# Supporting information

## Role of Residual Monomers in the Manifestation of (Cyto)toxicity by Polystyrene Microplastic Model Particles

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This supporting information contains 20 pages, 7 figures, and 1 table.

## 2.6. Methods

**2.6.1. Validation of accuracy and precision of UV-Method.** To test the reliability of the new UV-Method, we have validated its accuracy and precision. For styrene calibration, styrene solutions in mixed solvents with concentrations of 1.5 $\mu$g/mL, 2.5 $\mu$g/mL, 3.5 $\mu$g/mL were prepared. In the case of PS calibration, PS dispersions in Milli-Q water of 6 $\mu$g/mL, 10 $\mu$g/mL, 14 $\mu$g/mL were prepared. The recorded UV absorbance of those samples were used to calculate their concentrations according to the corresponding equations of calibration curves. The calculated concentrations were further used to obtain the accuracy and precision of the UV-Method of styrene (Figure S4E, 96.8±1.6%) and PS (Figure S4F, 94.5±2.0%).

**2.6.2. Diffusion of styrene in PS dispersions stored over time.** The styrene concentrations in the PS dispersions being stored in 4 °C fridge over time were detected by determining the residual styrene and PS concentration with the UV-Method. For styrene detection, 0.1 mL – 0.3 mL of PS dispersions were diluted; for PS detection, 0.1 mL of PS dispersions were diluted.

**2.6.3. NMR spectroscopy ($^1$H-NMR)**. $^1$H-NMR in $CDCl_3$ with 64 measurement scans was performed to characterize PS, using a 300 MHz Bruker Ultrashield 300 spectrometer.

**2.6.4. Gel permeation chromatography (GPC)**. To measure the molecular weight of PS, gel permeation chromatography (GPC) was performed using chloroform eluent on Agilent Technologies 1200 series machine. 20 $\mu$L of 1 g/L of PS in chloroform solution was injected into GPC machine. The flow rate is 0.5 mL/min. The molecular weight was obtained according to the calibration of standard PS.

**2.6.5. Scanning electronic microscopy (SEM)**. The size of PS particles was visualized by SEM FEI Quanta FEG 250 equipped with second electron detector. SEM images were recorded with conditions of voltage at 4.5 kV, spot 2.0. Before visualization, in-house synthesized PS particles

dispersion and standard PS model particles dispersion were diluted in methanol as solvent to a concentration of approximately 100 $\mu$g/mL. Then one drop of the diluted dispersion was added on the glass substrate cemented on top of a carbon tape that was stuck on the metal carrier, followed by drying in an oven at 50 °C under vacuum for 24 hours. The dried samples were sputter-coated to 1.5 nm thickness of platinum with a Cressington sputter coater (120 s, 40 mA). For each sample, mean diameter was determined by measuring 100 PS beads with ImageJ software.

**2.6.6. Dynamic light scattering (DLS) and Zetasizer**. The hydrodynamic diameter and size distribution measurements were performed by dynamic light scattering (DLS) using ALV/DLS/SLS-5022GF system. The Helium-Neon laser was set at 633 nm and 22 milli Watt (mW). The signal was acquired at a detector angle of 90°, at 21 °C. The samples were prepared in water. The data were evaluated by a Dullware AfterALV software.

The zeta-potential was determined with a Malvern Zetasizer Nano-ZS ZEN3600, samples were diluted in Milli-Q water to 50 $\mu$g/mL. The sample was transferred to a disposable folded capillary cell made of polystyrene latex (RI 1.59, absorption 0.01). Three measurements were taken for each sample at 25 °C, using the Smoluchowski model, and using dispersant viscosity as sample viscosity (water as dispersant, at 25 °C, viscosity 0.89 cP, RI 1.33, dielectric constant 78.5).

**2.6.7. Instrumental information of UV-vis measurement.** For quantification of styrene and PS in the PS particles dispersions, ultraviolet-visible spectroscopy (UV-vis) was conducted on a Jasco V-630 spectrophotometer equipped with light sources of Deuterium Lamp (D2 Lamp) and Tungsten Halogen Lamp (WI Lamp). All measurements were performed under the photometric mode of absorbance using both light sources of D2/WI with correction of the baseline of the blank solvent, which is methanol/water mixed solvent for styrene detection and water for PS. The UV-vis bandwidth is 1.5 nm.

To make sure the measurement is valid either for styrene or PS in the dispersions, a spectrum was recorded for each measurement, since styrene has an absorbance peak at 246 nm, and PS at 280 nm, these peaks are close enough to interfere with each other either due to failure of sample preparation or the UV-vis machine itself. A spectrum could tell if interference happened, hence avoiding invalid data. Spectra for styrene calibration, spherical PS particles calibration (using SIP, diameter ~500 nm), and contents detection of styrene and PS particles were recorded in a wavenumber range of 500 – 200 nm, with a scan speed of 400 nm/min.

**2.6.8. Cell culture**. The L929 cells (CCL-1, ATCC) were maintained in Eagle's Minimum Essential Medium (EMEM, Lonza, Visp, Switzerland) cell culture medium supplemented with 10% fetal calf serum (FCS, Sigma Aldrich, Taufkirchen, Germany), 1x Penicillin-Streptomycin mixture (Lonza, corresponding to 100 $\mu$g/mL streptomycin and 100 IU/mL penicillin), and 4 mM L-glutamine (Lonza). The cell culture is referred to as MEM10. Cells were cultivated at 37 °C in a humidified 5% $CO_2$ atmosphere.

**2.6.9. MTT assays**. The in vitro cytotoxicity of styrene monomers and PS particles dispersions (SIPA, SHPA, MIPA, MHPA) were evaluated via tetrazolium dye assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay) according to the protocol ISO 10993-5[1]. Briefly, L929 cells were seeded at a density of $1 \times 10^4$ cells per well in 96-well plates 24 hours before the experiment. On the experiment day, PS particles dispersions were diluted in MEM10 to concentrations ranging from 0.1 $\mu$g/mL to 1.9 mg/mL, and styrene was pre-diluted in dimethyl sulfoxide (DMSO, Sigma Aldrich, Taufkirchen, Germany) and further diluted with MEM10 to final concentrations ranging from $10^{-7}$ $\mu$g/mL to $10^3$ $\mu$g/mL. As controls, samples containing equal volumes of water or DMSO instead of the PS dispersion or styrene were prepared. In all cases, the water and DMSO volume never exceed 10 and 1% of the total cultivation volume. The cell culture

medium in each plate was replaced by 100 µL of diluted samples at different concentrations, and the cells were incubated for another 24 hours in the cell culture incubator (37 °C, 5% $CO_2$, 95% humidity). Thereafter, the medium was aspirated, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), and 50 $\mu$L freshly prepared MTT reagent (1 mg/mL MTT (Alfa Aesar, Thermo Fisher (Kandel) GmbH, Kandel, Germany) in Minimum Essential Medium (MEM) without phenol red) was added to each well. After 2 hours of incubation, the supernatant was removed, and 100 $\mu$L of isopropanol were added per well to dissolve the produced formazan crystals. After 5 min shaking at 600 rpm at room temperature, the absorbance at 570 nm (reference wavelength 680 nm) was measured using a TECAN GENios Pro plate reader (Tecan Austria GmbH, Gröding). The experiments were conducted in six biological replicates. Cells incubated with samples containing only water or DMSO were taken as 100% viability control. The cell viability was calculated from the ratio of $A_{treated}/A_{control}$, where $A_{treated}$ and $A_{control}$ represent the cells treated with and without PS particles dispersion or styrene, respectively. Curve fitting of styrene cytotoxicity was performed to determine the 50% lethal concentration $LC_{50}$. However, the dose-response results revealed more than one point of inflection (i.e., multiphasic curve) preventing the fitting of the data with a standard Hill equation. Therefore, we used an algorithm, incorporated in the Dr-Fit freeware (http://sourceforge.net/projects/drfit/), which automatically generates and ranks dose-response models with varying degrees of multiphasic features[2]. Data were analyzed using the one-way analysis of variance (ANOVA) with a Tukey post hoc test to determine whether data groups differed significantly from each other. Differences were considered statistically significant for * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

**2.6.10. Acute immobilization test of daphnids**. The experiments were conducted using *D. magna* clone BL2.2 which originates from a small pond (Oud Meren) in Leuven, Belgium. This clone is

in culture since 1997 and has been used in several studies with microplastic at the Chair of Animal Ecology I at the University of Bayreuth[3–5]. Daphnids were cultured in M4 medium[6] at 20 ± 0.5 °C and a 16 h : 8 h light: dark regime and fed *ad libitum* with the green algae *Acutodesmus obliquus.*

The experimental procedure was based on the OECD Guideline 202[7] for acute immobilization testing with *Daphnia* sp.. Due to the high number of daphnids required, the experiments were carried out in two runs, where each run had its own control group (n = 4). In the first run, the test was carried out for styrene treatments, and in the second run, for in-house synthesized PS particles dispersion (SIP, SHP) and standard PS model particles dispersion (MIP, MHP) treatments.

For the styrene treatment, a stock solution with M4 medium was prepared beforehand (10 $\mu$g/mL) which was then diluted with M4 medium to achieve the selected concentrations. The chosen concentrations for the styrene treatment were 1 $\mu$g/mL, 3 $\mu$g/mL, 5 $\mu$g/mL, 7 $\mu$g/mL, and 10 $\mu$g/mL. For each concentration, four replicates with 10 mL each were poured into the wells of six-well culture plates. For all other treatments, the PS beads were dispensed directly from the stocks into the well plates containing 10 mL M4, each concentration with 4 replicates. Special care was taken to avoid absorption of the PS onto the pipette tips. For that, pipette tips were rinsed several times with the stock solution, then the outside was thoroughly cleaned mechanically (with a wipe), and the pipette tip was rinsed several times with the medium in the well. After each well, a new pipette tip was used. The chosen concentrations for the PS beads were 5 $\mu$g/mL, 20 $\mu$g/mL, 50 $\mu$g/mL, 100 $\mu$g/mL, and 300 $\mu$g/mL.

Then age-synchronized (born within 24 h) third brood neonates were randomly placed in groups of five into the wells, each containing 10 mL M4 and the respective treatment concentration. According to the OECD Guideline 202, no food was added, and no medium exchange was done during the exposure (OECD 2004)[7]. In the first experimental run (styrene treatments), immobility

was checked after 24 h and 48 h. In the second experimental run (PS treatments), the test was prolonged to 96 h following the recommendation of Baumann *et al.*[8] Data normality and heteroscedasticity were checked using Shapiro-Wilk tests, Q-Q plots, and Levene's tests respectively. As assumptions for ANOVAs were not met, Kruskal-Wallis tests with post-hoc Dunn's tests with Bonferroni-Holm correction were calculated. Data analysis was conducted in R[9], half-maximal effective concentrations ($EC_{50}$) were calculated utilizing the package morse ("Modelling tools for Reproduction and Survival data in Ecotoxicology")[10]. Model evaluation was performed using the function "ppc" implemented in the package morse.

**2.7. Statistical analysis**.

Diameter values of PS particles were presented as mean ± SD. The quantitative equations of determining styrene and PS contents in the PS particles dispersions were performed with Origin 2018 software with a linear fitting. For cell viability study, significant differences between treatments with synthesized and standard PS were showed by one-way ANOVA with a Tukey post hoc test. 50% lethal concentration ($LC_{50}$) of styrene to L929 cell line was generated by Dr-Fit freeware (http://sourceforge.net/projects/drfit/)[2]. For acute immobilization test of *Daphnia*, significant differences between treatments with synthesized and standard PS were presented by a Kruskal-Wallis test followed by Dunn's test with Bonferroni-Holm correction. Its data analysis was performed in R[9], half-maximal effective concentrations ($EC_{50}$) were calculated utilizing the package morse ("Modelling tools for Reproduction and Survival data in Ecotoxicology")[10]. Model evaluation was performed using the function "ppc" implemented in the package morse. The p-value is noted in the manuscript and depicted in figure legends as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

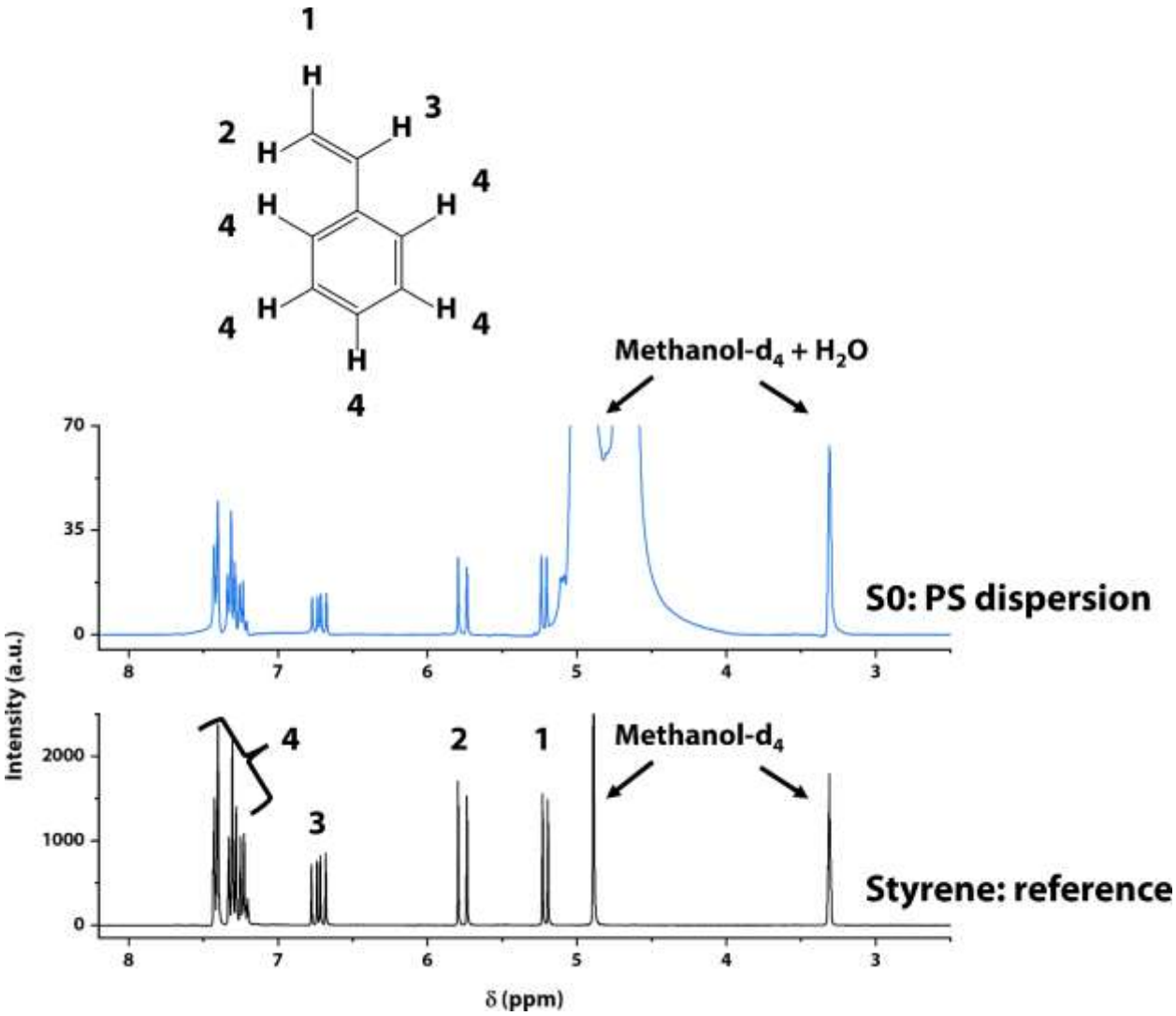


**Figure S1.** $^{1}$H-NMR spectra of monomer styrene in the PS particles dispersion (S0, in-house synthesized PS particles dispersion without purification). Methanol-$d_4$ was used as solvent. To confirm signals of the unreacted styrene in the PS dispersion (blue curve), the NMR spectrum of styrene as a reference (black curve) was recorded. In the reference spectrum, all signals were assigned to the protons in styrene molecule.

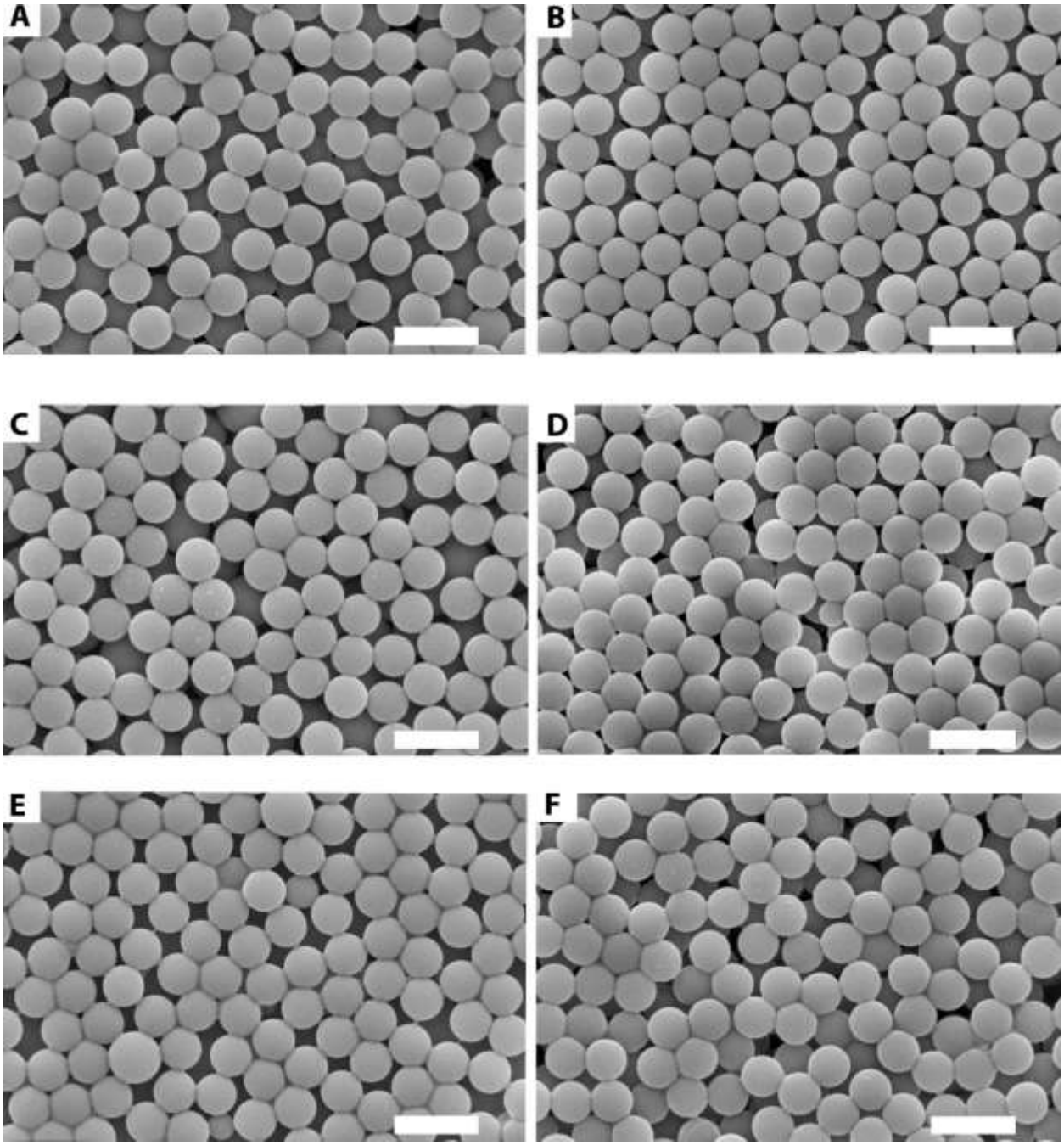


**Figure S2.** SEM images of PS particles. SIP, 515 ± 17 nm; MIP, 507 ± 7 nm; SEM images of SIP and MIP are presented in **Figure 1**. (A) SIPA, 510 ± 28 nm; (B) MIPA, 502 ± 6 nm; (C) SHP, 513 ± 20 nm; (D) MHP, 509 ± 7 nm; (E) SHPA, 509 ± 24 nm; (F) MHPA, 508 ± 10 nm; mean diameter ± SD by ImageJ, n = 100; scale bar 1 $\mu$m. MIP: standard PS model particles dispersion as received, PS 27400 $\mu$g/mL, styrene 241.8 $\mu$g/mL, purity 99.13%; MIPA: MIP after autoclave, PS 30500

176 $\mu$g/mL, styrene 65.4 $\mu$g/mL, purity 99.79%; MHP: MIP after rapid dialysis, PS 17300 $\mu$g/mL,
177 styrene 5.1 $\mu$g/mL, purity 99.97%; MHPA: MHP after autoclave, PS 21030 $\mu$g/mL, styrene 7.9
178 $\mu$g/mL, purity 99.96%; SIP: in-house synthesized PS particles dispersion after 40 days dialysis
179 against Milli-Q water, PS 19360 $\mu$g/mL, styrene 111.4 $\mu$g/mL, purity 99.43%; SIPA: SIP after
180 autoclave, PS 20780 $\mu$g/mL, styrene 48.9 $\mu$g/mL, purity 99.77%; SHP: SIP after rapid dialysis, PS
181 15510 $\mu$g/mL, styrene 4.3 $\mu$g/mL, purity 99.97%; SHPA: SHP after autoclave, PS 17260 $\mu$g/mL,
182 styrene 7.5 $\mu$g/mL, purity 99.96%. The rapid dialysis of MIP and SIP is against distilled
183 methanol/Milli-Q water (50/50 volumetric ratio) mixed solvents for 8 days then against Milli-Q
184 water for 12 days.

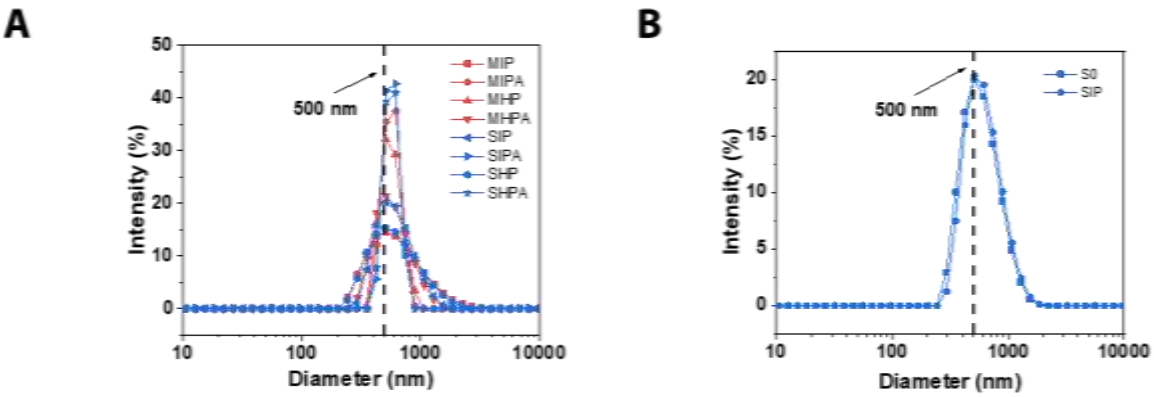


**Figure S3.** Hydrodynamic diameter determined by DLS. (A) size distribution of PS particles. (B) comparison of the size and size distribution of the in-house synthesized PS particles dispersion before and after dialysis against Milli-Q water. Dash line corresponds to 500 nm. The following is the Z-average determined by DLS, presented as mean diameter ± SD. MIP, 555 ± 157 nm; MIPA, 570 ± 103 nm; MHP, 553 ± 116 nm; MHPA, 553 ± 174 nm; SIP, 578 ± 173 nm; SIPA, 578 ± 81 nm; SHP, 551 ± 178 nm; SHPA, 575 ± 89 nm. MIP: standard PS model particles dispersion as received, PS 27400 $\mu$g/mL, styrene 241.8 $\mu$g/mL, purity 99.13%; MIPA: MIP after autoclave, PS 30500 $\mu$g/mL, styrene 65.4 $\mu$g/mL, purity 99.79%; MHP: MIP after rapid dialysis, PS 17300 $\mu$g/mL, styrene 5.1 $\mu$g/mL, purity 99.97%; MHPA: MHP after autoclave, PS 21030 $\mu$g/mL, styrene 7.9 $\mu$g/mL, purity 99.96%; S0 corresponds to the in-house synthesized PS without purification; SIP: in-house synthesized PS particles dispersion after 40 days dialysis against Milli-Q water, PS 19360 $\mu$g/mL, styrene 111.4 $\mu$g/mL, purity 99.43%; SIPA: SIP after autoclave, PS 20780 $\mu$g/mL, styrene 48.9 $\mu$g/mL, purity 99.77%; SHP: SIP after rapid dialysis, PS 15510 $\mu$g/mL, styrene 4.3 $\mu$g/mL, purity 99.97%; SHPA: SHP after autoclave, PS 17260 $\mu$g/mL, styrene 7.5 $\mu$g/mL, purity 99.96%. The rapid dialysis of MIP and SIP is against distilled methanol/Milli-Q water (50/50 volumetric ratio) mixed solvents for 8 days then against Milli-Q water for 12 days.

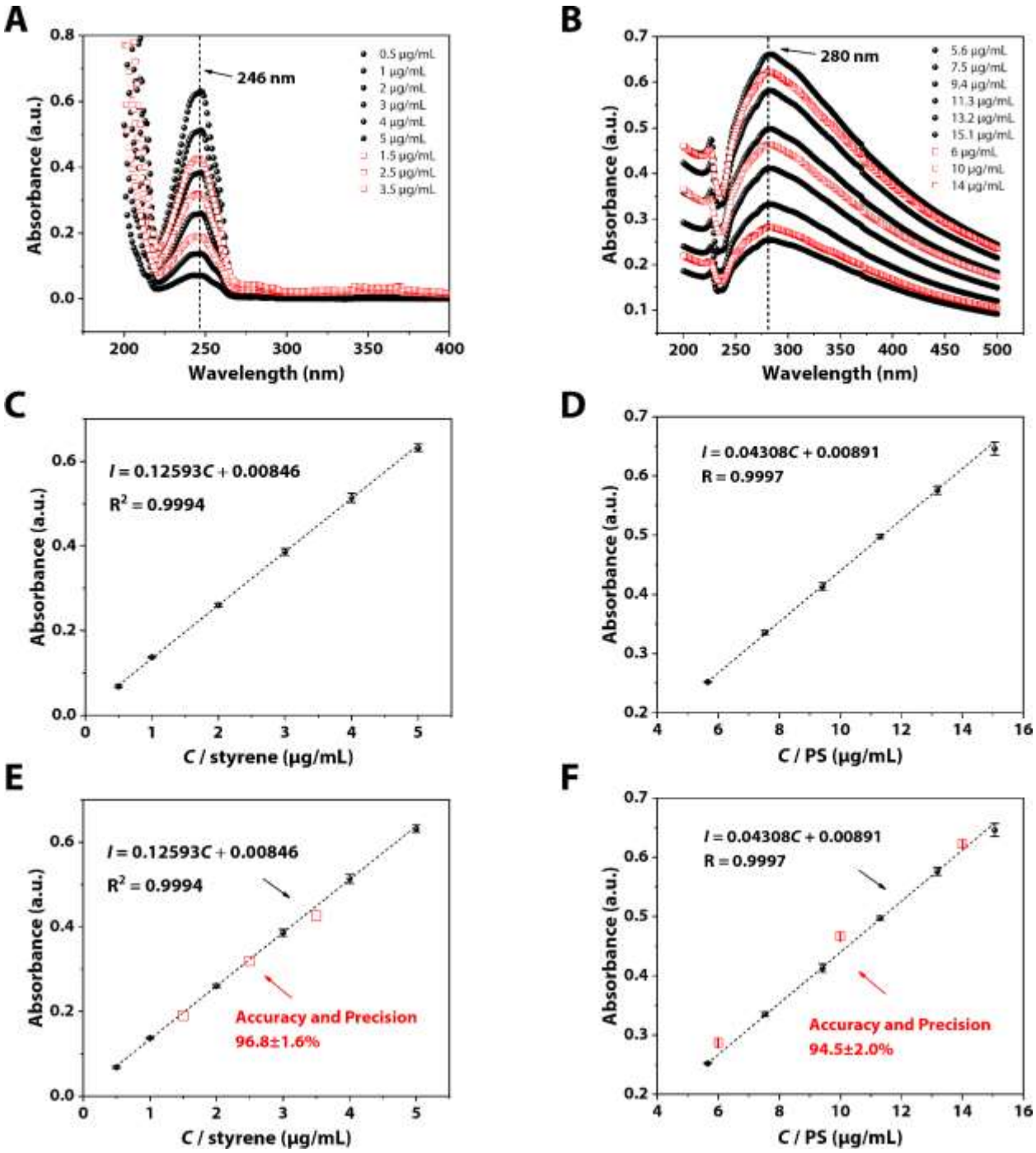


**Figure S4.** The calibration curves of styrene and PS particles by UV-vis spectroscopy (UV-vis method). (A) the UV-vis spectra of styrene calibration (black solid circle) and accuracy and precision determination of the calibration (red hollow square). For this, three independent solutions for each concentration of styrene were prepared by dissolving styrene in the mixed solvent of

distilled methanol/Milli-Q water (90/10 volumetric ratio). The absorbance values of the measurement for the solutions were read at peak 246 nm[11]. (B) the UV-vis spectra of PS calibration (black solid circle) and accuracy and precision determination of the calibration (red hollow square). Three independent dispersions for each concentration of in-house synthesized PS particles dispersion (SIP) were prepared by diluting PS dispersion in Milli-Q water. Their absorbance values were read at peak 280 nm. (C) the scatter plot of styrene calibration by UV-vis, $I_{246} = 0.12593C + 0.00846$ ($R^2 = 0.9994$). (D) the scatter plot of PS calibration by UV-vis, $I_{280} = 0.04308C + 0.00891$ ($R^2 = 0.9997$). Styrene and PS calibration plots were linearly fitted with Origin software, $I$ stands for absorbance intensity, and $C$ for concentration. (E) accuracy and precision determination of styrene calibration curve, absorbance values were plotted in the calibration curve. The accuracy and precision is 96.8±1.6%. (F) accuracy and precision determination of PS calibration curve, absorbance values were plotted in the calibration curve. The accuracy and precision is 94.5±2.0%.

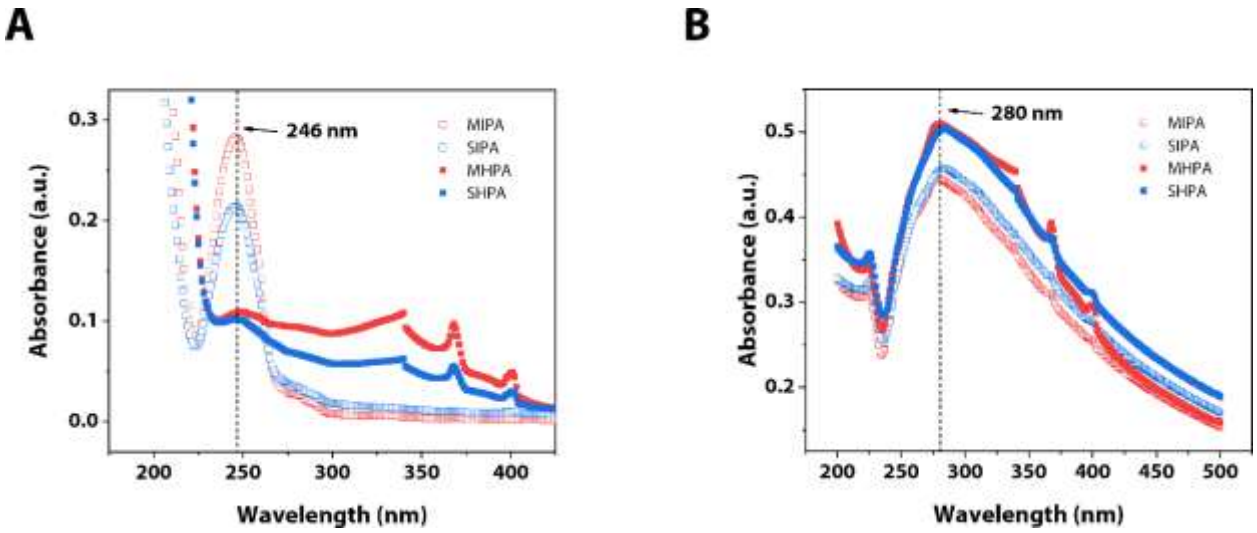


**Figure S5.** Representative UV-vis spectra of residual styrene and PS particles content detection by UV-vis spectroscopy. (A) UV-vis spectra of styrene in PS particles dispersions. The PS particles dispersions were diluted to a range of styrene concentration that can be covered within the styrene calibration curve. (B) UV-vis spectra of PS in PS particles dispersions. The PS particles dispersions were diluted to a range of PS particles that can be covered within the PS calibration curve.

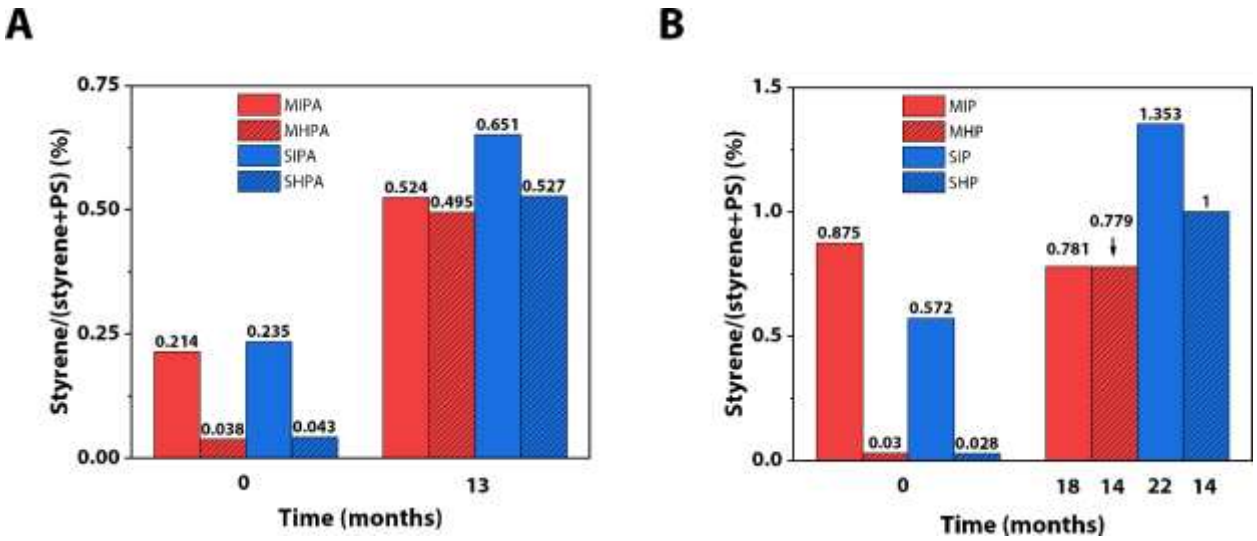


**Figure S6.** Residual styrene concentrations in PS particles dispersions being stored in 4 °C fridge over time. Measurements were performed at room temperature. (A) residual styrene in PS dispersions used in MTT assays. (B) residual styrene in PS dispersions used in acute immobilization test of daphnids. Numbers in horizontal axis such as 13, 14 are the duration (unit: months) of PS dispersions being stored when measurements were taken.

**Table S1.** Residual styrene contents at given PS concentrations of PS particles dispersions being stored in 4 °C fridge over time. Measurements were performed at room temperature.

| Nomenclature | PS ($\mu$g/mL) | Styrene (%) | Residual styrene at varying PS ($\mu$g/mL) | | | |
|---|---|---|---|---|---|---|
| | | | 300 | 500 | 1000 | 1900 |
| MIPA | 30500 | 0.214 | 0.64 | 1.07 | 2.14 | 4.07 |
| MHPA | 21030 | 0.038 | 0.11 | 0.19 | 0.38 | 0.71 |
| SIPA | 20780 | 0.235 | 0.71 | 1.18 | 2.35 | 4.47 |
| SHPA | 17260 | 0.043 | 0.13 | 0.22 | 0.43 | 0.83 |
| MIPA$^{13}$ | 31710 | 0.524 | 1.58 | 2.63 | 5.27 | 10.01 |
| MHPA$^{13}$ | 17840 | 0.495 | 1.49 | 2.49 | 4.97 | 9.45 |
| SIPA$^{13}$ | 19930 | 0.651 | 1.97 | 3.28 | 6.55 | 12.45 |
| SHPA$^{13}$* | 15810 | 0.527 | 1.59 | 2.65 | 5.30 | 10.07 |
| MIP | 27400 | 0.875 | 2.65 | 4.41 | 8.82 | 16.77 |
| MHP | 17300 | 0.030 | 0.09 | 0.15 | 0.30 | 0.56 |
| SIP | 19360 | 0.572 | 1.73 | 2.88 | 5.75 | 10.93 |
| SHP | 15510 | 0.028 | 0.08 | 0.14 | 0.28 | 0.53 |
| MIP$^{18}$ | 30250 | 0.781 | 2.36 | 3.94 | 7.87 | 14.96 |
| MHP$^{14}$ | 16280 | 0.779 | 2.36 | 3.93 | 7.85 | 14.92 |
| SIP$^{22}$ | 20200 | 1.353 | 4.11 | 6.86 | 13.72 | 26.06 |
| SHP$^{14}$* | 15630 | 1.000 | 3.03 | 5.05 | 10.10 | 19.19 |

* The superscript of 13 or 14 in the nomenclature for instance SHPA$^{13}$ or SHP$^{14}$ is the duration (unit: months) of SHPA or SHP being stored when measurements were taken.

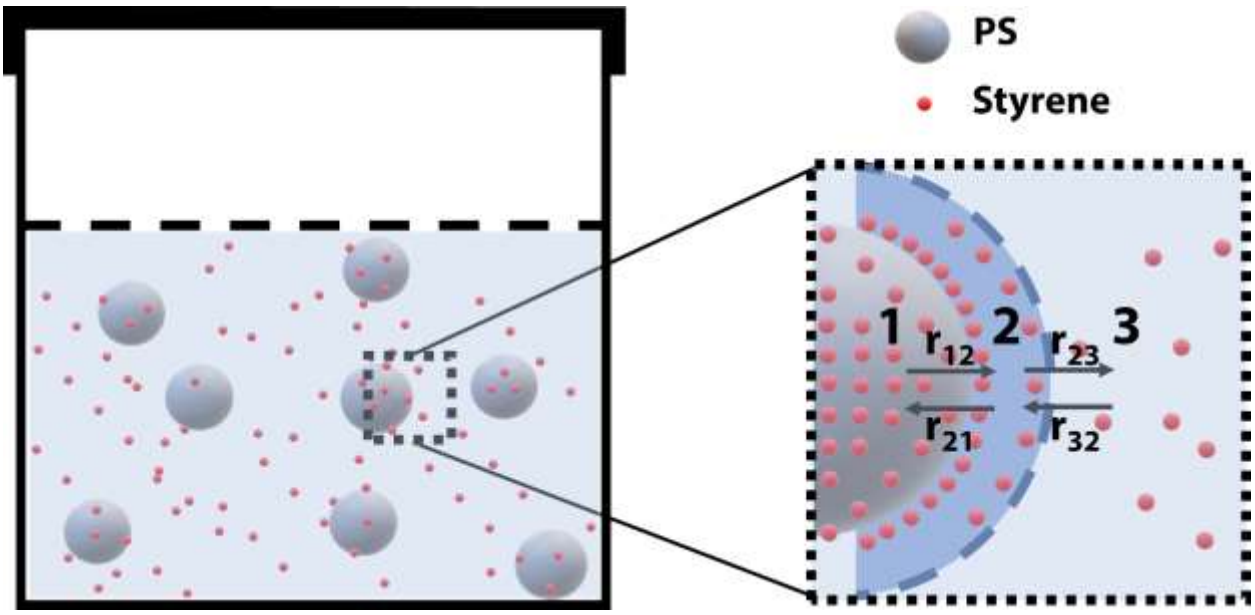


**Figure S7.** States of styrene in aqueous dispersions of PS particles and a proposed mechanism of styrene leaching. In a single PS particle and its vicinity, Zone1 (Z1) is the internal area of the particle, Zone2 (Z2) is the particle surface, Zone3 (Z3) is aqueous area out of Z2. If equilibrium is reached, the boundary of Z2 and Z3 is represented with a virtual dash line from which the rate of styrene diffusion to Z2 and to Z3 is equal, i.e., $r_{23} = r_{32}$. Diffusion of styrene takes place between Z1-Z2 and Z2-Z3. The "free" styrene, forming styrene-water complexes, are in Z3 and the boundary of Z2-Z3, while the "trapped" styrene, forming PS-styrene complexes, are in Z1 and Z2. Styrene diffuses along two paths, from Z1 to Z2 and to Z3, it is leaching of styrene from PS particles to water medium (abbreviated as $L_{PS\text{-}H_2O}$); or from Z3 to Z2 and to Z1, it is absorbing of styrene from water medium to PS particles (abbreviated as $A_{H_2O\text{-}PS}$). $L_{PS\text{-}H_2O}$ and $A_{H_2O\text{-}PS}$ take place simultaneously. The overall outcome of diffusion, either $L_{PS\text{-}H_2O}$ or $A_{H_2O\text{-}PS}$, is governed by the rate of diffusion. If $r_{12} > r_{21}$ and $r_{23} > r_{32}$, then it is $L_{PS\text{-}H_2O}$, or $A_{H_2O\text{-}PS}$ *vice versa*. The diffusion rate of styrene depends on the difference of styrene concentrations $C_1$, $C_2$, $C_3$ in different zones. The number of styrene molecules and the size of the PS particles in this diagram do not represent the real ratio or concentration.

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