Electronic supplementary information (ESI)

Correlation between the stability and toxicity of PFAS-nanoplastic colloids†

Katalin Viktória Bere,[a⌀] Zsolt Csenki-Bakos,[b⌀] Edit Kaszab,[c] Béla Urbányi,[d] István Szabó,[b*] and Istvan Szilagyi[a*]

[a]MTA-SZTE Momentum Biocolloids Research Group, Department of Physical Chemistry and Materials Science, Interdisciplinary Centre of Excellence, University of Szeged, 6720 Szeged, Hungary

[b]Department of Environmental Toxicology, Institute of Aquaculture and Environmental Safety, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary

[c]Department of Environmental Safety, Institute of Aquaculture and Environmental Safety, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary

[d]Department of Aquaculture, Institute of Aquaculture and Environmental Safety, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary

[⌀]Co-first authors.

*Corresponding authors.

† The electronic supplementary information (ESI) is available. See DOI:…

S1 Materials and methods

S1.1. Chemicals

Styrene (99 %) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AIBA) initiator (≥98.0 %) were bought from Acros Organics (Geel, Belgium). Laboratory grade HCl (37 %), NaCl (~99.5 %), and absolute ethanol (≥99.8 %) were obtained from VWR International (Debrecen, Hungary), while perfluorohexanoic acid (PFHxA, ≥97.0 %) was purchased from Sigma-Aldrich (MO, USA). Hellmanex III used as cleaning agent was acquired from Hellma (Müllheim, Germany). The samples were prepared with ultrapure water from an Adrona system (Riga, Latvia). The water for light scattering measurements was filtered with PVDF-based 0.1 μm syringe filters (Millex-VV, MilliporeSigma, Budapest, Hungary).

S1.2. Synthesis of NPL

The synthesis of the amidine functionalized polystyrene latex particles (NPLs) was performed in an additive-free emulsion polymerization with AIBA as initiator.[1] First, 375 mL water was stirred for 15 minutes and the temperature was increased to 80 °C followed by addition of 10 g of styrene monomer and stirring for another 15 minutes. Thereafter, 0.2 g initiator was dissolved in 115 mL deionized water and added to the reaction mixture. The temperature was kept at 80 °C for 24 hours under nitrogen atmosphere and then, the mixture was cooled to room temperature. The remaining monomer and AIBA were removed with repeated washing, centrifugation, and re-dispersing processes. The precipitate was washed with HCl, ethanol and water (three times 1 M HCl, once with ethanol, and three times with water) and dialyzed against water for a day. For the dialysis, cellulose ester membrane was applied with a molecular mass cut-off of 50 kg/mol (Spectrum). The final concentration of NPL was 10 g/L in the stock dispersion, which was stored in fridge thereafter.

Since AIBA was used as polymerization initiator, the particles possessed positive surface charge under the experimental conditions applied.[2]

S1.3. Electrophoretic light scattering (ELS)

The electrophoretic mobilities were determined with ELS using a Litesizer 500 device (Anton Paar, Graz, Austria) with a 700 µL Omega cuvette (Anton Paar, Graz, Austria). The light source of the instrument is a laser diode with a wavelength of 658 nm and a power of 40 mW. The applied voltage was 200 V throughout all the experiments. The 2 mL samples were prepared by mixing 1.8 mL of solutions containing calculated amount of PFHxA and/or NaCl with 0.2 mL NPL of 250 mg/L concentrations. The NPL stock dispersion was always homogenized by sonication to minimize the presence of particle aggregates. The samples were equilibrated at room temperature for 2 hours and transferred to the cuvettes. Thereafter, 8 consecutive measurements were implemented and the average of the values was reported.

S1.4. Dynamic light scattering (DLS)

A CGS-3 compact goniometer system (ALV GmbH, Langen, Germany) with a He/Ne laser of 633 nm wavelength (λ) was used to perform DLS measurements. The scattered light was collected at 90° angle (θ) and the analysis of the intensity autocorrelation function was carried out with the second order cumulant fit.[3] The translational diffusion coefficient (D) was determined from the decay rate (Γ) of the autocorrelation function:[4]

$$D = \frac{\Gamma}{q^2} \tag{S1}$$

where q is the magnitude of the scattering vector and can be calculated from the experimental setup:

$$q = \frac{4\pi n}{\lambda} sin\frac{\theta}{2} \tag{S2}$$

where n is the refractive index of the medium. Finally, the hydrodynamic radius (R_h) was obtained from the Stokes-Einstein equation:[5]

$$R_h = \frac{k_B T}{6\pi\eta D} \tag{S3}$$

where k_B is the Boltzmann constant, T is the absolute temperature and η is the dynamic viscosity of the medium.

To study the NPL aggregation quantitatively, time resolved DLS measurements were run, i.e., R_h versus time (t) data were recorded. The apparent aggregation rate constant (k_{app}) of the particle dimer formation was calculated as:

$$k_{app} = \frac{1}{N_0}\frac{1}{R_h(0)}\left(\frac{dR_h(t)}{dt}\right)_{t\to 0} \tag{S4}$$

where N_0 is the number concentration of the individual particles, $R_h(0)$ is their average hydrodynamic. The colloidal stability was expressed as stability ratio (W):[6]

$$W = \frac{k_{app,fast}}{k_{app}} \tag{S5}$$

where $k_{app,fast}$ is the apparent aggregation rate constant measured in 1 M NaCl solution. At this condition, the aggregation process is absolutely controlled by the diffusion of the particles and all particle collision results in dimer formation.[7]

During the time-resolved DLS experiments, 80 repetitions were carried out and each run was lasted for 20 seconds. The total volume of each sample was 2.0 mL in the borosilicate glass cuvettes (Kimble Chase, NJ, USA) and the particle concentration was always 25 mg/L. In a typical experiment, 1.8 mL of calculated volume of PFHxA and NaCl solutions as well as 0.2 mL of sonicated latex dispersion was mixed to reach the desired conditions. The measurements started

immediately after adding the particles and vortexing the sample. All light scattering experiments were run at 25±0.2 °C

S1.5. Raman spectroscopy

The Raman spectrum of NPL was recorded with a Bruker Senterra II Raman microscope (MA, USA) after centrifugation and drying the dispersion. The reported data were acquired by averaging 32 spectra with an exposition time of 4 seconds.

S1.6. Transmission electron microscopy (TEM)

The size and morphology of NPLs were investigated with TEM after drying the dispersions on a copper-coated carbon mesh TEM grid. The images were recorded via TECNAI G2 20 X-TWIN instrument (FEI, OR, USA) with 200 kV accelerating voltage and analyzed with the ImageJ software.

S1.7. Experiments on zebrafish embryos

Wild-type adult AB zebrafish were housed in the ZebTEC recirculating system (Tecniplast S.p.a., Italy) of the Institute of Aquaculture and Environmental Safety (MATE, Gödöllő, Hungary). Groups of fish were kept under the same microenvironmental conditions, such as 25.5 °C ± 0.5 °C, 14 hours light/10 hours dark photoperiod, pH 7.0 ± 0.2 and conductivity of 550 ± 50 µS/cm. Fish were fed with dry feed (ZEBRAFEED 400-600, Sparos Lda., Portugal) twice a day and twice a week with live *Artemia salina* nauplii. On the day before the initiation of toxicity tests, adult male and female zebrafish (3-3 each) were placed into specific breeding tanks supplied with a separator (Tecniplast S.p.a., Italy). On the day of the experiments, right after the light switched on, the separators were removed and the fish were allowed to spawn naturally. After 1 hour, eggs were collected using a tea strainer and were put into 100 mm diameter Petri dishes (Thermo Fisher Scientific, MA, USA) filled with egg water (60 mg NaCl in 1 L distilled water, pH 7.2). At about

2 hours post fertilization (hpf), normally developing (4 to 16 cell-stage) embryos were collected under a dissecting microscope (Leica Microsystems GmbH, Wetzlar, Germany) and were transferred directly into the test vessels.

For the individual substance treatments, the embryos were solely exposed to the NPLs (50; 100; 200; 300; 400; 500; 600; 700; 800; 900 and 1000 mg/L) and the PFHxA (200; 250; 300; 350; 400; 450; 500; 550 and 600 μM). In all cases, sterile egg water was used as a dilutant to prepare the solutions and no solvent was used. Embryo groups of five were treated with the listed concentrations of substances in four replicates (n=20) (ZETA (zebrafish embryo toxicity assay).[8] All tests were carried out in 24-well microtiter plates (1 mL solution/well) the entire volume of the solutions was changed daily. The test plates were kept at 26 ± 1 °C with a 14 hours-to-10 hours light-to-dark period. Mortality values for embryos were determined daily until 120 hpf on the basis of egg coagulation, the lack of somite formation and heart function. Dose-response curves were set and LC50, LC25, LC10, and LC1 values were calculated every day from the mortality percentages.

In the co-exposure experiments, NPL was applied at 25 and 100 mg/L fixed concentrations (25% of 120 hours' LC25 and LC25). Doses of PFHxA (100; 250; 300; 350 and 1000 μM) were selected based on the 120 hours' LC100, LC25, and LC1 values determined previously. In all cases, the treatment solutions were prepared in the following order: 1) PFHxA; 2) egg water; 3) NPL. The experimental setup was the same as it was described for the acute embryotoxicity test but in the co-exposure studies, three independent experiments were performed in each case. . Mortality values and sublethal effects of the treatments were determined after 120 hours of exposure. The combined effect of the mixtures was analyzed using the Combination Index (CI) method [9] based on the mortality results.

Prior to microscopic analysis, surviving embryos were anaesthetized in 0.02% MS-222 (tricaine methanesulphonate, Merck, Budapest, Hungary) solution and then, the larvae were oriented in 0.5% methyl-cellulose. Bright field images of embryos were captured in 30X magnification (Leica M205 FA, Leica DFC 425C camera, LAS X software).

Animal studies were performed in accordance with the Hungarian Animal Welfare Law (XIV-I-001/2303–4/2012) and the European directive (2010/63/EU) on the protection of animals used for scientific purposes. All experiments were completed before the treated individuals reached the free-feeding stage.

S1.8. Combination index (CI) method to determine joint toxicities

The CompuSyn software (The ComboSyn, Inc.) was used to determine CI values.[10] To determine synergism, additive effect, or antagonism, five concentration-response data points (LC10, LC20, LC50 LC80, LC95) were used for the combinations consisting of the two compounds. CI values were calculated by the software using the following equation as described by Chou[9] and Yang et al.[11] where:

$$n_{(CI)_x} = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\{[D]_j/\sum_1^n[D]\}}{(D_m)_j\{(f_a{}_x)_j/[1-(f_a{}_x)_j]\}^{1/mj}} \quad \text{(S6)}$$

where $^{n}(CI)_x$ – is the CI for n chemicals at a lethality rate of x%, $(D_x)_{1-n}$ is the sum of the concentrations of n chemicals causing a lethality rate of *x%* in the mixture, $\{[D]j/\sum_1^n[D]$ is the proportionality of the individual concentration of *n* chemical causing a lethality rate of *x%* in the mixture and $(D_m)_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/mj}$ is the concentration of each individual chemical causing a lethality rate of *x%*, where D_m is the median-effect concentration (antilog of the x-intercept of the median-effect plot), f_{ax} is the fractional lethality at *x%* inhibition, and *m* is the slope of the median-effect plot. The effects of mixtures were classified by the method of Chou[9] ranging CI as < 0.1 –

very strong synergism, 0.1-0.3 – strong synergism, 0.3-0.7 – synergism, 0.70-0.85 – moderate synergism, 0.85-0.90 – slight synergism, 0.9-1.1 – nearly additive, 1.1-1.2 – slight antagonism, 1.20-1.45 – moderate antagonism, 1.45-3.3 – antagonism, 3.3-10.0 – strong antagonism as well as >10.0 very strong antagonism.

S1.9. Statistics

Results were analyzed and graphs were plotted by GraphPad Prism 8 (GraphPad Software, CA, USA). Lethal concentration values were determined with non-linear regression model after normalization of mortality results (presented as percentages). Significant differences were verified by Kruskal-Wallis analysis with Dunn's multiple comparisons test.

Table S1. Raman spectrum peak assignment of the NPL.

Raman shift (cm^{-1})	**Assignment**
621	ring deformation
795	C-H out of plane deformation
1000	ring breathing
1030	C-H in plane deformation
1150-1200	C-C stretches
1447	CH2 scissoring
1597	C=C stretching vibration
1600	ring-skeletal stretch

Table S2. LCx ± SD values for NPL and PFHxA.

NPL	**LC1 (mg/L)**	**LC10 (mg/L)**	**LC25 (mg/L)**	**LC50 (mg/L)**
24h	322.30 ± 88.10	504.60 ± 53.50	619.60 ± 77.75	760.90 ± 155.45
48h	287.90 ± 44.25	360.20 ± 29.20	399.10 ± 20.70	442.20 ± 16.80
72h	211.80 ± 58.80	295.40 ± 42.95	344.10 ± 31.45	400.80 ± 23.85
96h	153.00 ± 28.10	229.40 ± 22.85	276.20 ± 17.70	332.50 ± 13.15
120h	-	92.11 ± 7.00	111.80 ± 6.55	135.60 ± 7.30
PFHxA	**LC1 (µM)**	**LC10 (µM)**	**LC25 (µM)**	**LC50 (µM)**
24h	252.60 ± 36.75	324.10 ± 23.10	363.40 ± 16.60	407.40 ± 18.10
48h	284.90 ± 28.55	338.10 ± 17.35	365.70 ± 11.85	395.60 ± 9.75
72h	291.20 ± 38.40	336.10 ± 22.80	359.00 ± 14.70	383.40 ± 11.05
96h	304.90 ± 28.60	337.90 ± 17.00	354.20 ± 11.50	371.20 ± 9.35
120h	292.30 ± 35.20	329.90 ± 20.30	348.70 ± 13.35	368.60 ± 9.45

Table S3. Mortality and combination index data at different NPL and PFHxA doses.

Sample	**Mortality Replicates (%)**				**Combination Index**
NPL 100 mg/L + PFHxA 1000 μM	100	100	100	100	0.63 (synergism)
NPL 100 mg/L + PFHxA 350 μM	100	100	100	100	0.78 (moderate synergism)
NPL 100 mg/L + PFHxA 300 μM	60	60	60	80	0.95 (nearly additive)
NPL 100 mg/L + PFHxA 250 μM	60	40	60	60	1.16 (slight antagonism)
NPL 100 mg/L + PFHxA 100 μM	0	20	20	0	1.31 (moderate antagonism)
NPL 25 mg/L + PFHxA 1000 μM	100	100	100	100	0.30 (synergism)
NPL 25 mg/L + PFHxA 350 μM	100	100	100	100	0.35 (synergism)
NPL 25 mg/L + PFHxA 300 μM	80	80	80	100	0.40 (synergism)
NPL 25 mg/L + PFHxA 250 μM	40	40	40	20	0.46 (synergism)
NPL 25 mg/L + PFHxA 100 μM	0	20	0	0	0.50 (synergism)
PFHxA 1000 μM	100	100	100	100	N/A
PFHxA 350 μM	0	20	40	40	N/A
PFHxA 300 μM	0	20	20	0	N/A
PFHxA 250 μM	0	0	0	0	N/A
PFHxA 100 μM	0	0	0	0	N/A
NPL 100 mg/L	20	40	0	20.	N/A
NPL 25 mg/L	0	0	0	0	N/A

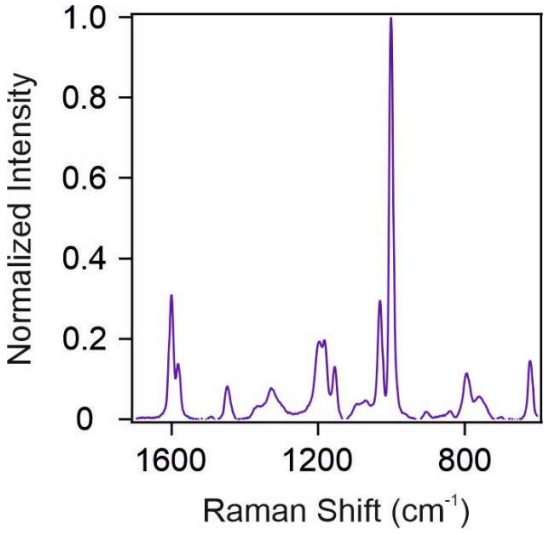

Fig. S1 Raman spectra of the synthesized NPL particles.

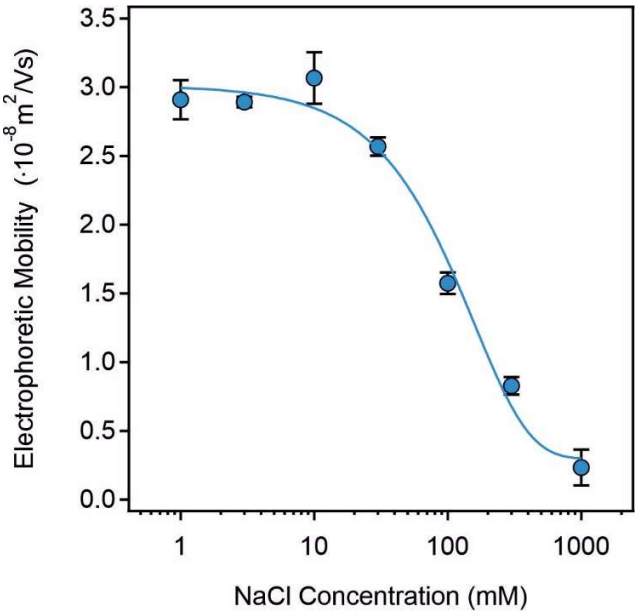

Fig. S2 Electrophoretic mobility of NPL as a function of the NaCl concentration at pH 4.

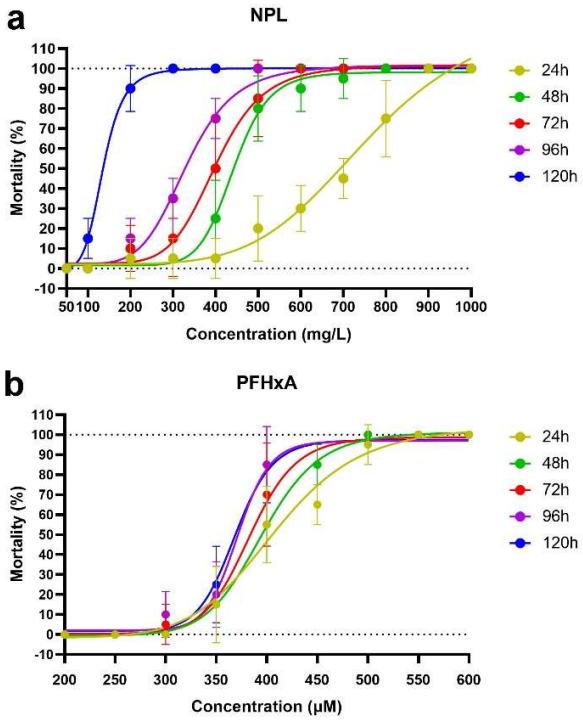

Fig. S3 Concentration-response curves for lethality at 24, 48, 72, 96 and 120 hpf zebrafish embryos for NPL (a) and PFHxA (b).

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