5679  | 84.777 65     |
|                 | Mtk         | Metchnikowin     | 0.988 9279  | NaN           |
| Stress response | Duox        | Dual oxidase     | 0.949 799 24 | 84.777 65     |
|                 | TotA        | Turandot A       | 0.908 471 35 | 84.777 65     |
|                 | Hsp70a      | Heat shock 70    | 0.858 6059  | 84.777 65     |
|                 | Hsp22       | Heat shock protein 22 | 0.915 7634 | 84.777 65     |
| Xenobiotics     | Cyp6t1      | Cyp6t1           | 1.242 8458  | 28.723 404    |
|                 | Cyp6a2      | Cytochrome P450-6a2 | 1.038 9432 | NaN           |

3.4. Longevity of adult flies exposed to nanowires

Percentage of surviving flies subjected to constant exposure to GaP or HfO$_x$ nanowires.

3.5. Somatic mutation and recombination test

In order to investigate a possible mutagenic effect of nanowires, we used a variant of the somatic mutation and recombination test (SMART), which uses the warts (wts) tumor repressor gene as a marker to visualize the loss of heterozygosity (LOH). In flies heterozygous for a wts loss of function allele, LOH results in the proliferation of mutant clones in imaginal disc epithelia that appear as tumor-like outgrowths. The tumors are visible on the fly cuticle and can be counted. This test has been shown to be more sensitive than the standard SMART assay using the recessive eye color marker white [24]. The proportion of flies with tumors reflects the mutational frequency, which was found to be 0.035 for control flies, 0.047 for nanowire-fed flies and 0.38 for irradiated positive control flies. These results confirm that x-ray irradiation increases the mutational frequency in flies, but no significant difference could be detected between nanowire-treated flies and the negative controls.

4. Discussion

To date, very little is known about the toxicity of GaP nanowires, as they have not been extensively investigated in model organisms. We have previously shown by local injection of short (2 $\mu$m) nanowires into rat brain that this tissue responds with initially increased astrocyte counts, but significant long-term differences compared to controls were not observed [25]. Our in vitro experiments showed that GaP nanowire substrates promote neurite outgrowth and neuronal attachment, and also limit the spreading of astroglial cells on the substrates [26–29], which suggested that GaP nanowires are a material of interest for the design of neural interfaces. Other studies have shown that high aspect ratio nanowires or nanotubes do not have any adverse effects on cultured cells [12, 30–33]. Recently, however, we have shown that arrays of long nanowires limit fibroblast motility, impede cell
division and cause DNA damage [34]. Here, we examined the effects of a systemic exposure to GaP nanowires in Drosophila melanogaster. Our results did not show a measurable response of Drosophila tissues to GaP and HfO₂-coated nanowire exposure through food ingestion. We cannot exclude that there might be a response at higher nanowire concentrations. However, it is noteworthy that, extrapolated to average human body mass, the concentration we used corresponds to an exposure to 50 mg of nanowires per day. The production of this amount would require a 1.5 m² nanowire substrate (assuming a typical 1 μm⁻² nanowire density), which is far in excess of what a clean-room worker would typically be handling at any given time.

The concentration we tested represents the maximum concentration of nanowires available, since these materials are made using MOVPE and cannot be produced in greater quantities due to technological limits and cost issues.

In our experiments, we did not observe penetration of the intestinal epithelium and uptake of nanowires into internal larval tissues. One reason for this could be the presence of a peritrophic membrane in the fly intestine. This chitinous membrane has a pore size of 2–10 nm and is designed to protect the insect from pathogen invasion [35]. However, this membrane is not 100% effective against pathogens [36] and has been shown to be permeable to gold nanoparticles that were larger than the membrane pores [18]. Ingestion of gold nanoparticles of diameter ranging from 5 to 80 nm has been shown to decrease Drosophila life span and fertility, and to induce stress responses [17, 18]. In the present case, it is possible that the peritrophic membrane effectively prevents nanowires from directly interacting with intestinal cells. In future studies, we will investigate other routes of exposure such as direct injection of nanowires into the fly hemolymph. In such an approach, nanowires will come into direct contact with internal tissues and cells of the immune system. A further advantage will be that significantly smaller amounts of nanowires are required.

5. Conclusion

We have exposed Drosophila melanogaster to GaP and HfO₂-coated nanowires contained in the food supply and investigated the effects of such exposure in terms of immune response, gene expression, longevity, fecundity, and gene mutation. Our results did not show a measurable response to nanowire exposure. Specifically, the number of crystal cells in third instar larvae was the same between nanowire-fed and control larvae. No significant gene expression changes could be measured as the result of exposure to nanowires through ingestion. The life span of flies exposed to nanowires during the larval stages or throughout adult life did not differ from the life span of control flies. Fly fecundity and basic mutation rate were not influenced by the nanowire exposure. Our results suggest that the Drosophila intestinal tract is relatively insensitive to nanowire exposure and protects the adjoining tissues well from environmental hazards. In the future, we will inject nanowires directly into the hemolymph in order to force a direct interaction between nanomaterials and Drosophila tissues and cells of the immune system.

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The authors have no competing interests.

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