in vitro Chemical and physical toxicity of polystyrene microplastics in human-derived cells

Daheui Choi a, Junah Bang b, Taeho Kim a, Yoogyeong Oh a, Youngdeok Hwang c,* and Jinkee Hong a,*

a Department of Chemical and Biomolecular Engineering, Yonsei University, Seoul 03722, Republic of Korea
b Department of Statistics, Sungkyunkwan University, Seoul 03132, Republic of Korea
c Paul H. Chook Department of Information Systems and Statistics, Baruch College CUNY, New York, NY, United States of America

*Corresponding authors.

Tel.: +82-2-2123-5748, Fax: +82-2-312-6401, E-mail: jinkee.hong@yonsei.ac.kr (Jinkee Hong)
Tel.:+1-646-312-3411, E-mail: Youngdeok.Hwang@baruch.cuny.edu (Youngdeok Hwang)
Abstract

Background

With the increase of plastics production, a variety of toxicological studies regarding the microplastics have been reported since the microplastics could be ingested by the human body and cause serious diseases. However, the previous studies have been mainly focused on the toxicity of sphere type microbeads, which may be different from that of the randomly-shaped microplastics in real environment. Here, we have conducted the in vitro toxicology for randomly-shaped microplastics following the hypothesis that (1) physical cytotoxicity is affected from nano-/micro-size roughness in polystyrene (PS) microplastics and (2) chemical toxicity is caused by chemical reagents from microplastics.

Results

To prepare random shape of PS microplastics, we produced microfragments by ball mill grinding, then analyzed them via various toxicity tests in chemical and physical aspects with various kinds of human-derived cells. Ground PS microplastics were sorted in 3 ranges: 5-25 μm, 25-75 μm and 75-200 μm, and treated up to 1 mg/mL to cells based on weekly human intake of microplastics. We have confirmed that the PS microfragments induced 20 times increased acute inflammation for immune cells, production of reactive oxygen species and cell-death for fibroblasts and cancer cells by release of chemical reagents from microplastics. In addition, when the PS microfragments were in direct contact with the fibroblast and red blood cells, they lead to the lactose dehydrogenase release caused by a cell membrane damage and hemolysis by physical stress of microfragments. This phenomenon was amplified as microfragments concentration and roughness increases, we quantitatively analyzed roughness differences between microplastics, demonstrating that there are strong relationship physical damage of cells and roughness of microplastics.
Conclusion

We found that the PS microfragments have chemical toxicity. Furthermore, the physical toxicity by PS resulted in cellular membrane damage and correlated with statistically quantified-shape roughness. Therefore, we newly suggested the additional physical toxicity of random shape of microplastics. This provides the evidence of environmental and biological risks on random shape of microplastics.

Keywords: Polystyrene, Secondary microplastics, Statistical shape analysis, Immune response, Cytotoxicity, Cell membrane damage
1. Background

Global use of plastics has been dramatically increasing since the initial plastic development in the middle of 1900’s, reaching to the 8.4 billion metric tons of plastic production in 2017 [1, 2]. Due to the strong advantages of plastics being durable, inexpensive, relatively lightweight, and flexible [3], the plastic production are expected to continuously increase, which consequently the plastic contamination at environments makes a serious problem [4]. Discarded plastics that were not recycled, entered into the ocean, and then physical and chemically degraded into small pieces of plastics (less than 5 mm) gradually over a long period of time, are defined microplastics [5, 6].

Besides the microplastics that is manufactured-microbead to meet the market demand, the secondary microplastics are degraded and formed from bulk plastics by several extrinsic stresses. First, discarded bulk plastics in ocean are mainly exposed to solar-UV radiation, which facilitates oxidative degradation. At the development stage of decomposition, the plastics become weak and discolored by mechanical forces like wind, wave action or animal bites, and then end up becoming plastic fragments with random shapes [7]. The plastic degradation and their fragment shapes highly depend on the various environmental factors such as weathering, plastic properties, irradiation and pH [8, 9, 10].

The microplastics could be exposed to human mainly in 3 routes, ingestion of foodstuffs, inhalation and dermal contact [11, 12, 13, 14], leading to the potential serious safety issues. Microplastics have large surface area and undegradability, which may induce the oxidative stress, inflammation and toxicity to tissue as accumulation, and cause potential chronic inflammation or cancer generation [11, 15, 16]. Furthermore, the adsorption of toxic reagents (persistent organic pollutants; POPs) or microorganisms from environments onto hydrophobic surface of microplastics increase their toxicity [17]. According to the previous reports on toxicity of
microplastics, it has been mainly demonstrated the toxicity of plastic beads depending on their size or plastic types [18, 19, 20, 21]. In secondary microplastics, however, over 50-65% in ocean environments are accounted by random shape of microfragments [22], hence the additional toxicity issue may be induced from shape differences. Although the cells could not uptake the micron-size plastics, the nano-/micro-sharpness and roughness in microplastics could be responsible for cellular toxicity like cell membrane disruption. Therefore, it is critical to investigate the in vitro toxicity of random shape of microplastics in a physical toxicity point of view.

In this study, we have demonstrated the cytotoxicity for random shape of secondary microplastics. Among the common plastics widely used in worldwide, we studied the polystyrene (PS). The PS is polymerized from a monomer styrene, can be solidified and foamed (Styrofoam) depending on their demands; used as container, rigid materials and thermal insulation materials. We have mimicked the one part of the procedure for secondary microplastic production; the physical degradation by wind, wave action or animal bites [7]. To accelerate the physical degradation of PS plastics, we used ball mill to grind plastics (fig. 1). The ground PS microplastics were sorted by 3 different ranges of size and we have carried out various kinds of cytotoxicity assays in different PS concentrations. We hypothesized that the random shape of PS microfragments have relatively rough and sharp morphology than other plastics due to the nature of high stiffness and hardness in PS, cause more harmful physical effect to prompt cell toxicity.

In fig. 1, we have investigated the cytotoxicity of the random shape of PS microfragments in two aspects; (1) chemical effect and (2) physical effect, In case of chemical toxicity of PS, we concluded that unpurified reagents during PS synthesis could be released and induce reactive oxygen species (ROS) generation by mitochondria damage or immune response at an initial cultivation of PS. At a long-term culture, some of damaged cells were dead. For physical toxicity
of PS, direct cell membrane damage and rupture are occurred by sharp and rough shape of PS
microfragments, resulting in hemolysis of red blood cells and LDH release from fibroblasts.

To examine this hypothesis, it is important to validate that physical characteristics that are
compared indeed can be considered to be different. Hence we first established that the PS
microfragments in different sizes have statistically different roughness. Then we found a strong
relationship between physical cell damage and shape of PS microfragments. Consequently, based
on our results, we concluded that both chemical and physical properties of the microplastics should
be considered to be important factors to determine toxicity of microplastics.
2. Methods

2.1. Preparation of PS microfragments

Polystyrene (PS) pellets (average molecular weight ~192,000 g/mL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PS pellets were smashed with hammer to facilitate breaking. The smashed PS pellets and zirconia ball were treated to liquid nitrogen for 10 min to make brittle state of PS. After 10 min, frozen PS and zirconia balls were placed into mini ball mill (Fritsch, Idar-Oberstein, Germany) and milled PS particle at 45 rpm, for 5 min. The milling process was repeated to 10 times to get a desired size of PS microfragments. The PS fragments were sorted by continuous sieving using 200 mm, 75 mm, 25 mm and 5 mm mesh size sieve, respectively. The filtered PS microfragments were treated methanol, followed by drying overnight to get 5-25 µm, 25-75 µm and 75-200 µm size PS microfragments. The size, morphology of microfragments were measured by scanning electron microscope (SEM) using an IT-500HR instrument (JEOL, Tokyo, Japan). According to SEM image, area distribution of each PS microfragment was obtained using an Image J software.

For in vitro analysis, PS microfragments were dispersed in cell culture media due to their hydrophobicity. In PBS or deionized water, the PS could not be dispersed well. For concentrations of PS solution, 1000 µg/mL, 100 µg/mL and 10 µg/mL were prepared.

2.2. Materials for in vitro analysis

Different types of cell culture media were used; L-glutamine containing RPMI-1640 for peripheral blood mononuclear cells (PBMCs) and KATO III cells, and high glucose containing Dulbecco's modified eagle medium (DMEM) for HeLa cells and human dermal fibroblasts (HDFs). All media were purchased from Gibco (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from
Welgene (Gyeongsan-si, Gyeongsangbuk-do, Korea). Mg\(^+\) and Ca\(^{2+}\)-free 10× phosphate buffered saline (PBS) trypsin-EDTA (0.25%) and Penicillin-Streptomycin (PS) were also obtained from Gibco. Lipopolysaccharide (LPS) from Escherichia coli, 10% Triton X-100 (Tx-100) in and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RBC lysis buffer was supplied from Merck (Darmstadt, Germany). For preparation of cell culture media, 10% of FBS and 1% of PS were added into 90% of DMEM or RPMI-1640.

2.3. PBMC culture

Uncharacterized human PBMCs were purchased from C. T. L. (Shaker Heights, OH, USA). The cells were selected randomly within Hispanic or Asian donors 20–40 years of age. For inhibition of additional immune response of PBMC, we have prepared the cell culture media with 90% of RPMI-1640, 10% of heat inactivated FBS (heat treated at 56°C for 30 min) and 1% of PS. Frozen 1 vial of PBMCs (approximately 1 × 10⁷ cells) were thawed and culture in 5% CO₂ incubator at 37°C for 1 day using 25 T-flask. Red blood cells (RBCs) were eliminated by RBC lysis buffer for 5 min prior to proceed in vitro assay.

2.4. Live-dead staining and viability analysis for PBMC

Cultured PBMCs were collected and centrifuged at 330 × g for 7 min. After RBC elimination, the cells were seeded onto 96 well-plate at a density of 5 × 10⁴ cells per 100 mL. After 1 day, 2 times concentrated PS microfragments were additionally added to each well-plate to get desired concentration (1 mg/mL, 100 µg/mL and 10 µg/mL), respectively. For negative control, 1% of Triton X-100 was treated. PS-treated cells were cultured for 1 day and 4 days. For live-dead staining, LIVE/DEAD™ viability/cytotoxicity kit for mammalian cells (Thermo Fisher Scientific, Waltham, MA, USA) were dissolved in 1 × PBS and treated to media-removed PBMCs for 30–45
min. Live (green)/dead (red) images were measured by fluorescence microscopy (Leica, Wetzlar, Germany). PBMC viability was deduced by calculating live/dead cell number ratio. Cell viability (%) was calculated by the equation 1 and normalized as follows (n = 3):

\[
\text{Cell viability (\%)} = \frac{\text{Live cell number}}{\text{Total cell number}} \times 100
\] (1)

2.5. Cytokine release

The RBC removed PBMCs were seeded onto 24 well-plate at a density of \(5 \times 10^5\) cells per each well. The PS microfragments were treated as mentioned above. And PS-treated PBMC were cultured for 1 day. For positive control (induction of maximum immune response), 50 ng/mL of LPS was treated. To measure pro-inflammatory cytokines (interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-\(\alpha\)) and IL-2 induced by PS microfragments, cell culture supernatant was collected by centrifugation. The concentration of released cytokines from PBMCs was detected by enzyme-linked immunosorbent assay (ELISA; BioLegend, San Diego, CA, USA).

2.6. HeLa, HDF and KATO III culture and viability analysis

HeLa, HDF and KATO III cells were cultured in a 100 mm culture dish (SPL) in a 5% \(\text{CO}_2\) incubation at 37°C using a culture media for each cell. The culture media was changed every 2-3 days. The cells were cultivated until cells reached over 95% confluency. Cells were detached using 0.05% of trypsin-EDTA for 3 min and centrifuged to collect cells. For viability analysis, cells were seeded at a density of \(5 \times 10^3\) cells/cm\(^2\) for 1-day viability and \(2.5 \times 10^3\) cells/cm\(^2\) for 4-day viability test. After 1-day cultivation, cell culture media was removed and PS microfragments dispersed media was treated. At the desired time point (1 and 4 days), 10% CCK-8 reagent (Dojindo, Kumamoto, Japan) was treated to the cells. The absorbance at 450 nm visible wavelength was
measured using a SpectraMax 340 PC plate reader (Molecular Devices, San Jose, CA, USA). The cells were treated with 20% DMSO as a positive control.

2.7. Hemolysis and L-lactate dehydrogenase (LDH) assay

For hemolysis, 1 mL of sheep blood (MBcell) were washed with 10 mL of 1× PBS and centrifuge at 2,000 rpm for 5 min to eliminate hemolyzed red blood cells (RBCs). This process was repeated until clear supernatant was obtained after centrifugation. Remained RBCs was diluted by 1× PBS and seeded into a 96 well-plate. PS microfragment solutions in cell culture media were treated to RBC. To analyze physical damage of PS microfragments to RBC, we put RBC-PS mixture in well-plate into shaker (Thermo Scientific) at 750 rpm for 2 hours to continuous contact between PS microfragments and RBCs. After 2 hours, the well-plate was centrifuged at 2,000 rpm for 7 min to get a supernatant which is hemoglobin from hemolyzed RBC. The supernatants were collected and measured visible wavelength absorbance at 540 nm. For positive control, 1% triton X-100 in PBS was used.

For LDH assay, HDFs were seeded on 96 well-plate at a cell density of 1 × 10^4 cells/well. Cells were incubated at 5% CO₂ incubator for 1 day. After 1 day, PS microfragments solutions were treated to cells and incubated for 1 day. To get LDH released solution from HDF, cell-PS mixture in well-plate was centrifuged at 1500 rpm for 3 min, then we collected 100 mL of supernatant. The supernatants were mixed with LDH assay reagent which is purchased Takara Bio Inc. (Shiga, Japan) and wait for 30 min in dark. The mixture changed to red color by a reaction of LDH was measured by plate reader (SpectraMax 340 PC; Molecular Devices, San Jose, CA, USA) at 490 nm visible wavelength. To minimize background color reaction (LDH reagent reacts with FBS),
we have used 2% FBS-contained cell culture media. For positive control, 1% triton X-100 in PBS was used.

2.8. Reactive oxygen species (ROS) assay

For fluorescence microscope measurement, the HDFs were seeded onto 48 well plate at a cell density of $2 \times 10^4$ cells/well. After 1-day incubation, PS microfragments dispersed in cell culture media was treated to cells and stored in 24 hours. 0.2 mM of $\text{H}_2\text{O}_2$ was treated for 6 hours for maximization of ROS release from cells (positive control). To detect ROS by fluorescence, 20 mM of 2’,7’-dichlorofluorescin diacetate (DCF-DA) (Sigma-Aldrich) in 1× PBS was treated for 30 min at 37°C. After staining, cells were washed with PBS and observed green fluorescence (Fluorescein filter) using fluorescence microscope (IX71-F22PH, Olympus) For flow cytometry analysis, HDFs were seeded onto 6 well-plate at a cell number of $2 \times 10^5$ cells per each well. The $\text{H}_2\text{O}_2$ and PS microfragments were treated as same as above process. To measure flow cytometry, cells were detached and filtered with 70 mm strainers to prevent the nozzles of the machine from clogging by PS microfragments. And cells were stained with 20 mM of CDF-DA for 30 min and measured flow cytometry (Guava®, Luminex). The cells were counted 5,000 cells per each group.

2.9. TEM analysis

To confirm cellular behavior under PS treatment, transmittance electron microscope. For preparation of cells for TEM, we have cultured HDFs in a 100 mm culture dish. At 95% of cell confluency, 100 $\mu$g/mL of 5-25 $\mu$m PS microfragments were treated to HDFs for 1 day. Then, cells were detached by 0.05% trypsin-EDTA and collected by centrifugation. 4% paraformaldehyde was used for cell fixation.

2.10. Statistical analysis
Numerical data represented in the graphs represents mean value with error bar. Differences between control (Ctrl) and the test groups were compared by unpaired t-test. A $p$-value $> 0.05$ was considered non-significant (ns). The *, **, and *** symbols indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. All the experiments were conducted at least three times.

2.11. **Statistical analysis of shape in PS microfragment**

2.11.1. **Local curvature**

In this section, we briefly introduced our methods to define the roughness, along with the statistical methods to compare the roughness measured from the microscopic images. First, let us introduce the definition of the *local curvature*. In two-dimensional space, local curvature $c$ at a pixel is defined by

$$c = \frac{|x'y'' - x''y'|}{\sqrt{(x')^2 + (y')^2|}}$$

(2)

where $x'$ and $x''$ are the first and second derivatives, respectively. In the analysis, the coordinates are rotated so that the downward direction in Euclidean space is toward the center of the debris when calculating the local curvature at every pixel. Local curvature values are obtained at each pixel around the boundary. Negative curvature value at a boundary pixel indicates that the boundary is bent outward (e.g., circle has constant negative curvature value along its boundary) at the pixel, whereas positive one means that it is bent inward (i.e., dent). Hence frequent changes in curvature over the boundary can be interpreted to be more “wiggly.” We calculated the values in (2) along the edge of the samples, and analyzed the values obtained the samples using a statistical hypothesis test.

2.11.2. **Hypotheses test**
When analyzing the local curvature values, it is difficult to make a typical assumption commonly used in statistical hypotheses such as normality or independent sampling in our setting, because of the spatial dependence inherent in microscopic images. Thus, we consider a nonparametric test that requires minimal assumptions and conditions. Our test relies on resampling procedure. For simplicity, we assume that same number of sample images are obtained for both plastic types, although it can be flexibly chosen with minimal modification. The procedure is summarized below.

1. For each sample \((i = 1, ..., I)\) and size type of the plastic \(j = 1 (5-25), 2 (25-75), 3 (75-200)\), randomly pick a partition of length \(K\) from the calculated curvature vector. Denote this partition \(y^{(b)}_{ij} = (y^{(b)}_{ij1}, y^{(b)}_{ij2}, ..., y^{(b)}_{ijK})\).

2. Within this partition, obtain the length \(K-1\) -vector containing the change at each point, denoted by \(\tilde{y}^{(b)}_{i1}, \tilde{y}^{(b)}_{i2}, \tilde{y}^{(b)}_{i3}, \ldots, \tilde{y}^{(b)}_{i8}\) and \(\tilde{y}^{(b)}_{i9}\), respectively.

3. Obtain the scaled curvature-changes, denoted by \(\tilde{y}^{(b)}_{ij} = \frac{\tilde{y}^{(b)}_{ij}}{s_{ij}}\), where \(s_{ij} = \frac{\sum_{k=1}^{K-1} (\tilde{y}_{ijk} - \bar{y}_{ij})^2}{K-2}\),
\[
\bar{y}_{ij} = \frac{\sum_{k=1}^{K-1} y_{ijk}}{K-1}.
\]

4. Define the length \(n = I \times (K-1)\) - vector containing scaled curvature-changes for \(j\), \(w^{(b)}_j = (\tilde{y}^{(b)}_{1j}, \tilde{y}^{(b)}_{2j}, \tilde{y}^{(b)}_{3j}, \ldots, \tilde{y}^{(b)}_{ij9})\).

5. Obtain the \(F^{(b)} = \frac{n \sum_{j=1}^{I} (\bar{w}^{(b)}_j - \bar{w}^{(b)})^2 / j - 1}{\sum_{j=1}^{I} n \sum_{l=1}^{n} (w^{(b)}_{jl} - \bar{w}^{(b)})^2 / j \times (n-1)}\), where \(\bar{w}^{(b)}_j = \frac{1}{n} \sum_{l=1}^{n} w^{(b)}_{jl}\), \(\bar{w}^{(b)} = \frac{1}{jn} \sum_{j=1}^{I} \sum_{l=1}^{n} w^{(b)}_{lj}\).

After repeating 1-5 \(B\) times, \(F^{(1)}, ..., F^{(B)}\) are available. These statistics can be used for other inferences, such as conducting a hypothesis test or constructing a confidence interval. Intuitively,
F values are small when there is little difference between different plastics in terms of curvature changes. In this paper, we focus on the comparison of the average curvature for different plastic types. Specifically, we conduct a hypothesis test with the following hypotheses:

H₀: PS 5-25 µm, PS 25-75 µm and PS 75-200 µm have same mean curvature-change.

H₁: All mean curvature-changes are not same.

2.11.3. Post-hoc test

When concluding to reject null hypothesis in the preceeding process, it becomes an interest to find the exact pair having different mean curvature-changes. The procedure is summarized below.

1. For each sample \((i = 1, ..., I)\) and size type of plastic \(j = 1, 2\), randomly pick a partition of length \(K\). Denote this partition \(y_{ij}^{(b)} = (y_{i(j1)}, y_{i(j2)}, ..., y_{i(jK)})\).

2. Within this partition, obtain the length \(K - 1\) -vector containing the change at each point, denoted by \(\bar{y}_{i1}^{(b)}\) and \(\bar{y}_{i2}^{(b)}\), respectively.

3. Obtain the average curvature change for \(j\), denoted by \(\bar{y}_{i}^{(b)} = \frac{1}{K-1} \sum_{l=1}^{K-1} \sum_{k=1}^{K-1} |\bar{y}_{i(jl)}^{(b)}|\), and define \(\bar{W}^{(b)} = (\bar{y}_{1}^{(b)}, \bar{y}_{2}^{(b)})\).

After repeating 1-3 by \(\binom{3}{2} = 3\) pairs \(g = 1(5 - 25 \text{ um}, 25 - 75 \text{ um}), 2(5 - 25 \text{ um}, 75 - 200 \text{ um}), 3(25 - 75 \text{ um}, 75 - 200 \text{ um})\) \(B\) times, \(\bar{W}_{g}^{(1)}, ..., \bar{W}_{g}^{(B)}\) are available. These statistics can be used for a hypothesis test with the following hypotheses:

H₀: PS 5-25 µm and PS 25-75 µm have same mean curvature-change.


268 H_{11}: PS 5-25 \mu m and PS 25-75 \mu m have different mean curvature-change.

269 H_{02}: PS 5-25 \mu m and PS 75-200 \mu m have same mean curvature-change.

270 H_{12}: PS 5-25 \mu m and PS 75-200 \mu m have different mean curvature-change.

271 H_{03}: PS 25-75 \mu m and PS 75-200 \mu m have same mean curvature-change.

272 H_{13}: PS 25-75 \mu m and PS 75-200 \mu m have different mean curvature-change.

273 Apparently, these tests are not readily available from the usual statistical software. We made an R package named ‘microplastics’ for analysis. The package can be installed and is publicly available at a webpage\(^1\) along with a detailed description.

\(^1\) https://ydhwang.github.io/supplement/microplastic_abs_pvc_suppl.html
3. Results and discussion

3.1. Characterization of PS microfragments

To demonstrate chemical and physical effects of PS microfragments to cells, we have prepared random shape of PS classified according to size range; 5-25 µm, 25-75 µm and 75-200 µm, respectively (fig. 1). We already have demonstrated the cytotoxicity of spherical PS microparticles, revealed that relatively small size of nanoparticles were shown cytotoxicity, hemolysis and immune response in vitro. For 40 mm and 100 mm of PS microparticles could not happen cellular uptake due to extremely their large size, resulting that it did not induce toxicity and immunity to cells (data not published). However, the random shape of microplastics (fragments) accounts for more than 50% of the total discarded microplastics in the real marine environments [22]; we believed that there has a possibility about additional effects of microfragments to cellular behavior which is different from that of sphere type of microplastics. Therefore, we have prepared random shape microfragments to confirm chemical and physical effects to cells (Fig. 1).

Fig. 1A illustrates the overall protocol for production of 3 different size PS microfragment. The PS pellets were cooled down to less than -196°C using liquid nitrogen in order to become more brittle, facilitating break-down of pellets. Below the glass transition temperature (Tg), the polymers showed increased modulus and strength like stiffness and hardness as temperature decrease [23]. Also, the increased hardness of PS plastics depending on temperature decrease up to -80°C was demonstrated [24]. Brittle and stiff plastic materials are apt to take place plastic fracture rather than deformation under the abrasive stress; therefore, it helps to make microplastic effectively. The brittle PS pellets were treated to ball mill machine and undergone physically breakage by the abrasive force generated from attrition and impact of ball. The ground PS...
Microplastics by ball mill grinding have random shape of fragments, which are intended to make secondary microplastics that are randomly decomposed by various mechanical stress such as wind, wave action and animal bite [7]. Then, the ground PS microfragments are sorted by 3 different sizes ranges.

In this study, we have demonstrated the toxicity of PS microfragments in two perspectives; (1) chemical and (2) physical aspects (fig. 1B). PS is normally synthesized by free radical polymerization of styrene (ethylbenzene) in a bulk, using benzoyl peroxide (BPO) as initiator [25, 26]. According to the previous reports about toxicity of styrene and BPO, these are regarded as toxic, mutagenic, and possibly carcinogenic potential [27, 28, 29, 30]. Therefore, even though purification process undergoes, unpurified reagents in PS plastics could be released. We speculated that these chemicals may induce cytotoxicity (Chemical effects). Additionally, we added one more toxicity variable which could arouse from physical shape of microplastics such as sharpness and roughness of edge site (Physical effects). For physical cytotoxicity, we have demonstrated the quantification of roughness (sharpness) at an edge of microfragments and correlated roughness and toxicity results.

In fig. 2, the SEM images for each size range of microfragments were shown. Compare to the shape of big microfragments (75-200 µm), that of small size microplastics (5-25 µm and 25-75 µm) were observed relatively rough and sharp. In our previous research about polypropylene (PP) microfragments toxicity, the ground PP have blunt edge than PS, nevertheless those two plastics have shown good ground property by ball milling [31]. We believed that the shape of ground plastics is related to hardness and brittleness. In PS, the hardness and brittleness are relatively higher than other plastics such as PP, polyethylene (PE) [32]. In hard and brittle materials, deformation energy is contained in the materials until threshold abrasive grinding forces stress
without deformation [33, 34]. Consequently, the breakage of materials is accomplished through propagation and interaction of cracks at threshold of stress [34]. While ductile materials generate severe deformation under grinding forces [34], resulting that blunt shape of microfragment were deducted.

The area distribution of each PS microfragment is displayed in fig. 2A-C, indicating highly broad distribution and standard deviation for all groups. Assuming the microfragments are square, the average area of each microfragment is 225, 2500 and 18906 µm², respectively, which are larger than real area average values. It is noted that slightly smaller size of microplastics is mainly distributed than the average size.

3.2. Cytotoxicity of PS in immune-related cells

For PS in vitro toxicity, we have tested using various kinds of cell types; immune-related mononuclear cells, red blood cells, fibroblasts and cancer cells. First, we have confirmed the toxicity of PS microfragments using immune-related cells, the peripheral blood mononuclear cells (PBMCs). In fig. 3, the live/dead ratio of PBMCs after treated in PS microfragments for 1 day (A) and 4 days (B), respectively. We normalized viability on the basis of control (non-treated group) as 100%, it is clearly seen that there is no cytotoxicity of PS microfragment at day 1 for all size and concentration variations. However, for 4 days cultivation of PBMCs with PS, higher concentration (1 mg/mL) of PS microfragments slightly induced cellular toxicity; 71.16%, 75.82% and 66.85% cells were alive for 5-25 mm, 25-75 µm and 75-200 µm treated groups, respectively. Supplementary fig. S1 and S2 are live-dead stained cell images at 1 day and 4 days PS treatment. For supplementary fig. S1, there is no significant differences of cell live/dead ratio for all groups, though green and red fluorescence was slightly blurred by PS microfragments at high
concentration. Also, the morphology of PBMCs were indicated suspension, which is non-activated state [35]. However, for 4 days culture (Supplementary fig. S2), the control cells differentiated and transformed to macrophages by activation of monocyte [35]. Normally, monocytes are in suspension state before immune response, and get differentiated to dendritic cells, mature macrophages when immune response is activated [35, 36]. However, cells were severe damaged and get lead to cell-death at 1000 µg/mL of PS treated groups, and still have a suspension cell morphology. For lower concentration, significantly similar cell morphology was revealed as compared to control.

We have demonstrated that PS microfragments did not affect the cell death for 1-day culture (fig. 3). At initial stage of cell damage by extrinsic materials, the immune cells release various kinds of cytokines that can start immune response and spread to other cells. Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are commonly well-known pro-inflammatory cytokines that is related to initial step of inflammation [37]. Therefore, we have measured the cellular behavior, especially immune response, for 1-day PS treatment before cell death. In fig. 4, normalized IL-6 and TNF-α released from PBMCs were displayed. In overall, the amount of cytokines release from the PBMC is strongly dependent on the concentration of PS microfragments we treated. For positive control, we have treated 5 ng/mL of LPS which is constituent of the outer membrane of gram-negative bacteria and promotes inflammatory responses effectively [38], indicating that 23.58 times (for IL-6) and 87.61 times (for TNF-α) higher responses were occurred. When we added PS microfragments, the inflammation got induced at higher concentration; specially almost similar IL-6 release amount was presented for 1 mg/mL of 75-200 µm PS as that of positive control. Consequently, we have demonstrated that PS microfragments induce release of proinflammatory cytokines, IL-6 and TNF-α, at the initial treatment, continuous release of
cytokine in cells transduces self-killing signal, leading to the cell-death [39]. In this result, we also found that larger size of PS microfragments promotes more cytokine release, which is also confirmed the PBMC viability at day 4 (fig. 3B). It is implied that roughness or sharpness edge of PS which is main factor for physical toxicity is not directly related to PBMC toxicity; chemical effect is considered as main contributor.

3.3. Cellular survival rate in vitro analysis under PS microfragments treatment

The cellular survival rates for human dermal fibroblast (HDF) and cervical cancer cells (HeLa) are displayed in fig. 5. Similar as viability of PBMCs, both cells were exhibited non-toxic behavior at day 1, getting worse their viability at day 4. 55-60% and 64-67% of cellular viability were shown in case of HeLa cells and HDFs, respectively. Surprisingly, some PS microfragments did not have cytotoxicity at higher concentration, which is due to the fact that small hydrophobic microfragments tend to form cluster in culture media condition at high concentration. Therefore, the PS clusters at high concentration might not effectively impact on cellular viability which means that physical toxicity of PS microfragments were reduced. However, the PBMC, which is immature immune cells, is extremely sensitive against extrinsic materials and pathogens than HeLa and HDF, resulting in toxicity at higher concentration of PS microfragments (fig. 3). Consequently, it is clearly confirmed that the PS microfragments have in vitro toxicity at long term culture regardless of their concentration and size.

We also demonstrated the in vitro toxicity of PS to KATO III cells, the gastric cancer stem cells. As shown in supplementary fig. S3, the cells still have higher viability though 4 days culture with PS microfragments, approximately 80% of survival rate at higher concentration. It is because cancer stem cells are typically drug or extrinsic material-resistant [40].
3.4. ROS generation and cellular damage under PS treatment

We next investigated the oxidative stress of cells induced by PS microfragments. Fig. 6 is intracellular ROS images from HDF measured by microscope. To prove intracellular ROS, we have treated DCF-DA, the cell permeable non-fluorescence reagent, to HDFs. The DCF-DA can be rapidly converted to the highly fluorescent 2,7, -dichlorofluorescein (DCF) by ROS or nitric oxide (NO) in cells [41]. Significant green fluorescence intensity differences between control (non-treated group) and H$_2$O$_2$-treated group were disclosed, indicating that the H$_2$O$_2$ can sufficiently induce oxidative stress to cells. When PS microfragments were treated, it is noticeably increased the intensity and number of stained cells by PS concentration dependent manner. Even though the PS microfragments make the image blur at high concentration, it is confirmed that more oxidative stress was induced in cells by PS-treated groups in overall. As shown in supplementary fig. S4A, which is ROS-positive fluorescence intensity in cells measured by flow cytometry, the percentages of positive green fluorescence population for control and H$_2$O$_2$-treated group were 21.6% and 92.8%, respectively, as same tendency as that of fig. 6. In case of PS treatment, we have found that the more cell population was ROS-positive at higher PS concentration; 83.1%, 40.2% and 99.1% of ROS-positive cells were observed for 5-25 µm, 25-75 µm and 75-200 µm PS treated, respectively. We also summarized cell population of green-positive (%) in supplementary fig. S4B.

When the PS is synthesized, styrene and BPO are commonly used. According to the previous studies about relationship between oxidative stress and styrene or BPO [42, 43]. Therefore, we have considered that the impurities in PS microfragments may occur mitochondria damages at short-term cultivation (1 day), resulting in ROS generation. It is noted that the viability of HDF at day 1 is not concentration dependent and still remain high level (fig. 5B), which is due to the fact that oxidative stress by PS is not main factor to induce cell-death. Also, a moderate oxidative stress
could induce mitochondrial metabolism is reported [44]. Therefore, it is not surprising that
moderate oxidative stress to cells get lead to mitochondrial metabolism, resulting that inverse
relationship between ROS generation and viability (measured by CCK-8 which basically confirms
level of mitochondria metabolism) was revealed.

To directly demonstrate the cellular response by PS microfragments, we have treated 5-25 mm
size PS to HDFs at a concentration of 100 µg/mL for 1 day and observed by TEM (fig. 7). In case
of control (non-treated cells), though vacuoles and apoptotic structure of cells were detected, most
of cells are seemed to be healthy. In the contrary, cells occurred apoptosis and there are have
abnormal vacancies (blue arrow) or autophagosome (red arrow) in cytosol for PS treated cells. We
have considered that autophagase is happened to overcome starvation and oxidative stress conditions
[45]. We did not find the microfragments in cells due to the size is over 5 µm.

3.5. Statistical shape analysis of PS microfragments and physical toxicity by their shapes

By statistically analyzing microplastic images, we assessed the roughness for 3 different size of
PS microfragments. From the original SEM image, we randomly sampled some of the PS
microfragments by size. The image size was adjusted, and the image was converted to matrices to
estimate the curvature along the boundaries (fig. 8A). In this graph, the concave and convex
regions are represented negative and positive slope of derivative of quadratic functions,
respectively. The local curvatures from PS microfragments are displayed as a series of local
curvature estimates. Local curvatures are estimated for each PS, where “id” indicates the index for
the boundary pixels (fig. 8B). The curvature deviation at each pixel is related to roughness of each
microfragments, in which some PS samples depicts highly rough region. Among the 3 different
sizes, 25-75 µm size of PS microfragments has more abrupt change points, which indicates that it
has more wiggly and rough boundaries. It is also plotted the density as a function of curvature changes (fig. 9), the mean curvature changes of middle size of PS showed 1.28 times rougher than that of large size of PS. We then conducted a hypothesis test using $F^{(1)}, \ldots, F^{(B)}$. With $K = 100$ and $B = 5000$. The resulting $p$-value $= 0 \approx 0.0001$, hence we can conclude that these 3 different types of PS have statistically significantly different shapes in terms of change of local curvature.

We next investigated the toxicity of PS microfragments according to their size and shape differences. Though the size of PS microfragments have extremely larger than that of cells, we have considered that cells could be damaged by edge of microfragments when the fragments are sufficiently rough and sharp. The sharp and rough edge of microfragments could attack the cellular plasma membrane, resulting in cell lysis and cell-death by necrosis. To demonstrate physical toxicity of PS microfragments, we have designed hemolysis assays using RBCs with a vigorous agitation culture (750 rpm) with PS microfragments for 2 hours, allowing that the PS can directly contact to cells and attack. In fig. 10A, the hemolysis which is the release of the hemoglobin in RBC are represented. The Tx-100 treated RBCs underwent severe hemolysis, we normalized it as 100%. When the PS microfragments were treated, it is clearly seen that the higher concentrations (1000 µg/mL) of PS occurred hemolysis compare to control group. Especially, the 25-75 µm of PS which is relatively rougher microfragments exhibited highest hemolysis. In fig. 10B, release amount of LDH in HDF by disruption of plasma membrane was demonstrated. The lower curvature changes of PS microfragments (75-200 µm) showed low LDH release manner. However rougher PS microfragments (25-75 µm) have higher LDH release property, demonstrated that the sharpness and roughness of microfragments could bring out the cellular damage by physical way. Consequently, we found that the microplastics could have not only chemical toxicity, but also
physical toxicity which is directly damage to cells membrane depending on their shape and roughness.

3.6. Chemical and physical toxicity of PS microfragments

The typical pathways to occur cytotoxicity by particles include interference of DNA synthesis or cellular organelles by cell uptake of several nanometer sized particles [46] and cell membrane rupture by the highly positive charged materials [47], which result in cell death. However, the PS microfragments used in this study have neither strong positive charge nor cellular uptake property (see fig. 7), it is needed to demonstrate cytotoxicity in a different aspect. Therefore, we have tried to interpret the cytotoxicity of PS microfragments in a chemical and physical points of view. Even though the plastics are highly stable and durable which are the strong advantages in plastics, we have considered that monomers and initiators in PS microfragments could be released at a cell culture condition (37 °C and dispersed in cell culture media) and negatively affect the cells. Indeed, Z. Amirshaghagi et al reported that approximately 0.35 mg/cm² of styrene monomers were released from a PS food packaging (dish) for 7 days incubation at 40 °C [48]. We believed that the monomer release in micron sized PS fragments may be occurred more rapidly due to the large surface area. According to previous studies on cytotoxicity of styrene and BPO, those induce oxidative stress in tissue and cancer [27, 28, 29, 30]. Therefore, it is considered that some released chemical reagents from PS microfragments may chemically and negatively affect the cells.

In previous our study, we also have investigated the cytotoxicity of PS nano- and microsphere and found non-cytotoxicity for micron size of particles. It is implied that the shape of microplastics, specially roughness and sharpness, could impact on cellular behaviors. In this study, we have tried to demonstrate the physical toxicity on rough structure of PS microfragments by vigorous agitation.
culture to directly contact PS and cells, resulting that the lysis of cells (hemolysis and LDH release) was proportional to the roughness and concentration of PS microfragments. Also, to correlate shape difference of microfragments and cytotoxicity, we suggested the quantification of roughness for random shape of PS by statistical methods. It is meaningful that it is possible to compare the quantified roughness of materials in edge site, even though it is not perfectly fitted in 3-dimensional microfragments.

4. Conclusion

In this study, we have demonstrated the in vitro toxicology of polystyrene (PS) microplastics using various types of cells. To produce the secondary source of microplastics degraded by physical and chemical ways, we have prepared the random shape of PS microfragments by ball mill grinding process and sorted 3 different size ranges of microfragments. We hypothesized that the cellular damaged by PS microfragments were happened in two different ways; cell damage by release of the chemical reagents used when PS is synthesized from the microfragments (Chemical effects) or the direct disruption of the cellular membrane by rough and sharp edge of microfragments (Physical effects). For the chemical toxicity of PS, we have found that ROS generation and immune response from cell were happened at an initial culture with PS microfragments, and finally cell death was induced over time (long term culture). This trend was not size dependent, but concentration dependent manner. Furthermore, in case of the PS microfragments with high roughness and sharpness, it caused physical cellular membrane damage, resulting in hemolysis and LDH release in cytosol. To quantify roughness of random shape on PS microfragments, rapid or gradual curvature change along the edge was confirmed by statistical and mathematical models,
and it was confirmed that all three sizes of microfragments have different roughness and sharpness, and accordingly it showed different physical cell toxicity. Finally, to demonstrate toxicity of microplastics, we have shown that chemical and physical properties of microplastics should be considered.
Declaration

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors consent to publication of this manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that there are no conflicts of interest.

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Authors’ contributions
D. Choi performed all of toxicology assays and prepared manuscript. J. Bang and Y. Hwang demonstrated statistical shape analysis of PE particles. T. Kim and Y. Oh have prepared spherical and ground PE particles. Y. Hwang and J. Hong have supervised the project and examined the final version of manuscript.

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Fig. 1. Schematic illustration on (A) preparation procedure of PS microfragments that is mimicked secondary source of microplastics and (B) their toxicity to cells in two ways; (1) Chemical effects induced by chemical reagents in PS and (2) Physical effects directly damage plasma membrane of cells. To demonstrate between physical cellular toxicity and shape of microfragments, we statistically quantified PS morphology.
Fig. 2. (A-C) Morphology of PS microfragments in 3 different size distributions (5-25 µm, 25-75 µm, and 75-200 µm) measured by SEM. Overall area distribution of PS microfragments calculated from SEM images (n= 50). The size of each microfragments was measured by Image J area analysis.
Fig. 3. Normalized live/dead ratio of PBMC under PS treatment for (A) 1 day and (B) 4 days.
Statistical analysis was performed by unpaired t-test (n=3). *, **, *** and non-significant indicate “p < 0.05”, “p < 0.01”, “p < 0.001” and “no mark” compared to Ctrl (Control), respectively.

Fig. 4. Normalized (A) IL-6 and (B) TNF-α release amounts from PBMC after 1-day PS treatment.
5 ng/mL of LPS was used as maximum inducer for immune response. Statistical analysis was performed by unpaired t-test (n=3). *, ** and non-significant indicate “p < 0.05”, “p < 0.01”, and “no mark” compared to Ctrl (Control), respectively.
**Fig. 5.** Cellular metabolic toxicity (viability) of (A) HeLa and (B) HDF for 1 and 4 days after PS treatment measured by CCK-8. For positive control 20% DMSO was used. Statistical analysis was performed by unpaired *t*-test (n=3). *, **, *** and non-significant indicate “*p* < 0.05”, “*p* < 0.01”, “*p* < 0.001” and “no mark” compared to Ctrl (Control), respectively.
**Fig. 6.** Fluorescence microscopic images of ROS release assay in HDF for PS microfragments treatment.

**Fig. 7.** Cell-TEM images for HDF. (Left) Control HDF (non-treated group), (Right) 1-day PS microfragments (5-25 µm, 100 µg/mL) treated HDFs. Blue and red arrows indicate abnormal vacancies and autophagosome, respectively.
**Fig. 8.** Local curvature of 3 different PS microfragments (A) local curvature from background removed SEM image of PS fragments and (B) curvature change as a function of pixel of each sample.
**Fig. 9.** Average distribution of curvature changes for 3 different size PS microfragments.

**Fig. 10.** (A) Hemolysis (%) of RBC under agitation culture with PS microfragments. Hemolysis was normalized by 100% for triton x-100 treatment group. (B) Normalized LDH release from HDF after PS treatment. Statistical analysis was performed by unpaired \( t \)-test (n=3). *, **, ***, non-significant indicate “\( p < 0.05 \)”, “\( p < 0.01 \)”, “\( p < 0.001 \)” and “no mark” compared to Ctrl (Control), respectively.