microRNAs control of in vivo toxicity from graphene oxide in Caenorhabditis elegans

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Abstract

The molecular basis for in vivo graphene oxide (GO) toxicity is still largely unclear. We here used Caenorhabditis elegans to investigate the microRNAs (miRNAs) control of GO toxicity. With the aid of SOLiD sequencing, we identified 23 up-regulated and 8 down-regulated miRNAs in GO-exposed nematodes. Gene ontology and KEGG pathway database analysis implied that these identified miRNAs might be involved in control of many biological processes, and some of them suggest the possible new functions of GO. Functions of the identified miRNAs in regulating the GO toxicity on lifespan were confirmed in the available miRNAs mutants. Moreover, we provide the evidence to raise a hypothesis that GO may reduce lifespan through influencing the functions of insulin/IGF signaling, TOR signaling, and germline signaling pathways. Our results will be helpful for understanding the molecular basis for GO toxicity, and finding clues for useful surface modifications to reduce GO toxicity.

From the Clinical Editor: In this study, toxicity of graphene oxide is studied in a Caenorhabditis elegans model via microRNA analysis. The authors report that multiple important pathways are influenced by GO and raise a hypothesis that GO may reduce lifespan through influencing the functions of insulin/IGF signaling, TOR signaling, and germline signaling pathways.

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Key words: Caenorhabditis elegans; Graphene oxide; microRNAs; Toxicity; Lifespan

Graphene oxide (GO), one of the important derivatives of graphene, has attracted the interests from different areas including drug and gene carrier, and bioimaging.1–3 After exposure, GO could be distributed and accumulated in the body of animals.4,5 In mice, most of the GO could be accumulated in the lung or liver.4,6 Moreover, both in vitro and in vivo studies have demonstrated that the pristine GO tends to be toxic to organisms in a dose-dependent manner.7–11 The in vivo data suggest that GO exposure can result in a series of adverse effects on animals, such as the pulmonary toxicity.4,5,12–15

So far, it has been considered that at least the oxidative stress, inflammatory activation, and apoptosis induction are important cellular and molecular mechanisms for GO toxicity.5–7,12 The thorough understanding of biological behavior and mechanism of engineered nanomaterials (ENMs) will be helpful for guaranteeing the sustainable nanotechnology.16 The induction of toll-like receptor 4 may be the predominant molecular mechanism underlying GO-induced macrophagic necrosis.8

With the aid of proteome analysis, the molecular targets for GO at the translation level have been systematically investigated in an in vitro assay system.17 In contrast, we still know little about the molecular mechanism for GO toxicity at the level of microRNAs (miRNAs).

Nematode Caenorhabditis elegans, a well-known model animal, can be easily cultivated in a laboratory and reproduced in thousands of individuals.18 The completion of C. elegans genome has revealed that approximately 45% of the genes have human homologues, including numerous disease-related genes.19,20 C. elegans has been served as an important alternative toxicity assay system for toxicants, and can offer a system best suited for asking in vivo questions with relevance at the organism level.19,20 C. elegans has been further used for toxicological study of ENMs including metal oxide-nanoparticles (NPs) or metal-NPs, quantum dots (QDs), and carbon ENMs.21–31

In C. elegans, it has been determined for the toxicity from acute (from adult for 24-hr) or prolonged (from L1 larvae to adult) GO exposure and the possible underlying cellular and...
chemical mechanisms. However, the molecular mechanism of GO toxicity is still largely unclear in nematodes. miRNAs are a large class of short noncoding RNAs found in many plants, animals, and humans, and usually act to post-transcriptionally inhibit gene expression. Herein, in the present study, we employed the assay system of *C. elegans* to perform the systematic study on the identification of the possible miRNAs targets for GO using the SOLiD sequencing technique and the examination of functions of candidate miRNAs in regulating the GO toxicity. Our data reveal the candidate miRNAs targets for GO in the used in vivo assay system, which provides the important molecular basis at the miRNAs level for the in vivo GO toxicity.

**Methods**

The complete methodology description is available at www.nanomedjournal.org.

**Results**

**Physicochemical properties of prepared GO**

The prepared GO was black, and the transmission electron microscopy (TEM) and atomic force microscopy (AFM) images demonstrated the sheet-like shape of the prepared GO (Figure 1, A-C). After sonication, the aggregation size of GO (100 mg/L) in K-medium was 164 ± 34 nm (Figure 1, B). The used different concentrations did not significantly influence the GO aggregation sizes (0.1 mg/L, 135 ± 36 nm; 1 mg/L, 142 ± 18 nm; 10 mg/L, 154 ± 26 nm; 100 mg/L, 164 ± 34 nm). After sonication, the size distribution of GO in K-medium was similar to that in water (Figure S1). The height image from AFM indicates that the thickness of the prepared GO was approximately 1.0 nm in topographic height, corresponding to the approximately one layer (Figure 1, C). Fourier transform infrared spectroscopy (FTIR) spectrum of GO...
showed that the peaks at 1406 and 3414 cm$^{-1}$ attributed to O–H stretching vibration, the peak at 1618 cm$^{-1}$ attributed to C = O stretching vibration, and the peak at 1094 cm$^{-1}$ attributed to vibration of C–O (alkoxy) (Figure 1, D). Raman spectroscopy showed that D-band signal appeared after treatment with sulfuric acid and KMnO$_4$, demonstrating the introduction of disorder into the graphite layer (Figure 1, E). Zeta potential of GO (100 mg/L) in K-medium was $-22.5 \pm 1.4$ mV.

Toxic effects of prolonged exposure to GO on lifespan of nematodes

In C. elegans, lifespan is an important endpoint for toxicity assessment of toxicants. After prolonged exposure from L1 larvae to adult, GO at concentrations of 0.1-1 mg/L did not significantly affect the lifespans of nematodes; however, GO at concentrations of 10-100 mg/L significantly reduced the lifespans of nematodes compared with control (Figure 2, A and B).

Moreover, we investigated the aging-related properties in nematodes exposed to GO. Firstly, we examined the effects of prolonged exposure to GO on intestinal autofluorescence and ROS production. Intestinal autofluorescence is caused by lysosomal deposits of lipofuscin, and can accumulate over time in aging nematodes. For wild-type N2 nematodes, the population of dead animals and the accumulation of intestinal autofluorescence usually increase sharply after adult day 10. At adult day 10, prolonged exposure to 0.1 mg/L of GO did not induce the noticeable intestinal autofluorescence or intestinal ROS production compared with control; however, prolonged exposure to 10-100 mg/L of GO obviously resulted in the intestinal autofluorescence or intestinal ROS production (Figure 2, C and D). Prolonged exposure to 1 mg/L of GO
Among these miRNAs, 23 up-regulated miRNAs and 8 down-regulated miRNAs were acquired by comparing the miRNA sequences with the databases of Genbank and miRbase databases expressed genes were acquired by comparing the miRNA use of a 2.0-fold change cutoff. Annotations of differentially dysregulated miRNA expression in GO-exposed nematodes was examined with the fold change analysis, and Dysregulated miRNA expression in GO (10 mg/L)-exposed nematodes. We found the possible 163 targeted genes, the biological processes involved in the in vivo GO toxicity were evaluated by the DESeq. The significantly influenced gene ontology terms were mainly classified into the several categories, which at least involved the biological processes of development, reproduction, cell adhesion, cell cycle, cellular localization and transportation, cell communication, response to stimulus, immune response, and cell metabolism (Figure 4, A).

Prediction of targeted genes for dysregulated miRNAs in GO-exposed nematodes and the assessment of gene ontology

With the aid of TargetScan database, we predicted the possible targeted genes for the dysregulated miRNAs in GO-exposed nematodes. We found the possible 163 targeted genes for down-regulated miRNAs and 49 targeted genes for up-regulated miRNAs in GO-exposed nematodes, respectively (Tables S2 and S3). Gene ontology analysis provides the ontology of defined terms and gene product properties. Based on the dysregulated miRNAs and their predicted targeted genes, the KEGG pathway mapping is used to map the molecular data sets in genomics, and the related signaling pathways were extracted by the pathway mining tool. We identified 49 signaling pathways for up-regulated miRNAs and 47 signaling pathways for down-regulated miRNAs in GO-exposed nematodes (Figure 4, B). The influenced signaling pathways possibly by in vivo GO exposure mainly focused on the signaling pathways related to

miRNAs participant in the control of in vivo GO toxicity

To determine the possible in vivo miRNAs targets of GO, the SOLiD sequencing used for analysis of miRNA expression profiling was performed to compare the miRNA expression profiling between control and 10 mg/L of GO exposure from L1 larvae to adult. We performed the clustering analysis according to length and chromosome location for the detected miRNA sequences. The detected miRNA sequences appeared as the size of 18-25 nucleotides (Figure S3, A), and among them, most of the detected SOLiD sequences were found to be distributed between 20 and 23 nucleotides, which was considered as mature miRNAs by subsequent miRNA database blasting (Figure S3, A). The miRNAs detected in the SOLiD sequencing were located on all chromosomes including the sexual chromosome X of nematodes (Figure S3, B). Most of the detected SOLiD sequences were localized onto chromosomes II and X (Figure S3, B). These results suggest the feasibility of this RNAomics study, and demonstrate the possible involvement of miRNAs in regulating the in vivo GO toxicity.

Dysregulated miRNA expression in GO-exposed nematodes

The dysregulated expression of miRNAs in GO-exposed nematodes was examined with the fold change analysis, and developed for further analysis based on statistical significance and use of a 2.0-fold change cutoff. Annotations of differentially expressed genes were acquired by comparing the miRNA sequences with the databases of Genbank and miRbase databases (Figure 3, A, Table S1). We identified 31 differentially expressed miRNAs in GO-exposed nematodes compared with control (Table S1). Among these miRNAs, 23 up-regulated miRNAs and 8 down-regulated miRNAs were identified (Figure 3, B-C, Table S1). The up-regulated miRNAs were mir-259, mir-1820, mir-36, mir-82, mir-239, mir-246, mir-247, mir-392, mir-4806, mir-2217, mir-360, mir-4810, mir-4807, mir-1822, mir-4805, mir-800, mir-1830, mir-236, mir-244, mir-235, mir-4937, mir-4812, and mir-43, and the down-regulated miRNAs were mir-1834, mir-800, mir-231, mir-5546, mir-42, mir-2214, mir-2210, and mir-73 (Figure 3, C, Table S1). Thus, the expression patterns of miRNAs can be globally influenced by GO exposure in nematodes.

Distribution and translocation of GO in nematodes

Again, we used the molecular probe of Rho B to label GO to further examine the distribution and translocation of GO in nematodes. After prolonged exposure to GO, although most of the labeled GO was located in pharynx and intestine, a great amount of GO was already translocated into the spermatheca and gonad in body through the primary targeted organ of intestine (Figure S2, A). Therefore, GO could be distributed in both the primary and the secondary targeted organs in nematodes. Compared with the distribution of GO-Rho B in nematodes, exposure to Rho B caused the relatively equable distribution of fluorescence in all tissues of nematodes (Figure S2, B).

Dysregulated miRNAs in GO-exposed nematodes and the assessment of gene ontology

To examine the molecular basis of miRNAs differentially expressed in GO-exposed nematodes, we also used the KEGG pathway database to identify the possible related signal pathways mediated by the predicted targeted genes for the dysregulated miRNAs. KEGG pathway mapping is used to map the molecular data sets in genomics, and the related signaling pathways were extracted by the pathway mining tool. We identified 49 signaling pathways for up-regulated miRNAs and 47 signaling pathways for down-regulated miRNAs in GO-exposed nematodes (Figure 4, B). The influenced signaling pathways possibly by in vivo GO exposure mainly focused on the signaling pathways related to...
development, cell cycle, cell death, neuronal degeneration, transcription regulation, oxidative stress response, DNA damage and repair, vesicle transportation, immune response, and cell metabolism (Figure 4, B), which was largely consistent with the data from gene ontology above.

Functions of miRNAs in regulating the GO toxicity in nematodes

To further determine the role of dysregulated miRNAs in regulating GO toxicity formation, after prolonged exposure to 10 mg/L of GO, we employed the available mutants for the identified dysregulated miRNAs to compare their lifespan with that of wild-type N2. With the aid of lifespan as the endpoint, interestingly, we found that GO-exposed \textit{mir-244} and \textit{mir-235} mutants showed the significantly decreased lifespan compared with that of GO-exposed wild-type N2 (Figure 5). The GO-exposed \textit{mir-360}, \textit{mir-81/82}, \textit{mir-246}, and \textit{mir-259} mutants had the similar lifespan to that of GO-exposed wild-type N2 (Figure 5). These data suggest that some miRNAs are involved in the control of GO toxicity formation in nematodes.

Mutations of miRNAs can alter the aging-related properties in GO-exposed nematodes

We further investigated the aging-related properties in some miRNA mutants exposed to 10 mg/L of GO. Besides the changes of lifespan, we also found that GO-exposed \textit{mir-244} and \textit{mir-235} mutants showed the significantly increased induction of intestinal autofluorescence or intestinal ROS production compared with that of GO-exposed wild-type N2; however, GO-exposed \textit{mir-247/797}, \textit{mir-73/74}, and \textit{mir-231} mutants exhibited the significantly decreased intestinal autofluorescence or intestinal ROS production compared with that of GO-exposed wild-

![Figure 4. Assessment of gene ontology terms and signal pathways. (A) Gene ontology terms with gene counts based on dysregulated miRNAs in GO-exposed nematodes. (B) The predicted KEGG signal pathways based on dysregulated miRNAs in GO-exposed nematodes.](image-url)
type N2 (Figure S4, A and B). Moreover, GO-exposed mir-244 and mir-235 mutants showed the significantly decreased head thrash or body bend compared with that of GO-exposed wild-type N2; however, GO-exposed mir-247/797, mir-73/74, and mir-231 mutants had the significantly increased head thrash or body bend compared with that of GO-exposed wild-type N2 (Figure S4, C). Therefore, miRNAs are involved in the control of both the lifespan and the aging-related properties in GO-exposed nematodes.

Molecular mechanism for dysregulated miRNAs in regulating the in vivo GO toxicity in nematodes

To determine the molecular mechanism for miRNAs in regulating the in vivo GO toxicity, we searched the predicted targeted genes involved in the control of longevity for dysregulated miRNAs in nematodes. We found that daf-16, daf-18, pdk-1, akt-2, sgk-1, smk-1, hcf-1, aak-2, unc-51, daf-15, raga-1, rheb-1, pha-4, daf-9, daf-12, and kri-1 genes are possible targeted genes for dysregulated miRNAs in GO-exposed nematodes (Table S4), and these genes are involved in the molecular control of longevity (Table S5). We further performed the quantitative analysis for these genes in control and 10 mg/L of GO-exposed nematodes by real-time PCR. Interestingly, we found that expression patterns of daf-16, daf-18, pdk-1, sgk-1, smk-1, daf-15, and kri-1 genes were significantly altered in nematodes exposed to 10 mg/L of GO compared with control (Figure 6, A). After exposure to 10 mg/L of GO, expression levels of pdk-1, and daf-15 genes were significantly altered.
increased, whereas expression levels of daf-16, daf-18, sgk-1, smk-1, and kri-1 genes were significantly decreased in nematodes (Figure 6, A). In C. elegans, the aging process is under the control of three major endocrine- and nutrient-sensing signaling pathways, the insulin/insulin-like growth factor (IGF), target of rapamycin (TOR), and germline signaling pathways. In C. elegans, daf-16, daf-18, pdk-1, and sgk-1 genes encode the insulin/IGF signaling pathway, smk-1 genes encodes a DAF-16 transcriptional coregulator, daf-15 gene encodes a component for TOR signaling pathway, and kri-1 gene encodes a component for germline signaling pathway.

Effects of GO exposure on expression pattern of genes required for miRNAs biogenesis

In C. elegans, primary miRNA transcripts are processed into pre-miRNAs in the nucleus by the Microprocessor complex containing RNase III enzyme Drosha and its binding partner, the dsRNA binding proteins of DRSH-1 and PASH-1. The pre-miRNA is presumably exported into the cytoplasm by a homolog of exportin 5, possibly IMB-4, and further cleaved by the RNase III enzyme Dicer (DCR-1) into a miRNA and its partner strand. The Argonauta protein ALG-1 is required for the Dicer cleavage, and ALG-1 and ALG-2 are in the same complex with DCR-1. After prolonged exposure to 10 mg/L of GO, we found that GO exposure did not significantly affect the expression levels of algi-1, alg-2, and imb-4 genes (Figure S5). In contrast, GO exposure significantly decreased the expression levels of drsh-1, pash-1, and dcr-1 genes in nematodes (Figure S5). Therefore, GO exposure may influence the molecular machinery of miRNAs biogenesis in nematodes.

Discussion

Our data indicate the possibility of prolonged exposure to GO at concentrations more than 10 mg/L to reduce the lifespan of nematodes. In C. elegans, silica-NPs could also induce the reproductive senescence after translocation into the body of nematodes. Moreover, we found that, during the aging process, prolonged exposure to GO can further significantly increased both intestinal autofluorescence and intestinal ROS production, and decreased locomotion behavior (Figure 2, C-F), suggesting the severely adverse effects of prolonged exposure to GO on both lifespan and aging-related properties. Considering the important contributions of intestine, neurons and reproductive organs to longevity control in nematodes, these results further support our previous observations on the effects of GO exposure on functions of primary or secondary targeted organs of nematodes to a certain degree.

In C. elegans, previous studies have investigated the possible molecular targets at the mRNA level for some ENMs such as Ag-NPs and Au-NPs. In organisms, expression of a large number of mRNAs is controlled by a limited number of miRNAs. In the present study, we performed the SOLID sequencing to systematically investigate the possible miRNAs targets for GO in the C. elegans assay system. miRNAs have been systematically studied in the model animal of C. elegans, which contributes greatly to our understanding the biological function and regulation of miRNAs in organisms. Based on our SOLID sequencing and the following annotations of differentially expressed genes acquired by comparing the miRNA sequences with the databases of Genbank and miRbase databases, we identified 23 up-regulated miRNAs and 8 down-regulated miRNAs in GO-exposed nematodes compared with control (Figure 3, B-C, Table S1). Expression patterns of these 31 miRNAs were confirmed by the further quantitative analysis of qRT-PCR (Figure 3, D). Moreover, expression of the differentially expressed miRNAs was in a concentration-dependent manner in GO-exposed nematodes (Figure 3, D).

The further bioinformatics analysis provides more information for the identified 31 differentially expressed miRNAs. Both the gene ontology analysis and the KEGG pathway database analysis have implied that the identified differentially expressed miRNAs might be involved in the control of a series of biological processes including development, reproduction, cell adhesion, cell cycle, cell death, neuronal degeneration, DNA damage and repair, cellular localization and transportation, vesicle transportation, cell communication, transcription regulation, oxidative stress response, response to stimulus, immune response, and cell metabolism in GO-exposed nematodes (Figure 4). These data are largely consistent with previous studies in nematodes and other biological assay systems. Our data here further imply the new possible effects of GO on organisms, and these effects at least contain vesicle transportation, cell communication, and cell metabolism. However, we still do not know the exact properties of these effects. Previous study have identified the possible protein targets for GO in an in vitro assay system, and the identified protein targets were associated with the calcium binding, cell skeleton, metabolism, and growth. These information and the further confirmation of targets for the identified miRNAs in the in vivo assay system will greatly contribute to our deep understanding of the molecular basis or mechanism for biological effects from GO. In C. elegans, many of the miRNAs mutants are available for the aim of genetic study. Interestingly, we found that GO-exposed mir-244 and mir-235 mutants showed the significantly decreased lifespan; however, GO-exposed mir-235/797, mir-73/74, and mir-231 mutants exhibited the significantly increased lifespan compared with wild-type (Figure 5). Moreover, GO-exposed mir-244 and mir-235 mutants exhibited the significantly decreased locomotion behavior, and the significantly increased intestinal autofluorescence, and intestinal ROS production; however, GO-exposed mir-247/797, mir-73/74, and mir-231 mutants had the significantly increased locomotion behavior, and the significantly decreased intestinal autofluorescence, and intestinal ROS production (Figure S4). Therefore, our data confirm the possible functions of miRNAs in regulating in vivo toxicity formation from GO exposure. Nevertheless, among the identified 31 differentially expressed miRNAs, we did not examine the possible roles of other 22 miRNAs in regulating the GO toxicity because of the unavailability of their mutants. Among the examined 9 mutants, we confirm the exact role of mir-231, mir-244 and mir-235 in regulating the GO toxicity. In contrast, we still cannot completely confirm the functions of mir-73 and mir-247 in regulating the GO toxicity, because the examined mutants still have the mutations of other miRNAs such as mir-74 or mir-797.
To examine the possible molecular mechanism for miRNAs in regulating GO toxicity, we integrated the information from bioinformatics analysis and the molecular analysis for genes required for aging control. Among the genes serving as the candidate molecular targets for the identified differentially expressed miRNAs, we found that expression levels of pdk-1, and daf-15 genes were significantly increased; however, expression levels of daf-16, daf-18, sgk-1, smk-1, and kri-1 genes were significantly decreased (Figure 6). Among the three signaling pathways regulating longevity, insulin/IGF signaling pathway contains proteins of DAF-16, DAF-18, DAF-2, AGE-1, AKT-1, AKT-2, PDK-1, SGK-1, PRMT-1, RLE-1, SMK-1, HCF-1, HSF-1, SKN-1, AAK-2, and UNC-51, TOR signaling pathway contains proteins of PHA-4, DAF-15, RAGA-1, RHEB-1, and RICT-1, and DAF-9, and TCER-1 and KRI-1 which are specific components for the germline signaling pathways. Therefore, prolonged exposure to GO may reduce the lifespan of nematodes through influencing functions of the insulin/IGF signaling, TOR signaling, and germline signaling pathways controlled by miRNAs (Figure 6, B).

For the mechanisms explaining the influence of GO exposure on expression levels of miRNAs biogenesis, we raised two possibilities. One of the possibilities is that GO exposure may affect the molecular machinery of miRNAs biogenesis. In nematodes, GO exposure caused the decrease in expression levels of drsh-1, pash-1, and dcr-1 genes (Figure S5), implying that long-term GO exposure might affect the processing from the primary miRNA transcripts into the pre-miRNAs and the miRNAs maturation. The second possibility is that GO may affect the germline development and the related biological processes, because GO could be translocated into the gonad and the embryo of exposed nematodes.

In conclusion, with the aid of *C. elegans* as an *in vivo* assay system, we performed the systematical identification of candidate miRNAs targets for GO, and obtained a limited number of candidate miRNAs targets for GO. These identified miRNAs have been implied to be involved in the regulation of a series of biological processes. The bioinformatics analysis implied the possible new functions of GO on organisms. The functions of identified differentially expressed miRNAs in regulating the GO toxicity were confirmed by the analysis of endpoints in the available mutants for the identified miRNAs in GO-exposed nematodes. Moreover, we raise a hypothesis here that GO may affect the germline development and the related biological processes. The presented data here will be helpful in further deeply understanding the molecular basis for GO toxicity. Our results also provide an important clue for further design of surface modifications to reduce the toxicity of GO.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.04.005.

References


