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Unmodified Polystyrene Nanoparticles Induce Inflammatory and Oxidative Stress Responses in Human Lung Epithelial Cells

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Abstract

Polystyrene (PS) is one of the commercially used polymers considered as a pollutant in both aquatic and terrestrial environments. Moreover, PS-NPs are used in nanosensors and nanocarrier for the drug. Thus, it is crucial to determine the potential toxicological effect of PS-NPs to cells. Here we used varying concentrations of unmodified PS-NPs of 60±12 nm in size and analyzed their cytotoxic effect and stress and toxicity gene expressions in NCI-H292 cells. NCI-H292 cells were exposed to minimum of 5 µg/mL and maximum of 50 µg/mL of PS-NPs; 50 µg/mL of PS-NPs showed about 50% cell viability. Moreover, unmodified PS-NPs and induce expressions of stress-related genes, such as those encoding interleukin 6 (IL 6), colony-stimulating factor 2 (CSF 2), C-X-C motif chemokine 10 (CXCL 10) responsible for inflammation and heme oxygenase 1 (HMOX 1) responsible for oxidative stress in NCI-H292 cell lines. Our findings suggest that PS-NPs are considered as a potential toxicological compound to the human cell line.

Keywords: Nanoparticles, Polystyrene, Nanotoxicology, Lung epithelial cells, gene expression, inflammation

1. Introduction

Nanoparticles (NPs) possess 1 to 100 nm in diameter at least in one dimension [1]. The unique characteristics of NPs such as their nano size and high surface area avail their applications in numerous areas like electrical [2], agricultural [3], pharmaceutical [4], and medical fields [5, 6]. However, limited information is available on their impact on human health and the environment. Thus, necessary consideration is to be made to identify the potential toxicity of NPs to both cells and organisms. Moreover, it has been suggested that the nanosize and huge surface area are the major determinants of NP toxicity [7]. The complete understanding of nanomaterials interaction with cells necessitates the proper evaluation of NPs and their safety measures to cells and organisms.

The size of the NPs is considered as crucial criteria to determine its toxicity. For example, bulk gold is suggested to be safe, however, gold nanoparticles (Au NPs) are potentially toxic and accumulated in blood and other tissues. For example, Au NPs with diameters of 1.4 nm was accumulated in the nucleus and bind to DNA and then provoked mitochondrial damage, necrosis and oxidative stress in melanoma cells [8]. Another report states that Au NPs between 8 and 37 nm causes severe toxicity and death in mice within three weeks of administration [9]. Besides, larger titanium dioxide NPs (TiO_2 NPs) aggregates are

considered more cytotoxic compared to smaller ones [10] eventually the cytotoxicity of TiO_2 NPs could be reduced by surface modification with polyethylene glycol [11]. The mode of incorporation of NPs is another crucial aspect to resolve its toxicity. The absorption of ultrafine particles in the gastrointestinal tract stimulates phagocytosis [12]. Intravenous administration of Au NPs accumulated in lungs, liver and the spleen *in vivo* mouse model [13].

Polystyrene (PS) is considered as a hardly biodegradable polymer commercially used in the manufacturing of laboratory equipment and also in the food and medical industry. Over the past decade, the economic usage of PS increases steadily. Recently, polystyrene nanoparticles (PS-NPS) of 50-100 nm in size focused on its applications in numerous areas such as in optical sensor, drug carrier, food storage and paint coating due to their nano size, spherical shape, and uniform particle size. Depend on the surface coating with functional groups, PS-NPs are categorized into cationic (-NH2), neutral (unmodified) and anionic (-COOH) surfaces. For drug delivery application, the surface coating method was utilized to penetrate PS-NPs through the cell membrane. However, positively charged PS-NPs showed a higher level of absorption and internalization than anoinic or neutral PS-NPs [14].

Jangsun et al. [15] reported that 50 nm PS-NPs showed high blood brain barrier penetration and disturb nervous system compared with 100 nm PS NPs. Recently, Jeong et al [16] reported that PS particles of 10–100 μm were not toxic to human cells. However, small PS particles of 460 nm and 1 μm affected RBCs. Jeong et al. [17] reported that PS microparticles of 10 μm causes neurotoxicity by induces seizure-related genes such as *c-fos* and *pvalb5 in* zebrafish embryos. On the other hand, the aggregation behavior of PS-NPs determines the localization of the NPs in mice models. For example, less aggregated particles might have been localized on the liver and strongly aggregated particles distributed throughout the organ [18].

This study showed the effects of unmodified PS-NPs (60±12 nm) both at cellular and gene expression levels. We carried out cell viability test and analysis of mRNA expression in human lung epithelial cell lines, NCI-H292 to assess the toxicity induced by PS-NPs. Our results indicate that PS-NPs reduced cell viability as well as induced inflammatory and oxidative stress-related mRNA in NCI-H292 cells. Based on our findings, we suggest that PS-NPs is considered as a potential toxicological compound to human cell line.

2. Materials and Methods

2.1. Synthesis and Characterization of Polystyrene Nanoparticles (PS-NPs)

Polystyrene nanoparticles were synthesized via emulsion polymerization technique according to the previous report [19]. Briefly, purified styrene, divinylbenzene (DVB), sodium dodecyl sulfate (SDS) were added into distilled water maintained at a constant temperature. The polymerization process was initiated by adding potassium persulfate (KPS) with nitrogen purging in a 3- neck round flask at 70ºC for 5 h. The mixture was then cooled down followed by irradiation with sonication processor probe for 15 minutes.

The average particle size was analyzed by Delsa™ nanoparticle analyzer (Beckman Coulter, Inc., CA, USA). The particle morphology was obtained using a scanning electron microscope (SEM, HITACHI S-4800, Japan) operated with 5- 25 kV.

2.2. Cell Culture

Human lung epithelial cells, NCI-H292 were cultured in RPMI 1640 medium (Invitrogen) contains 10% fetal bovine serum (FBS, Biowest), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

The cells were maintained under 5% CO_2 and humidity at 37°C.

2.3. Cell Viability Assay

For cell viability, the cells were seeded at a density of 1.0×10^4 NCI-H292 cells per well in 96-well cell culture plate. After 24 h incubation, the cells were exposed with PS-NPs of different concentrations ranging from 0.5 µg/mL to 50 µg/mL at the volume ratio of 5:100 (PS-NPs: medium) and incubated for 6 and 24 h. Cell viability was determined depend on the concentration of cytoplasmic ATP using a Cell titer-Glo luminescent cell viability assay kit (Promega, W.I, USA) with a luminescent cell viability assay reader.

2.4. Gene Expression Analysis

2.4.1. PCR Array

For PCR array, 1.3×10^5 NCI-H292 cells/cm^2 were seeded in a cell culture dish and exposed to a suspension of PS-NPs at a final concentration of 25 µg/mL. The cells were collected after 6 h followed by wash with PBS and the expressions of 84 genes were analyzed for human stress and toxicity using PCR array. Briefly, total RNA was extracted by RNeasy Kit (Qiagen, Japan) and purified with DNaseI (Takara, Japan) to remove DNA contamination. cDNA was synthesized from the purified RNA (4 µg of total RNA) with random hexamer primer by PrimeScript II 1^{st} strand cDNA Synthesis kit (Takara). A standard reaction was also maintained the same 96-well plates having forward and reverse primers for few genes whose presence is an indication of stress and toxicity (SA Bioscience, Qiagen). A control reaction was also performed using cDNA prepared from cells that were not exposed to NPs. PCR array was performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Singapore).

2.4.2. Quantitative Real-time (q-RT) PCR

For mRNA expression analysis, 1.3×10^5 NCI-H292 cells/cm^2 were seeded in cell culture dishes and treated with PS-NPs at a concentration of 25 µg/mL for 6 h. The cells were the cells after the incubation period and washed with PBS. The expression of marker genes was determined by quantitative real-time PCR (qRT-PCR). For that, extraction of RNA was carried out and cDNA was synthesized as mentioned in the previous section. Quantitative real-time PCR (qRT-PCR) was performed with SYBR Premix Ex Taq II (Takara) with an ABI PRISM 7000 sequence detection system (Applied Biosystems) in the following condition. The thermocycling conditions were 95ºC- 30 s, 95ºC- 5 s and 60ºC- 34 s for 40 cycles. Normalization of data were done with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control in the same reaction. The following primers were used for this study (Table 1).

Table 1: List of primers were used for gene expression analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	CCCCCACCACACTGAATCTC	GCCCCTCCCCTCTTCAAG
CCL 4	GCTTCCTCGCAACTTTGTGGTAG	GGTCATACACGTACTCCTGGAC
CRYAB	CTTTGACCAGTTCTTCGGAG	CCTCAATCACATCTCCCAAC
CSF 2	TCTCAGAAATGTTTGACCTCCA	TCTGTGCCTGCAGCTTCGT
CXCL 10	GAACTGTACGCTGTACCTGCA	TTGATGGCCTTCGATTCTGGA
HMOX 1	GGGTGATAGAAGAGGCCAAGA	AGCTCCTGCAACTCCTCAAA

HSP70B'	CCGGCCCCATCATTGAG	CCCATAGCATAGCCCTGACAGT
IL 6	TGAGTACAAAAGTCCTGA	GCCCTTGAGC TTGGTGAG

2.5. Statistical Analysis

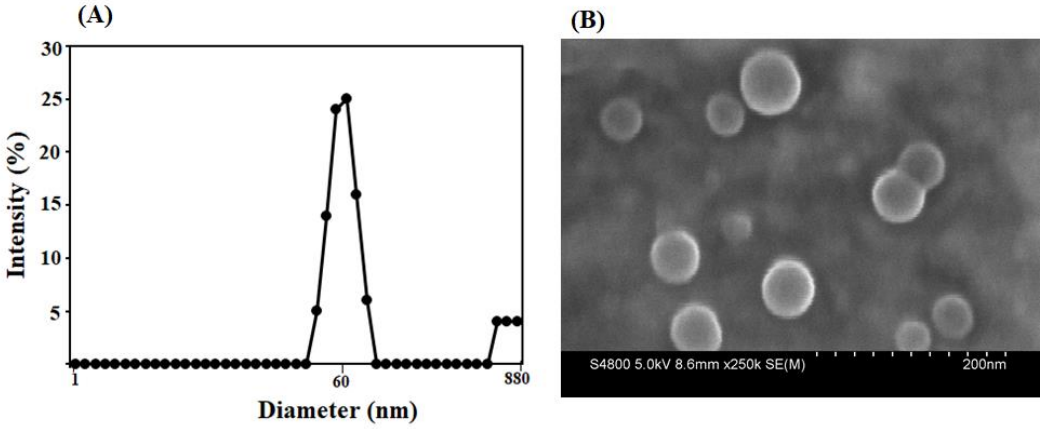
Data were expressed as mean ± SD, (n = 3). The significance of differences between the treated groups and control groups were analyzed by Student t-tests (Graph Pad, California, US).

3. Results and Discussion

3.1. Characterization of PS-NPs

PS-NPs suspension was diluted in distilled water at a final of 0.5 mg/mL and mixed by thorough shaking for particle size analysis. The unmodified PS-NPs possess the size of 60±12 nm (Fig. 1. A) with a polydispersity index (PDI) = 0.19 ± 0.05. Three independent DLS experiments were carried out to obtain the average value. The zeta-potential of PS-NPs in distilled water showed the value of +42.3 ± 2 mV. The morphology of PS-NPs was analyzed by SEM has shown in Fig.1. B. possesses spherical morphology. The results indicate that unmodified PS-NPs are having similar in their particle size and shape.

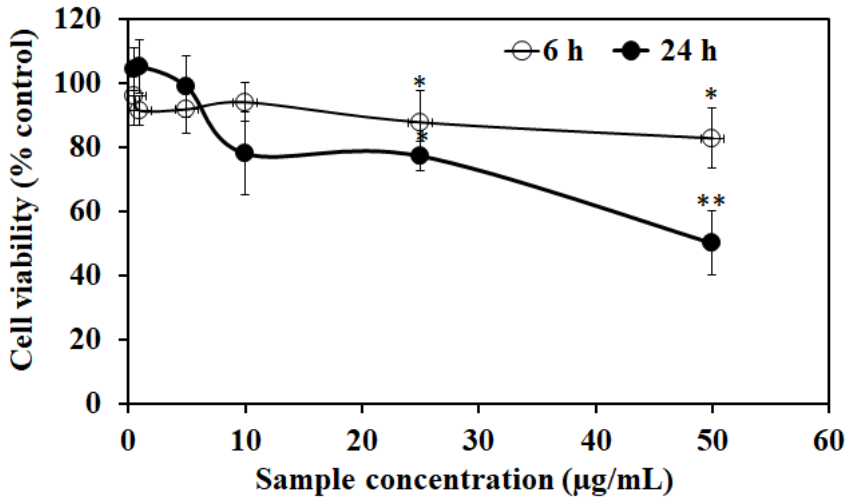
Figure 1: Characterization of unmodified PS-NPs. (A) PS-NPs size distribution by dynamic light scattering (DLS) and (B) morphology of PS-NPs by scanning electron microscopy (SEM).



3.2. Viability of NCI-H292 Cells Exposed to PS-NPs

The effect of unmodified PS-NPs to NCI-H292 cells were understood by analyzes the viability of cells after the exposure of PS-NPs. To understand the concentration-dependent cytotoxic effect of PS-NPs, we treated the cells with various concentrations of PS-NPs and analyzed the cell viability at 6 h and 24 h. About 10% and 50% of the cells were losses its viability at a high concentration of PS-NPs (50 µg/mL) at 6h (open circles) and 24 h (closed circles) respectively (Fig. 2). On the other hand, the viability of the cell increases by a decrease in the concentration of PS-NPs. This suggests that PS-NPs affect the cell in a concentration-dependent manner. It is suggested that the loss of cell viability is due to the exposure of high concentration of PS-NPs (50 µg/mL) may induce inflammation and oxidative stress in the human lung epithelial cells.

Figure 2: Cell viability of NCI-H292 cells exposed with indicated concentrations of PS-NPs for 6 h (open circles) and 24 h (closed circles). Each data represents mean± SD, n=3 for each concentration. *p = 0.05, **p = 0.01.



Recently, Philip et al. [20] reported cell-dependent cytotoxic effect of 20 nm-sized carboxyl-modified PS-NPs which induced less cytotoxic effect in HaCaT cells than HeLa cells. Another report states that aminated polystyrene amine nanoparticles (AmPs NPs) increase the intracellular reactive oxygen species (ROS) production, cell cycle arrest at G2/M phase results in cell death by caspase-mediated apoptosis in HeLa cells [21]. Amine modified PS-NPs were absorbed to the cell surface of the living yeast cell and subsequently cell death [22]. Studies on the exposure of 50-100 nm of unmodified, amine-modified and carboxyl-modified PS latex NPs induces platelet aggregation and increased cardiovascular risk [23,24]. Our result suggests that less concentration of PS-NPs are safe to use for human cell lines.

3.3. mRNA Expression Analysis of PS-NPs Exposed Cells

To identify potential biomarkers of PS-NPs toxicity, we conducted a human stress and toxicity pathway finder PCR array analysis for 84 genes whose presence is an indication of stress and toxicity. Some of the genes were identified as candidate biomarkers whose expression is up or down- regulated are CCL 4, CCNG1, CRYAB, CSF 2, CXCL 10, HMOX 1, HSP70B', IL 6, LTA, TNF, and UGT1A4 (Table 1). Among those genes, only up-regulated genes were selected as the candidate biomarker for further study.

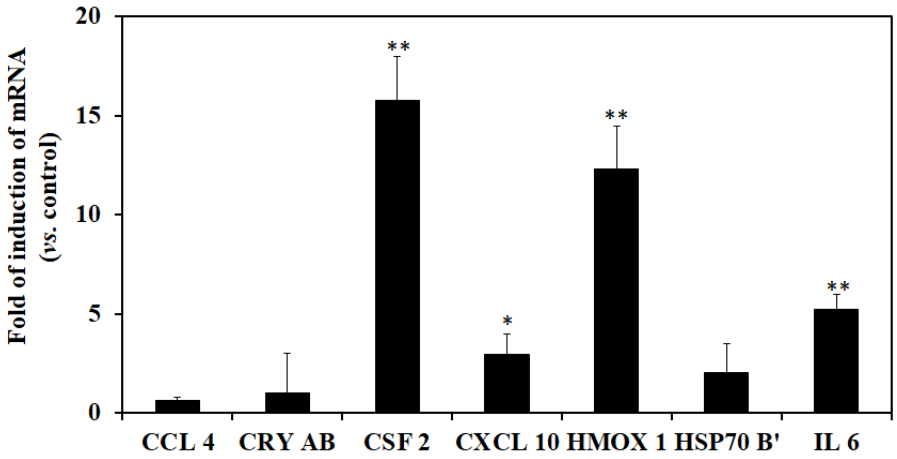
Table 2: Lists for genes showed up or down regulation of mRNA expression in PS-NPs exposed NCI-H292 cells after 6 h in PCR array

Symbol of the genes	Description of the genes	Gene Function	Fold-regulation
CCL 4	Chemokine (C-C motif) ligand 4	Inflammation	1.82
CCNG1	Cyclin G1	Proliferation and carcinogenesis	-2.42
CRYAB	Crystallin alpha B	Oxidative stress	7.59
CSF 2	Colony stimulating factor 2	Inflammation	4.88
CXCL 10	C-X-C motif chemokine 10	Inflammation	4.30
HMOX 1	Heme oxygenase 1	Oxidative stress	14.58

HSP70B'	Heat shock 70kDa protein 7	Heat shock	2.03
IL 6	Interleukin 6	Inflammation	8.98
LTA	Lymphotoxin alpha	Inflammation	-1.94
TNF	Tumor necrosis factor	Apoptosis signaling	-1.24
UGT1A4	UDP glucuronosyltransferase family 1 member A4	DNA damage and repair	-0.31

Next, we investigated the level of mRNA expression of up-regulated stress- and toxicity-associated biomarkers in PS-NPs exposed cells using qRT-PCR. The candidate biomarkers are oxidative markers such as CRYAB and HMOX 1, inflammatory markers such as CCL 4, CSF 2, CXCL 10 and IL 6 and heat shock marker such as HSP70B' (Fig. 3).

Figure 3: Expression of marker mRNA associated with stress and toxicity in PS-NPs exposed NCI-H292 cells for 6 h. Each bar represents mean ± SD, n = 3 for each marker. *p= 0.05, **p = 0.01.



Among the biomarkers identified by PCR array, few biomarkers showed a significant in their expression level. The inflammatory markers such as CSF 2, CXCL 10 and IL 6 increased 15.5, 2.9 and 5.2 fold respectively and oxidative marker HMOX 1 increases 12.3 fold than the control at 6 h. This suggests that PS-NPs induce inflammatory and oxidative stress in NCI-H292 cell lines.

In this study, we focused on the effects of PS-NPs on the cytotoxicity and gene expressions on NCI-H292 cell lines. NCI-H292 cells showed concentration dependent effect cell viability upon the exposure of PS-NPs, indicating that PS-NPs exposure induces stress in NCI-H292 cells. We also conducted PCR array analysis of 84 genes that are indicative of stress and toxicity in human cell lines. qRT-PCR analysis was performed for mRNA expression analysis based upon the result obtained from PCR array. NCI-H292 cells responded quickly to PS-NPs exposure within 6 h with induction of the inflammatory cytokines such as CAF 2, CXCL 10 and IL 6 and oxidative stress marker such as HMOX 1.

Reports showed that pulmonary exposure of PS-NPs provokes adjuvant effect on immune responses and (Inoue et al. 2009) lipopolysaccharide (LPS) related lung inflammation [25]. Some studies showed that increased expression of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 (IL 1), and IL 6 are related to oxidative stress which result from increased in the generation of reactive oxygen species (ROS) followed by the induction of cardiac dysfunction [26].

Most of the NPs enter the cell via endocytosis. Moreover, the mode of entry of NPs into the cells depends upon the size and surface properties [27] and the presence of appropriate cell membrane receptors.

It is shown that positively charged PS-NPs are penetrate effectively via the cell membrane than the negatively charged and unmodified PS-NPs [28] and causes DNA damage followed by accumulation of G0/G1 phase in HeLa and NIH-3T3 cells [29]. Another evidence showed that positively charged Au NPs absorbed more by the cells than the negatively charged particles [30]. It is suggested that PS-NPs possibly enter the cells through endocytosis and induces cytotoxicity and induces inflammatory and oxidative stress response in human cell line. Further studies are necessary to interpret the internalization of PS-NPs to the cells.

Conclusions

Here, we showed the effects of unmodified PS-NPs (60±12 nm) both at cellular and gene expression levels. We conducted a cell viability test and gene expression analysis in human lung epithelial cell lines, NCI-H292 to assess the toxicity induced by PS-NPs. Our results suggest that PS-NPs reduced cell viability as well as induced inflammatory and oxidative stress-related mRNAs in NCI-H292 cells. Based on our findings, we suggest that PS-NPs are considered as a potential toxicological compound to human cell line. However, it is considered as lower concentration of PS-NPs is safe to use for biological applications.

Conflict of Interest

No conflict of interest

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