Role of MicroRNAs in regulation of DNA damage in monocytes exposed to polystyrene and TiO$_2$ nanoparticles

Moyan Hu, Dušan Palić*

Chair for Fish Diseases and Fisheries Biology, Faculty of Veterinary Medicine, Ludwig Maximilian University of Munich, Munich, Germany

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ABSTRACT

The release of nanoparticles into the environment can interfere with the health of the exposed organisms. MicroRNAs have been suggested as potential toxicology biomarkers. The expression of potential zebrafish nanotoxicity biomarker miRNAs in our previous study was validated in THP-1 human monocytic cell line after exposure to polystyrene (PSNPs) and ARS labeled Titanium dioxide nanoparticles (nano-TiO$_2$-ARS). miRNAs expression post exposure to PLGA nanoparticles and *E. coli* BioParticles was used to exclude potential activation and engagement of miRNAs through phagocytosis or pro-inflammatory specific responses. miR-155-5p showed the highest potential to be used as biomarker for PSNPs and nano-TiO$_2$-ARS induced toxicity. To determine effects of PSNPs and nano-TiO$_2$-ARS on genotoxicity, time and dose dependent DNA damage profile was established. Severe DNA damage was triggered by both nanoparticles, and expression of DNA damage repairing genes was elevated post nano-TiO$_2$-ARS, but not post PSNPs exposure, questioning the utility of the comet assay as universal assessment tool for genotoxicity induced by nanoparticles in general. Transfection of miR-155-5p mimic influenced the expression of miR-155-5p related, DNA damage responsible genes post both nano-TiO$_2$-ARS and PSNPs exposure. Transfection results suggest significant involvement of miR-155-5p in gene repair mechanisms triggered by adverse effects of PSNPs and nano-TiO$_2$-ARS on monocytes.

1. Introduction

Increase in household and industrial application of nanoparticles (NPs) is accompanied by rise of annual production and also variety of NPs [1]. Such rapid changes in nanoparticle use are raising awareness about their potential adverse effects on environmental fate and public health. Major mechanisms underlying the concern for NPs induced toxicity are their inflammatory potency, oxidative stress induction, and ability to trigger genotoxicity [2].

Potential genotoxicity of nanoparticles is a major concern as inducible mutagenesis highly increases risk of carcinogenicity. Regulatory bodies are increasing their actions and activities in addressing risk of potential mutagenic carcinogens. Exposure thresholds for this type of toxic agents doesn't exist, and only "Margin of Exposure approach" that are derived from reference exposure values are applicable according to EFSA (European Food Safety Authority) guidelines [3]. Furthermore, the ECHA (European Chemicals Agency) updated the guidance for classification, labelling and packaging (CLP) of substances and mixtures with an emphasis on genotoxic carcinogens by classifying and labeling this kind of toxins separately [4]. Even with recent

increase of studies addressing nanoparticle genotoxicity, contradicting statements from those reports prevent development of clear guidelines to be used in risk assessment and regulations [5,6].

Titanium dioxide nanoparticles (nano-TiO$_2$) are widely used in sunscreens and pigments worldwide. Potential adverse effects of nano-TiO$_2$ have been reported to cause health concerns such as interfere with inflammatory responses, environmental biofilms, and can also cause DNA damage in experimental animals [7–10]. Comet assay is used as standard toxicological assay for evaluating DNA strand breaks *in vitro* [11], including DNA damage induced by nano-TiO$_2$. [5]. It was observed that DNA strands damage is triggered in presence of nano-TiO$_2$ as low as 1 µg ml^{-1} [12]. Crystalline structure of nano-TiO$_2$ is strongly related to toxicity, and anatase form exhibits higher toxicity than the rutile phase due to different surface properties [13]. However, it also appears that a mix of crystalline forms of nano-TiO$_2$ (anatase, and also 80 % anatase with 20 % rutile) induced comparable DNA strand damage and breaks [14].

Polystyrene is known to be one of the most frequently used organic polymers in our daily life. Multiple studies addressing risks of polystyrene contamination or pollution focused on the environmental

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perspective because of the long half-life and its persistence in the environment [15]. Polystyrene microspheres recently prompted further research, as their potential for adverse effects of oxidative stress and genotoxicity was discovered in the aquatic models [16,17]. However, risk of genotoxicity induced by nano-sized polystyrene particles (PSNPs) remains unclear as there is limited information available [6,18]. An intracellular dynamic imaging study indicated that cationic functionalized PSNPs could result in a prolonged G0/G1 phase in the cell cycle during mitosis in NIH 3T3 cells, therefore indicating potential for DNA damage and the interference with checkpoint control activation [18]. Paget et al. indicated that non-functionalized PSNPs did not induce a general genotoxicity except at the highest tested dose of after 8.1 µg/cm^2 exposure for one hour [6]. Therefore, the potential for genotoxicity of PSNPs, regardless of their surface modification, requires further investigation. We are not aware of any studies that reported activation of molecular regulatory mechanisms at transcriptional level post PSNPs exposure. Therefore, we also conducted a study to investigate transcriptional level of molecular regulation underlying potential PSNPs induced genotoxicity.

MicroRNAs (miRNAs) are endogenous gene regulatory molecules in various organisms playing an important role in diverse biological processes including DNA damage repair. miRNAs are involved in multiple regulatory pathways of DNA damage repair (DDR) and it is frequently observed that a single miRNA can regulate multiple DDR mechanisms. For example, one of miR-155 targets (*WEE*1) is involved in cell cycle checkpoint regulation [19]. *WEE*1 was up-regulated after exposure to gold NPs and reported to be involved in MAPK pathway activation following cellular stress event [20,21]. These studies indicate there is significant potential for the use of miRNAs tools and biomarkers in the nanoparticle toxicity risk assessment. Complexity of the miRNA-mRNA regulations in the process of DNA damage and repair suggests that an intricate web of miRNA regulations underlying nano-toxicity is likely. Therefore, further research is needed to elucidate multiple possible correlations between miRNAs and nanoparticle-induced DNA damage.

Objective of this study was to determine level of DNA damage induced by *in vitro* exposure of human monocytic cell line (THP-1) to polystyrene and titanium-dioxide nanoparticles at different concentrations and time points. Furthermore, expression of top six *in silico* predicted miRNAs in our previous study [22] to be used as biomarkers of nanoparticle toxicity was validated, and functional investigation of miRNAs with highest potential to regulate PSNP nano-TiO$_2$-ARS toxicity responses was performed.

2. Materials and methods

2.1. Nanoparticles preparation and characterization

Polystyrene Nanoparticle (Cat. #17149-10) was purchased from Polysciences, Inc. (Warrington, PA, USA). The stock solution of polystyrene nanoparticles (PSNPs) was in a form of 2.5 % aqueous water suspension with internally fluorescent labeling (Fluoresbrite Yellow Green; ex./em. 480/520 nm) and a nominal mean diameter of 0.05 µm. Original PSNPs were centrifuged and resuspended with Hank's balanced salt solution with Ca, Mg, no phenol red (HBSS; HyClone Laboratories Inc, USA). Anatase nano-TiO$_2$ powder with a primary nanoparticle diameter below 25 nm (Sigma-Aldrich Corp, USA) was used in our experiment. Fluorescent dye Alizarin Red S (ARS; Sigma-Aldrich Corp, USA) was used to fluorescent label nano-TiO$_2$ [23]. Briefly, ARS was diluted in distilled water (4 mM, pH = 5.7–6), mixed with nano-TiO$_2$, and stirred for 2 h at room temperature. Mixture was vortexed for 1 h and rinsed three times in fresh HBSS to remove unbound dye. ARS labeled nano-TiO$_2$ (nano-TiO$_2$-ARS) has been used as described in subsequent experiments.

Characterization of nano-TiO$_2$-ARS and PSNPs was kindly performed by Dr. Frits Kamp using dynamic light scattering (DLS) (Zetasizer, Malvern Instruments, UK) for determination of nanoparticle

size distribution and zeta-potential. Unlabeled nano-TiO$_2$ were characterized as previously described [7].

2.2. Cell culture

THP-1 monocytic cell line (American Type Culture Collection, ATCC reference number TIB-202TM) was used as *in vitro* model of monocyte responses to nanoparticles (a kind gift from Prof. Peter Nelson). Briefly, THP-1 cells were cultured in RPMI 1640 medium (Sigma-Aldrich Corp, USA) supplemented with 10 % fetal bovine serum (FBS) and 1% L-glutamine-streptomycin-penicillin at 37 °C in 5% CO$_2$. THP-1 monocytes were seeded in cell seeding plates and differentiated to macrophages by 24 h stimulation with 30 ng ml^{-1} phorbol myristate acetate (PMA; Sigma-Aldrich Corp, USA). Adherent cells were washed three times with PBS before all subsequent experiments. The viability of cells was evaluated with hemocytometer and trypan blue (1% in HBSS), was always > 90 %, and was not statistically different between treatment and control groups.

2.3. Single cell gel electrophoresis (Comet) assay

THP-1 cells were seeded and differentiated in 24-well plates (Thermo Scientific, USA) at a density of 5×10^4 cells/well for the comet assay [24]. The adherent cells were exposed to PSNPs or nano-TiO$_2$-ARS at concentrations of 10, 100, and 500 µg ml^{-1} for 4, 8, 12, and 24 h. Cells that were exposed to hydrogen peroxide (H$_2$O$_2$, 10 µM) were used as positive control groups, and HBSS was used for vehicle control groups. Each treatment and control group was seeded in triplicate wells in two separate plates and experiment was repeated on at least two different days. After exposure, cells were harvested with 0.05 % trypsin-EDTA (Thermo Scientific, USA) and re-suspended in culture medium supplemented with 10 % FBS. Slides were pre-coated with 1% normal melting point agarose, coded to remove experimental group information (blind evaluation) and harvested cells were mixed with low melting point agarose before loading on the slide (Fisher Scientific, USA).

For alkaline comet assay, one slide was prepared from each well (three wells per each concentration at each time point per plate per day) and kept overnight at 4 °C in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH10) with 1% Triton X-100 (Sigma-Aldrich Corp, MO, USA). The slides were covered with freshly prepared electrophoresis buffer (1 mM EDTA sodium salt, 300 mM NaOH, pH > 13) for DNA unwinding and electrophoresis was performed at 0.7 V/cm and 400 mA at 4 °C for 30 min. The excess alkali was neutralized with Tris buffer (400 mM, pH 7.5), slides were air dried at room temperature, stained with 20 µg ml^{-1} ethidiumbromide (EtBr) and stored at 4 °C in a slide box until microscopy imaging and scoring. For pH neutral comet assay, the preparation of slides with agarose and sample was similar to the above. The slides were incubated overnight at 4 °C in lysis solution followed by DNA unwinding and electrophoresis in 4 °C TBE electrophoresis buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.5). Fifty comets per slide were scored with CometScore 2.0 software (TriTek Corp, USA). The intra-assay variation was determined between wells, plates and days of experiment within each treatment group, and no significant differences were noted.

2.4. Nanoparticles exposure and MicroRNA qPCR

PSNPs and nano-TiO$_2$-ARS were prepared as previously described. THP-1 cells were seeded and differentiated in the 12-wells plate (Thermo Scientific, USA) at a density of 1×10^5 cells per well and incubated with either PSNPs or nano-TiO$_2$-ARS at 10 µg ml^{-1} for 8 h. THP-1 cells were also exposed to PLGA nanoparticles (Phosphorex Inc, USA) or *E. coli* BioParticles (Thermo Scientific, USA) at 10 µg ml^{-1} for 8 h to determine expression profile of miRNAs activated during nanoparticle engulfment/phagocytosis and PAMP specific pro-inflammation

Table 1
MicroRNA specific primers used in our study.

MicroRNA	miRBase Accession number	Primer sequence (5′ – 3′)
hsa-miR-124-3p	MIMAT0000422	TAAGGCACGCGGTGAATGCC
hsa-miR-124-5p	MIMAT0004591	CGTGTTCACAGCGGACCTTGAT
hsa-miR-144-5p	MIMAT0004600	GGATATCATCATATACTGTAAG
hsa-miR-144-3p	MIMAT0000436	TACAGTATAGATGATGTACTAAA
hsa-miR-148a-5p	MIMAT0004549	AAAGTTCTGAGACACTCCGACT
hsa-miR-148a-3p	MIMAT0000243	TCAGTGCACTACAGAACTTTGT
hsa-miR-155-5p	MIMAT0000646	TTAATGCTAATCGTGATAGGGGT
hsa-miR-155-3p	MIMAT0004658	CTCCTACATATTAGCATTAACAA
hsa-miR-19a-5p	MIMAT0004490	AAAGTTTTGCATAGTTGCACTACA
hsa-miR-19a-3p	MIMAT0000073	TGTGCAAATCTATGCAAAACTGA
hsa-miR-223-5p	MIMAT0004570	CGTGTATTTGACAAGCTGAGTT
hsa-miR-223-3p	MIMAT0000280	TGTCAGTTTGTCAAATACCCCA

cascades, respectively.

Cells were harvested with TRI-Reagent (Thermo Scientific, USA) and miRNA was isolated by mirVana™ miRNA Isolation Kit (Thermo Scientific, USA) following manufacturer's instruction. The quantity and purity of the miRNA was determined with Spectra Max M5 microplate reader (Molecular Devices, USA). The reverse transcription of miRNA samples was performed by using Qiagen miScript II RT kit (Qiagen, Germany) following manufacturer's recommendation and 500 ng of miRNA was reverse transcribed into cDNA for each sample. Primers for each miRNA Quantitative real-time PCR (QPCR) reaction were composed by a Universal Primer provided by Qiagen miScript SYBR Green PCR kit (Qiagen, Germany) and a miRNA specific primers were designed by using Premier Primer 6.0 software (PREMIER Biosoft, USA). In total 12 miRNA primers were designed for 12 miRNAs' expression detection pre-selected with *in silico* analysis [22]. miRNA specific primer sequences are listed in Table 1.

miRNA qPCR was performed by Stratagene MX 3005 system (Thermo Scientific, USA), 2 μL miRNA reverse transcribed cDNA were added for a total volume of 25 μL and the following PCR protocol was started: denaturation step at 95 °C for 15 min, cycling program (95 °C, 15 s; 55 °C, 30 s; 70 °C, 30 s) for 40 cycles then goes melting curve analysis. U6 was selected as the housekeeping gene for each miRNA QPCR and the relative changes of miRNA expression level were analyzed by using the $2^{-\Delta\Delta Ct}$ method [25].

2.5. MicroRNA mimic transfection

The MicroRNA mimics for hsa-miR-155-5p and the negative control mimic were ordered from Sigma (Sigma-Aldrich Corp, USA) and diluted into a stock concentration of 10 μM. THP-1 macrophages were transfected with different concentrations of miR-155-5p mimic (0, 1, 5, 10 nM) for 24 h for optimization of mimic transfection concentration and 10 nM concentration was selected for further experiments. miR-155-5p mimic or miRNA negative control mimic (10 nM each) were transfected to pre-differentiated THP-1 macrophages with Lipofectamine RNAiMAX reagent (Thermo Scientific, USA). Transfection reagent was replaced with 1640 medium with FBS after 8 h of incubation. After 24 h, transfected cells were exposed to either PSNPs or nanoTiO2-ARS (10 μg ml^{-1} for 8 h). All samples were harvested with TRI-Reagent for downstream mRNA QPCR experiments.

2.5.1. Total RNA isolation and mRNA qPCR analysis

Total RNA extraction was performed according to the acid guanidinium thiocyanate-phenol chloroform extraction protocol using TRI-Reagent [26]. The concentration of the total RNA was identified by Spectra Max M5 microplate reader. Extracted mRNA was reverse transcribed into cDNA by reverse transcriptase and oligo-dT primer (Promega, Germany). The expression of two DNA damage biomarker genes (*ATM, ERCC1*) and two hsa-miRNA-155-5p was selected by *in silico* predicted targeting (http://www.targetscan.org/vert_72/), while

DNA damage-responsible genes (*TAOK1, TRIP13*) were identified by the mRNA QPCR. In addition to the above, three genes experimentally proven to be targeted by miRNA-155-5p while engaged in the DNA damage repair mechanism were selected: *WEE1, APAF-1* and *RAD51* [19,27,28]. The primers were designed by using Premier Primer 6.0 software and PrimerBank (https://pga.mgh.harvard.edu/primerbank/). Primers are listed in Table 2. *β-actin* was selected as the internal reference control gene and QPCR was performed in following steps: one cycle (95 °C, 10 min) and 40 cycles (95 °C, 30 s) each followed by 1 min at the gene-specific annealing temperature. Fluorescence signals were read at the end of each cycle and melting curve analysis was performed subsequently. Completed QPCR datasets were analyzed by using the $2^{-\Delta\Delta Ct}$ method comparative to the control of each group.

2.5.2. Statistical analysis

All data were presented as the mean ± standard deviation (SD). All treatments and controls were randomly assigned to different wells within a plate in triplicate. The Student *t*-test was used for paired or unpaired observations. One-way analysis of variance (ANOVA) was used for comparison between the mean values of groups. P-value of < 0.05 was considered to be statistically significant unless specified differently. SPSS software (IBM Corp, USA), R language package (GGplot 2) and Origin version 8.0 software (OriginLab, USA) were used for data analysis and figure drawing.

3. Results

3.1. Nanoparticle characterization

The PSNPs (stock solution in water, 25 mg ml^{-1}) with 50 nm in primary mean diameter holding a mean hydrodynamic diameter of

Table 2
Primers for mRNA Quantitative PCRs.

Primer Name	Primer sequence (5′ – 3′)
ATM - Forward	CCGCGGTTGATACTACTTTGACC
ATM - Reverse	GCAGCA GGGTGACAATAAACAAGTAA
ERCC1 - Forward	GGGAATTTGGCGACGTAATTC
ERCC1 - Reverse	GCGGAGGCTGAGGAACAG
TAOK1 - Forward	TGCACGAGATGTGCGTACC
TAOK1 - Reverse	TGTGTTCACGTAAATAACAGCCT
TRIP13 - Forward	ACTGTTGCACTTCACATTTTCCA
TRIP13 - Reverse	TCGAGGAGATGGGATTTGACT
β-actin - Forward	CATGTACGTTGCTATCCAGGC
β-actin - Reverse	CTCCTTAATGTCACGCACGAT
WEE1- Forward	ATTTCTCTGCGTGGGCAGAAG
WEE1 - Reverse	CAAAAGGAGATCCTTCAACTCTGC
Apaf-1 - Forward	GGGTTTCAGTTGGGAAACAA
Apaf-1 - Reverse	CACCCAAGAGTCCCAAACAT
RAD51 - Forward	CAGTGATGTCCTGGATAATGTAGC
RAD51 - Reverse	TTACCACTGCTACACCAAACTCAT
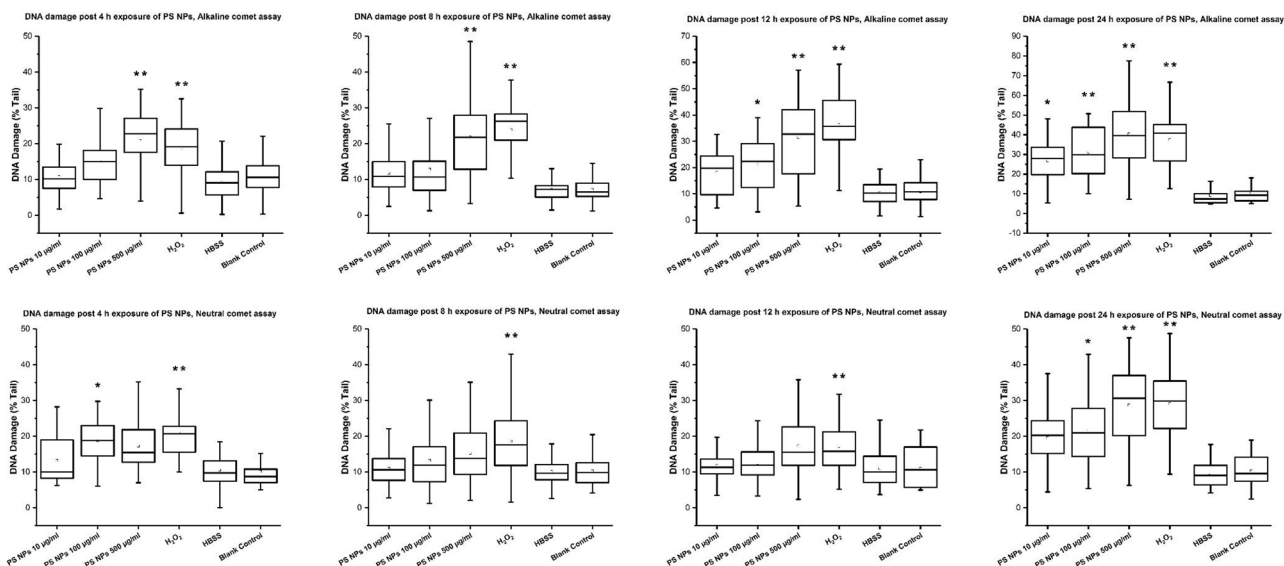
Fig. 1. Time and dose dependent DNA damage profile induced by Polystyrene Nanoparticles. DNA damage was evaluated by comet assay in alkaline and neutral pH environment ($100 < n < 200$ comets per group). Asterisk (*) indicates $p < 0.05$, double asterisks indicate $p < 0.01$. All data compared to untreated control.

33.68 ± 4.47 nm with a single peak at 37.88 nm was used. The nano-TiO_2-ARS (5 mg ml^{-1}, HBSS dissolved) was characterized with a size distribution of 43.49 ± 19.26 nm. The zeta potential of PSNPs was -69.30 ± 2.44 mV while the zeta potential of nano-TiO_2-ARS was -3.23 ± 1.19 mV. Characterization of PSNPs indicated low aggregation and nano-TiO_2-ARS indicated the higher aggregation preference (data not shown).
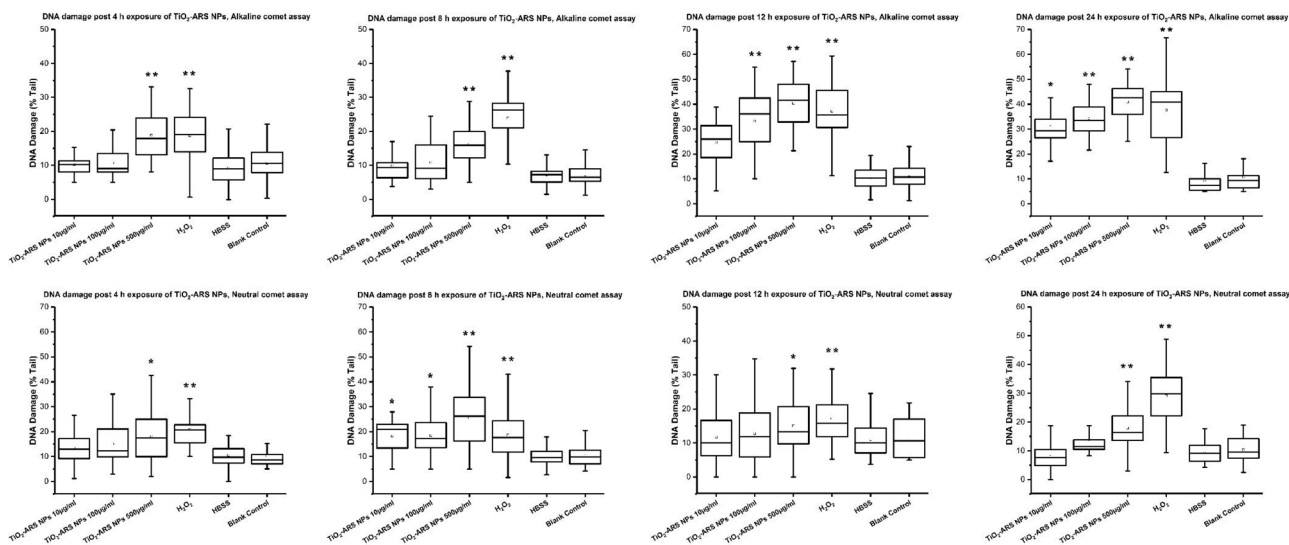
3.2. Time and dose dependent DNA damage profiling post nanoparticle exposure

Total of 3622 images were analyzed with the comet assay analytical software to establish *in vitro* DNA damage profile in THP-1 monocytic cell line after different time and dose exposures to PS NPs or nano-TiO_2-ARS. The percentage of comet "Tail" (% tail DNA) was referred to as the quantitative DNA damage parameter for comet assay in both alkaline and neutral conditions. The complete DNA damage profile is presented in Figs. 1 and 2.

Increased DNA damage was observed post 24 h incubation with 10 µg ml^{-1} PSNPs in alkaline comet assays. As for the cells treated with 100 µg ml^{-1} of PSNPs, the observable significant tail percentage increase ($p < 0.05$) was detected for the first time at 12 h post exposure, with significance level observed at $p < 0.01$ at 24 h of exposure in the alkaline comet assay. The only time point when a significant DNA damage occurred in the neutral comet assay for 100 µg ml^{-1} PSNPs exposed cells was at 24 h post exposure. Post PSNPs exposure at a concentration of 500 µg ml^{-1}, severe DNA damage ($p < 0.01$) was detected at all exposure time points (4 h, 8 h, 12 h and 24 h) in the alkaline comet assay comparing to the blank control, while the significant tail content increase was detected at 24 h of exposure in the neutral comet assay.

Dose of 500 µg ml^{-1} of nano-TiO_2-ARS was able to induce significant comet tail percentage increase at every exposure time point in both alkaline and neutral comet assays (Fig. 2). Concentration of 100 µg

Fig. 2. Time and dose dependent DNA damage profile induced by ARS labeled Titanium Dioxide Nanoparticles. DNA damage evaluated by comet assay in alkaline condition and neutral pH environment ($100 < n < 200$ comets per group). Asterisk (*) indicates $p < 0.05$, double asterisks indicate $p < 0.01$. All data compared to untreated control.
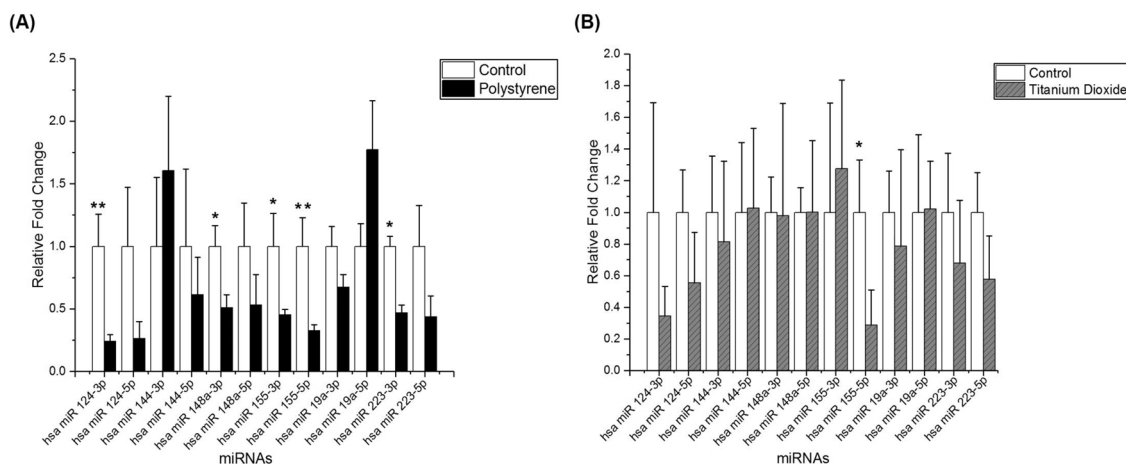
Fig. 3. Relative expression level of selected miRNAs in THP-1 Human monocytic cell line after the exposure of Polystyrene nanoparticles and ARS labeled Titanium Dioxide Nanoparticles. Relative expression level of all groups compared with the control. Single asterisk (*) indicates $p < 0.05$, double asterisks (**) indicate $p < 0.01$.

ml^{-1} of nano-TiO$_2$-ARS induced a severe SSB at both 12 and 24 h post exposure while significant DSB was observed only at 8 h post exposure. For nano-TiO$_2$-ARS exposure at concentration of 10 μg ml^{-1}, a significant tail content increase occurred at 24 h post treatment in the alkaline comet assay and 8 h post-treatment in the neutral comet assay.

3.3. MicroRNA expression after nanoparticles exposure

Six miRNA-mRNA regulation networks were constructed using *in silico* analysis approaches and data summary and six zebrafish miRNA categories were selected as most promising candidates for further studies: dre-miR-124, -144, -148, -155, -19a, -223 [22]. *In vitro* expression profile of these six inter-species conserved miRNAs was analyzed post PSNPs or nano-TiO$_2$-ARS exposure using human THP-1 monocytic cell line. The relative expression level of these miRNAs compared with control is presented in Fig. 3. Post 8 h exposure of PSNP (10 μg ml^{-1}) five mature miRNA strands showed significantly down-regulated expression compared to control: hsa-miR-124-3p, -148a-3p, -155-3p, -155-5p and -223-3p ($p < 0.05$). Among them, miR-124-3p and miR-155-5p were most significantly down-regulated ($p < 0.01$). Expression of miR-144-3p and miR-19a-5p was elevated, but not significantly up-regulated post PS NPs exposure.

After exposure to nano-TiO$_2$-ARS, miRNAs expression patterns suggested that half of the investigated mature miRNA strands were not significantly different from the controls (miR-144-3p, -144-5p, -148a-3p, 148a-5p, -19a-3p and -19a-5p) and a non-significant elevation trend was observed in miR-155-3p. The exposure to nano-TiO$_2$-ARS significantly down-regulated only miR-155-5p expression.

Based on the results from [22], the expression profile of top six predicted miRNA after exposure to nanao-TiO$_2$-ARS and PSNPs was determined. Only miR-155-5p was significantly down-regulated after exposure to both nanoparticle types. We also identified the miRNA expression patterns after stimulation of cells with PLGA nanoparticles, and *E. coli* BioParticles, in order to exclude miRNAs that functionally engaged in endocytosis (PLGA) or pro-inflammatory (BioParticles) responses. The miRNA expression profiles after the exposure to all four particle types (PSNP, nano-TiO$_2$-ARS, PLGA, and BioParticles) was presented as a heat-map in Fig. 4. miR-155-5p was the only miRNA biomarker candidate that was not significantly affected after the exposure of THP-1 cell line to PLGA nanoparticles and *E. coli* BioParticles, and at the same time it was down-regulated by PSNPs and nano-TiO$_2$-ARS. The results supported that miR-155-5p is the best candidate for functional validation (Fig. 4).

3.4. DNA biomarker expression and potential miR-155-5p targeting genes expression

As hsa-miR-155-5p appears to be a potential biomarker for PSNPs and nano-TiO$_2$-ARS (Fig. 4), miRNA functional study was performed by transfecting miR-155-5p mimic to simulate miRNA overexpression in THP-1 cell line. The result from our preliminary experiment indicated that the miR-155-5p was significantly up-regulated after transfection with 10 nM mimic, compared to the negative control mimic (data not shown). Then, we checked the expression of three categories of genes: 1) DNA damage biomarker genes (*ATM, ERCC1*), 2) genes *in silico* predicted to be targeted by miR-155-5p and responsible for DNA damage repairing (*TAOK1, TRIP13*), and 3) experimentally validated miR-155-5p target genes engaged in a variety of DNA damage repairing processes (*WEE1, APAF-1* and *RAD51*). The expression of selected genes was measured in the following conditions: 1) THP-1 cells exposed with PS NPs or nano-TiO$_2$-ARS; 2) THP-1 cells transfected with miR-155-5p mimic; and 3) Mimic transfected cells with the exposure of PS or nano-TiO$_2$-ARS. All data were presented as relative expression fold change comparing to their control. Post nano-TiO$_2$-ARS stimulation, five out of seven genes were significantly affected in expression levels while only *TAOK1* was significantly up-regulated post PS NPs exposure (Fig. 5). This result may indicate that DNA damage repairing (DDR) processes are initiated by nano-TiO$_2$-ARS, but not by PSNPs.

TAOK1, TRIP13, WEE1, APAF-1 and *RAD51* are either experimentally proven, or predicted with *in silico* analysis to be targeted by miR-155-5p. In our experiment, a majority of these genes are significantly ($p < 0.01$) suppressed in their expression level after the transfection of miR-155-5p mimic (*TAOK1, WEE1, ATM, TRIP13* and *ERCC1*) and the rest of the genes show overall down-regulation trend ($p < 0.05$) except *RAD51* (Fig. 6). Moreover, significant gene expression suppressions were observed in *ATM* and *ERCC1* post miR-155-5p overexpression. This finding is interesting, because the relations between miR-155-5p and these two genes were not reported previously. Our results suggest that miR-155-5p, may be directly targeting *ATM* and *ERCC1*, and could also be a part of the network to regulate the function of these genes. On the other hand, strong activation of *ATM, TAOK1, TRIP13, APAF-1* and significant attenuation of *ERCC1* was detected in miR-155-5p mimic transfected cells post PS NPs exposure while significant up-regulation of *ATM, APAF-1* and *RAD51* was found in transfected cells after TiO$_2$-ARS NPs (Fig. 7). The different gene expression profiles between mimic transfected and mimic un-transfected cells post NPs exposure indicate significant engagement of miR-155-5p in the NPs induced adverse outcomes such as DNA damage.
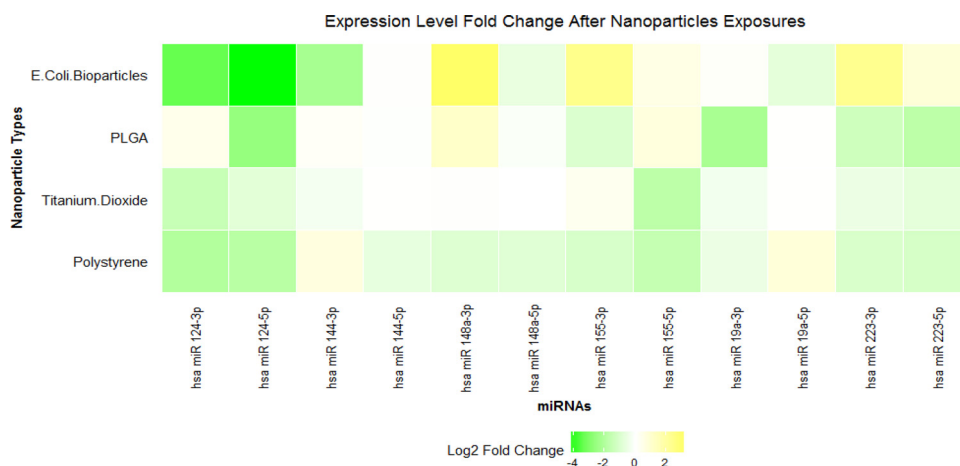
Fig. 4. Expression alteration of selected miRNAs after exposure of Polystyrene nanoparticles, ARS labeled Titanium dioxide nanoparticles, PLGA nanoparticles and *E. coli* bioparticles in THP-1 cell line. Relative expression level presented as the Log_2 Fold Change comparing with control in our heat-map.

4. Discussion

The potential of nanoparticles to possibly induce DNA damage has been recently raising attention since they are increasingly used in cosmetics and pharmaceuticals. One of the major categories of NPs that are of high concern is the titanium dioxide (TiO_2) NPs due to their observed potential for triggering carcinogenicity [29–31]. However, the genotoxicity of TiO_2 NPs remains controversial as EFSA panel concluded that nano-TiO_2 is unlikely to raise genotoxic concern *in vivo* as a food additive even though direct aggregation of nano-TiO_2 around the cell nuclei was observed in the vicinity of the endoplasmic reticulum *in vitro* [32,33]. Moreover, another type of nanoparticle that is used extensively are nano-sized plastic beads, majority of them with polystyrene core (PSNPs). The genotoxicity induced by PSNPs is rarely reported and the toxicity of PSNPs is not fully understood [6,18]. In present study, we used comet assay to determine if different exposure times and doses of both TiO_2 and PS nanoparticles could cause significant DNA damage in attempt to reduce the knowledge gap regarding the PSNPs genotoxicity and also further investigate mutagenesis potency of the nano-TiO_2 with and without fluorescent dye (ARS) labeling.

Nano-TiO_2-ARS nanoparticles could generally induce a dose and exposure time dependent DNA strand damage as shown with comet assay (Fig. 2) and in concert with majority of studies [34,35]. Starting at 4 h post exposure (hpe), 500 µg ml^{-1} TiO_2-ARS NPs (ARS labeled)
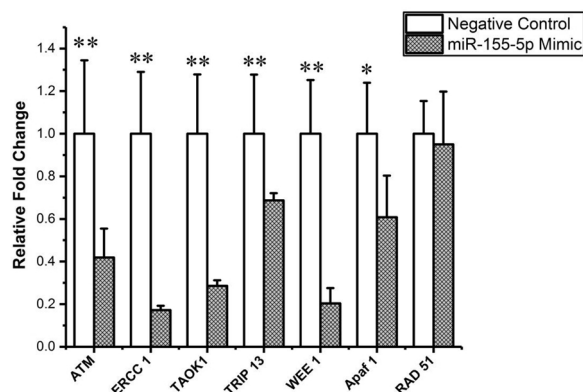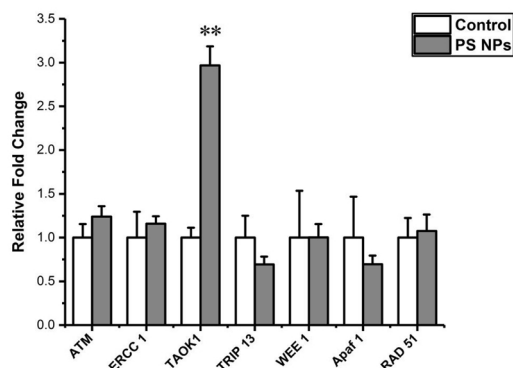

Fig. 6. The expression alteration of DNA damage repairing genes after miR-155-5p transfection in THP-1 cells. Negative control indicates the negative control mimic. All expressions of genes were comparing with the negative control transfected group. Single asterisk (*) represents $p < 0.05$, double asterisks (**) indicate $p < 0.01$.

induced significant increase in both single strand DNA (ssDNA) and double strand DNA (dsDNA) damage, and severe ssDNA damage was also observed post 100 µg ml^{-1} nano-TiO_2-ARS exposure after 12 h (Fig. 2). Similarly, exposure to nano-TiO_2 caused significant ssDNA
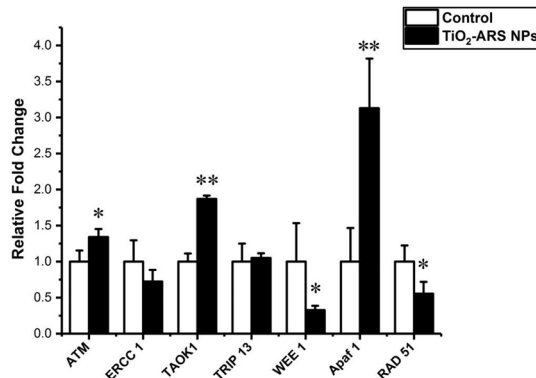
Fig. 5. DNA damage repairing responsible gene expression alteration post the exposure of PSNPs or nano-TiO_2-ARS in THP-1 cells. These genes are DNA damage biomarkers (*ATM, ERCC1*), DNA damage repairing genes that are *in silico* predicted targeted (*TAOK1, TRIP13*) or experimental proved targeted (*WEE1, APAF-1* and *RAD51*) by hsa-miR-155-5p. Single asterisk (*) represents $p < 0.05$, double asterisks (**) indicate $p < 0.01$.
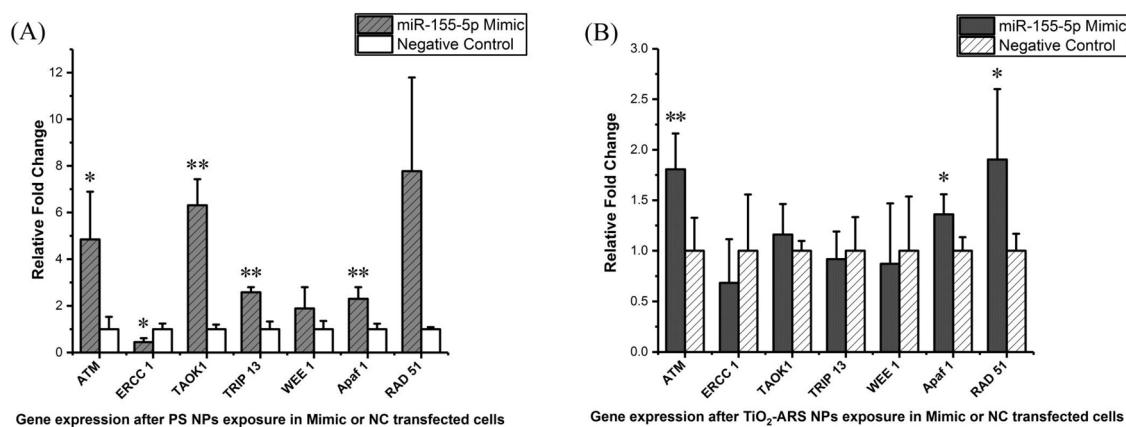
Fig. 7. Expression changes of DNA damage repairing genes after miR-155-5p transfection in miR-155-5p mimic transfected cells or negative control transfected cells. Mimic stands for the miR-155-5p mimic and NC stands for the negative control. Single asterisk (*) represents $p < 0.05$, double asterisks (**) indicate $p < 0.01$.

fragmentation at a concentration of 25 μg ml^{-1} at 3 hpe [36]. For dsDNA breaks, our pH neutral comet assay detected that the most severe DNA damage was observed at 8 hpe at 10 and 100 μg ml^{-1} of nano-TiO$_2$-ARS (Fig. 2). This finding is supported by significant dsDNA damage after 3 h of 20 μg ml^{-1} of nano-TiO$_2$ exposure reported by Saquib et al. [37]. The concentration used in the experiments is comparable to concentrations found in animal models after parenteral or dietary exposures to equivalent nano-TiO$_2$ amounts found in consumer products such as food additives. Furthermore, the bioaccumulation of environmentally relevant nano-TiO$_2$ concentrations is reported to reach the organismal levels within ranges used in the above experiments, for details see [38].. Therefore, these results indicate that tested concentrations of nano-TiO$_2$-ARS and unlabeled nano-TiO$_2$have similar potential to induce DNA damage *in vitro,* and can be relevant for health and environmental risk assessments.

PSNPs were shown to induce a significant ssDNA damage starting from 4 h post exposure (hpe) and a significant dsDNA damage at 24 hpe was observed in the highest concentration tested (500 μg ml^{-1}) (Fig. 1). This concentration of PSNPs is not environmentally relevant, however, due to bioaccumulation and bioconcentration processes it can reach the levels within an organism that are high enough to induce the cellular injury including the ssDNA damage and the double strand DNA damage [39,40], and in Fig. 1. The dose of PS NPs of 100 μg ml^{-1} induced ssDNA damage from 12 hpe and dsDNA damage from 24 hpe. This phenomenon implies that PSNPs are able to induce a severe DNA damage in both manners of SSB and DSB at a high dose towards the THP-1 cell. However, this result is different from the previous study of Paget et al. [6] where non-functionalized PSNPs in concentration of 8.1 μg cm^{-2} could not induce a significant raise of γ-H2Ax-foci counts in THP-1 cells except at 1 hpe [6]. One of the possible reasons for this discrepancy is that nanoparticle genotoxicity is generally dose dependent, and the concentration of 8.1 μg/cm^2 is considerably lower than our 100 μg ml^{-1} tested dose [41,42].

Interestingly, low concentration exposure with PSNPs (10 μg ml^{-1}) did not induce a significant increase of the DNA damage during pH neutral comet assay, but did induce a significant increase of DNA damage level in the alkaline comet assay environment (Fig. 1). This result may indicate that PSNPs had higher ability to trigger the DNA single strand damages, rather than double strand breaks, as the alkaline comet assay primarily detects SSBs (Single Strand Breaks) that are directly induced or formed as a result of BER (Base pair excision repair) and alkali labilization of AP sites while the comet assay in neutral conditions allows for the detection of DNA double-strand breaks (DSB) [43,44]. However, as this result differs from the well-known low-toxicity that this kind of nanoparticles could potentially induce, other possible explanations should be considered [18,45]. During incubations

prior to the comet assay, "Naked DNA" is exposed due to the degradation of nuclear membranes, while the cell internalized nanoparticles remain and persist in the lysosome [46], and it was reported that interaction between nanoparticles and the DNA outside of the cell environment results in additional DNA strand breaks [47].

Most studies investigating potential genotoxicity induced by polystyrene nanoparticles or micro-particles used γ-H2Ax-foci assay or transcriptomic analysis, but not comet assay [6,16], even though comet assay is directly quantifying the amount of breaks occurring in the DNA [48]. When comet assay was used to test PSNPs genotoxicity, functionalized PSNP concentration of 50 μg ml^{-1} applied for 48 h exposure time caused significant DNA damage increase in HeLa cells, similar to our study [49]. Those authors were also questioning the use and reliability of comet assay in the mutagenesis evaluation of nanoparticles, and it was suggested that the outcome of comet assay could suffer from direct interference of NPs and DNA, as strong interaction was indicated [49].

To further investigate possible issue with comet assay use, we analyzed the expression of DNA damage biomarker genes to compare the results of comet assay and the DNA damage post exposure to nano-TiO$_2$-ARS and PSNPs. In the follow-up gene expression experiments, DNA damage repairing related genes were not significantly activated after the stimulation with PSNPs. This appears supportive to concerns of other authors about use of the comet assay in assessment of nanoparticles induced genotoxicity. However, our study also revealed that *TAOK1* (thousand and one kinase 1) was strongly activated after the stimulation of PSNPs, and since TAOK1 is involved in the activation of JNK pathway in response to DNA damage [50], we can't completely exclude the possibility that PSNPs can cause DNA damage in living cells, and that results of the comet assay can at least partially be attributed to the DNA damage caused by PSNPs.

It has been reported that the expression of miRNA could be altered either by engaging in the endocytosis or by the pro-inflammatory responses that induced following the phagocytosis of PAMPs (Pathogen Associated Molecular Patterns) in monocytes and their differentiated macrophages [51]. In attempt to exclude potential interference of pro-inflammatory reactions following PAMP related phagocytosis, an additional experiment was performed by exposing the THP-1 cells with a type of nanoparticles that could trigger endocytosis but without induction of pro-inflammatory responses or toxicity responses (PLGA nanoparticle) [52]. Similarly, to exclude possible PAMP derived miRNA induction, a bacterial derived bio-particle (*E. coli* BioParticles) was used. So combining all the existing results, hsa-miR-155-5p shows the most promise to be used as possible biomarker of regulatory mechanisms activated by PSNPs and nano-TiO$_2$-ARS.

MicroRNAs normally regulate mRNA function through direct

binding with mRNAs and formation of the RNA-induced silencing complex, effectively suppressing the transcription process [53]. Transfection of miRNA mimics into the cell is used to simulate an over-expression of mature miRNA strands [54]. In our experiment, the transfection of miR-155-5p mimic lead to significant down-regulation of the genes that were either experimentally proven by others, or predicted *in silico*, to be targeted by this miRNA [22]. Our results strongly suggest that miR-155-5p has regulatory function in expression of these genes.

Significant induction of *ATM, TAOK1, TRIP13* and *APAF-1* was detected in miR-155-5p over-expressed cells post PSNPs exposure while strong up-regulation of *ATM, APAF-1* and *RAD51* was found in miR-155-5p mimic transfected cells after exposure to nano-TiO$_2$-ARS. This phenomenon not only implies that miR-155-5p regulated these genes after exposure with nano-TiO$_2$-ARS or PSNPs but also indicates that existence of miR-155-5p is necessary for regulation of multiple DNA damage repairing processes that were induced by nano-TiO$_2$-ARS, or potentially triggered by PSNPs. One example is the involvement of APAF-1 (apoptotic protease activating factor 1) in regulation of DNA damage-induced apoptosis by acting as a p53 downstream factor [55]. In addition, miR-155-5p was identified to target *APAF-1* supporting its engagement in DNA damage regulation and apoptosis *via* mitochondrial apoptotic pathway [28]. In our experiment, the expression of *APAF-1* was elevated in miR-155-5p over-expressed cells after both nano-TiO$_2$-ARS and PSNPs exposure, suggesting that activation of APAF-1 apoptotic pathway could be induced by different nanoparticles *via* interaction between miR-155-5p and *APAF-1*.

5. Conclusion

The role of nano-TiO$_2$-ARS in DNA damage was supported with results of time and dose dependent DNA damage assessment taken together with DNA damage repairing related genes expression profile. However, the role of polystyrene nanoparticles as possible causative agent of DNA damages remained unclear. Over-expression of miR-155-5p in PSNPs treated cells increased expression of *ATM, TAOK1, TRIP13,* and *APAF-1*, and attenuated the expression of *ERCC1* while *ATM, APAF-1* and *RAD51* were strongly activated post nano-TiO$_2$ARS exposure in miR-155-5p mimic-transfected cells. We conclude that there is significant potential of miR-155-5p to be used as a biomarker of nano-particle induced toxicity. Possible future applications include research and regulatory toxicology in different levels of biological complexity, becoming a valuable tool in One Health approaches to monitor potential nanoparticle effects.

CRediT authorship contribution statement

Moyan Hu: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Visualization. **Dušan Palić:** Conceptualization, Methodology, Writing - review & editing, Validation, Supervision, Resources.

Declaration of Competing Interest

The authors declare no conflict of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.05.007.

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