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Synthesis of $^{14}\text{C}$-labelled polystyrene nanoplastics for environmental studies

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Available analytical methods cannot detect nanoplastics at environmentally realistic concentrations in complex matrices such as biological tissues. Here, we describe a one-step polymerization method, allowing direct radiolabeling of a sulfonate end-capped nano-sized polystyrene (nPS; proposed as a model nanoplastic particle representing negatively charged nanoplastics). The method, which produces nanoplastics trackable in simulated environmental settings which have already been used to investigate the behavior of a nanoplastic in vivo in a bivalve mollusc, was developed, optimized and successfully applied to synthesis of $^{14}\text{C}$-labeled nPS of different sizes. In addition to a description of the method of synthesis, we describe the details for quantification, mass balance and recovery of the labelled particles from complex matrices offered by the radiolabelling approach. The radiolabelling approach described here, coupled to use of a highly sensitive autoradiographic method for monitoring nanoplastic body burden and distributions, may provide a valuable procedure for investigating the environmental pathways followed by negatively charged nanoplastics at low predicted environmental concentrations. Whether the behaviour of the synthetic nPS manufactured here, synthesised using a very common initiator, represents that of manufactured nPS found in the environment, remains to be seen.

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**Results and discussion**

**Radioabelling strategy.** As described in Fig. 1, the reaction was initiated by formation of a KPS radical at 65–75 °C in water (Fig. 1a, 1). The presence of such a radical initiated the formation of styrene radicals (Fig. 1a, 2), which created a polymerisation cascade (Fig. 1a, 2).

In this study, nominal 20 nm nPS was formed by the polymerisation of 14C-styrene in the presence of SDS in an emulsion of water and hexane (7:3), mixed by mechanical shear (i.e. 150 r.p.m.). SDS reduced the surface tension of water, producing nano-sized particles of polystyrene, defined by micro-emulsions with dispersed domain diameters varying from 1 to 100 nm. Thus, in the presence of SDS, nPS monomers nucleated inside SDS-micelles swollen with the input of monomers, reaching sizes up to 100 nm (Fig. 1b). In the absence of surfactants, the nucleation is thought to occur by the collapsing of water-insoluble polymer chains, forming precursor particles.

These unstable precursors (i.e. solidified nanoparticles of ~10 nm) that become monomers of larger particles are believed to aggregate until they produce enough surface charge to be individually stable (Fig. 1c). Such aggregates can then swell by incorporating more monomers, forming mature particles (up to ~500 nm). Both sizes of particles are colloidally stabilised electrostatically by the persulfate end groups of the initiator at the surface of the particles.

**Syntheses optimisation.** Syntheses of both sizes of non-labelled nPS (i.e. 20 and 250 nm) were also performed with 14C-styrene in order to provide materials for transmission electron microscopy (TEM) measurements. (Safety regulations prevented use of radioactive nPS for TEM investigations.) Results from TEM analyses indicated that the size of the nominally 20 nm polystyrene particles (nPS20) remained fairly constant, even when the concentrations of styrene or SDS were changed substantially (Fig. 2a, c). Particles reached an overall average size of 20.6 ± 4.9 nm (n > 100) over the different styrene concentrations: 24.8 ± 12.8 (n > 100) with changes in SDS and 21.6 ± 3.0 nm (n > 100) with changes in SDS concentrations. Interestingly, at the highest KPS concentration of 50 mM (Fig. 2b), the particles showed a tendency to reach larger sizes (47.5 ± 9.3 nm), which might be due to the high concentrations of initiator, perhaps initiating more polymerisation and increasing the aggregation of nucleus per drop of monomer. Also, according to the results of dynamic light scattering (DLS) measurements, particles formed at low styrene concentrations were estimated at about 70 nm, whereas they were measured at about 20 nm by TEM. Such results show that the particles probably clustered when produced under such conditions.

The larger nPS particles were produced using a technique employed industrially to produce polymeric particles with large diameters (i.e. d > 100 nm) and narrow size distributions. The
syntheses of these larger polystyrene particles (nominally nPS$_{250}$) showed that, over all the synthesis conditions tested, particles reached minimum sizes of 198 ± 43 nm, with 1 wt% styrene, as measured by TEM (Fig. 2d), but the average size also increased with increases in the concentration of KPS, up to 451.9 ± 186.8 nm (Fig. 2e) where the concentration of initiator (i.e. KPS) promoted particle growth. The absence of surfactant in the syntheses of nPS$_{250}$ simplified the synthesis (i.e. no nitrogen atmosphere was needed).

**Particles purification and characterisation.** To avoid potential toxicological effects associated with SDS and unreacted...
chemicals (e.g. KPS and styrene monomer), purification by dialysis (exclusion size of membrane 30,000 g mol$^{-1}$, ca. $d = 2$ nm$^{27}$) was performed until the conductivity of the surrounding water remained constant at a value of $<1.5 \mu$S cm$^{-1}$ and $^{14}$C counting reached background levels. Interestingly, keeping a KPS concentration below ~15 mM also allowed the preparation of particles with a narrow size distribution (i.e. a range of SD < 30 nm), which is uncommon in systems with no stabilising agent$^{24-28}$.

Besides the potential to produce NP for ecotoxicological studies, the strength of the method lies in its simplicity and robustness: the method corresponds to a one-step synthesis and requires only a 20 mL vial sealed with a rubber septum, a magnetic stirrer and an oil bath. Following the TEM measurements of unlabelled styrene and optimisation of the reaction conditions (Fig. 2), nPS particles were produced using $^{14}$C-styrene. Based on the activity measured in the reaction medium before and after synthesis and purification, the yields for ‘small’ and ‘large’ nPS were estimated at 98% and 99%, respectively. A specific activity of 0.16 MBq$^{14}$C/mgC NP was found after synthesis and the nPS suspensions were stable for weeks (i.e. no deposition was observed). The particles were stabilised by the negative charges of the sulfonic groups (i.e. derived from the initiator). At pH 6 in sodium chloride (5 mM), their $\xi$-potentials were $-129 \pm 10$ and $-83.6 \pm 11.9$ mV, while their sizes, as determined by DLS performed on a dedicated Zetasizer (Malvern Panalytic®), were 146 ± 29 and 275.9 ± 55 nm, for nominal $^{14}$C-nPS$_{20}$ and...
14C-nPS\textsubscript{250}, respectively. These measurements, as expected, almost certainly overestimated the particle sizes. DLS intensity values tend to overestimate particle size and results are influenced by the few large aggregates and/or clusters that likely occur. The TEM images clearly showed that ~20 nm particles were indeed produced (Fig. 2), but in the absence of surfactant, several clustered and may have been measured as one by DLS, explaining the systematic offset of these data, but in order to give further details of the measurement methods, we present additional, unpublished images, from the same experiments herein. Tissue sections were collected in duplicate. The autoradiogram produced from a scallop collected after 6 h exposure to waterborne nominal 14C-nPS\textsubscript{20} is shown in Fig. 3 and illustrates a tissue distribution typical of the duplicates. Most tissues were labelled (including muscles, gonads, mantle, gills, intestine and kidney), but the hepatopancreas exhibited the highest 14C concentration.

These and previous data\textsuperscript{2} indicate the great advantage of QWBA analysis, viz applicability to the study of anatomical structures, including those which may be very difficult to dissect (e.g. due to their small sizes, their location, or fragility). To achieve such quantitative analyses herein, calibration curves were produced according to a standard operation procedure\textsuperscript{11,29} using 20 different screens on which were randomly distributed all the scallop and 14C standard sections (both in CMC gel sections of 50 μm produced by the same procedure using a cryomicrotome). This corresponds to 200 calibration data points. The digital light intensity was plotted against the 14C concentrations of the standard spots. The limits of detection (LOD; 0.19 Bq g\textsuperscript{-1} = 0.28 ng g\textsuperscript{-1} = 6.4 × 10\textsuperscript{-6} nPS\textsubscript{250} particles g\textsuperscript{-1} = 3.3 × 10\textsuperscript{4} nPS\textsubscript{250} particles g\textsuperscript{-1} and quantification (LOQ; 0.56 Bq g\textsuperscript{-1} = 0.85 ng g\textsuperscript{-1} = 1.3 × 10\textsuperscript{4} nPS\textsubscript{200} particles g\textsuperscript{-1} = 6.5 × 10\textsuperscript{4} nPS\textsubscript{250} particles g\textsuperscript{-1} of nPS particles were calculated as corresponding to 3 and 10 times the standard deviations (SD) of the intercepts of the calibration curves, respectively. The phosphor screens used in QWBA are 20 times more sensitive than X-ray films and their linear dynamic range extends over four to five orders of magnitude, allowing capture and quantification of both very low and very high activities, in a single exposure.\textsuperscript{30} Quantification data from two scallops per aquarium for three different replicates (i.e. six scallops) for 14C-nPS obtained by QWBA analyses of the different organs (shown in Fig. 3) are given in Table 1. The use of 14C-labelled nPS allowed a mass balance and indicated that recoveries of 81–84% were possible (Table 2). We suspect that the remaining 16.0–18.6% of the 14C-nPS had become incorporated into the faeces or pseudofaeces, or both. One could quantify the amount of particles ending in this compartment, or on the aquarium walls, by collecting them and producing wipes of the walls that could be measured by liquid scintillation counting (LSC).

In summary, a method was developed herein which allowed efficient one-step synthesis of radiolabelled sulfonate end-capped plastic nanoparticles (nPS). Particle synthesis conditions from earlier studies of non-labelled nPS\textsuperscript{26,31} were improved in order that only small amounts of expensive isotopically labelled monomers were needed. Also, a better control over nPS sizes was obtained and the method provided 14C-nPS with a specific activity suitable for studies at predicted environmentally relevant concentrations in the parts per billion range\textsuperscript{11,12}. As an illustrative example, data showed that waterborne 14C-nPS were readily taken up by the scallop, P. maximus. Autoradiograms revealed a high accumulation of the radiolabelled nPS in the digestive gland. Using QWBA, the quantification of uptake, elimination and biodistribution of plastic particles proved possible, as published in detail elsewhere\textsuperscript{11}. The use of a radionuclide also allowed characterisation of the biodistributions in small and fragile organs in the scallop, such as the kidney, anus and intestine. Such detailed distribution images will be highly valuable for improving the understanding of the physiological processes governing the bioaccumulation of micro- and nanoparticles in marine bivalves and other organisms and may help to guide further research work. For example, fundamental biotic and abiotic mechanisms of degradation and assimilation of NP may be studied at environmentally relevant concentrations.

**Methods**

**Chemicals and reagents.** Styrene (≥99%, extra pure, stabilised) from ACROS Organics™ was used without purification. Styrene [methylene-14C] in hexane with a specific activity of 2.22 GBq mmol\textsuperscript{-1} (Mw = 106.14 g mol\textsuperscript{-1}, American radiolabelled chemicals Inc.) was used as received. Sodium dodecyl sulfate (SDS, ≥99%) obtained from Alfa Aesar™ was used without purification. Potassium persulfate (KPS, ≥99%, ACS reagent) from ACROS organics™ was purified by crystallisation in water. Sodium hydroxide (NaOH, ≥98%) from Honeywell Fulka™. Water used in...
In a second step (Exp 1.2), to assess the impact of the limiting amount of 14C-styrene on particle size, the concentration of styrene ([Sty]) was considered. Consequently, styrene was decreased from 7.8% to 1 wt% (i.e. 7.8, 5, 3, 2, 1 wt%). Emulsion system was fixed with water 90.2 wt%, SDS 2 wt%, and KPS 3.6 mM at 70 °C.

In a third step (Exp 1.3), several concentrations of the initiator KPS ([KPS]) were tested as the following: 0.5, 1, 3.6, 14.6, 50 mM with the emulsion system fixed at styrene 1 wt%, SDS 2 wt%, and KPS 3.6 mM at 70 °C. The solution turned white with the formation of nPS when synthesis was complete. The initial synthesis (Exp 1.1) was a conventional emulsion system with styrene 7.8 wt%, SDS 2 wt%, water 90.2 wt%, and KPS 3.6 mM was also polymerised at 70 °C.

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In a second step (Exp 2.2), to assess the impact of the limiting amount of 14C-styrene on particle size, the concentration of styrene ([Sty]) was considered. Concentrations tested were 1, 2, 3, 5 and 20%. The system was fixed with NaOH 2 wt%, water 76 wt% and KPS 14.6 mM at 70 °C.

In a third step (Exp 2.3), several concentrations of the initiator KPS ([KPS]) were tested as the following: 0.07, 2.7, 14.6 and 50 mM. The system was fixed with styrene 1 wt%, NaOH 2 wt% and water 76 wt% at 70 °C.

Radiolabelling synthesis of 20 and 250 nm nPS (Exp 3) used the optimised parameters from Exp 1 and 2. For comparison both size target and radiolabelling purpose, styrene proportion was decreased at 1 wt% and the volume of synthesis at 10 mL. Both synthesised, the reactions mixture was vigorously stirred at 70 °C overnight.

Radiolabelled 20 nm nPS (14C-nPS20) was prepared (Exp 3.1) by a conventional emulsion system with styrene 1 wt%, SDS 2 wt%, water 90.2 wt%, and KPS 3.60 mM in a 40 mL vial closed with a rubber cap. The vial was purged with nitrogen gas for 30 s and then heated at 70 °C. Once 70 °C was reached, 14C-styrene was a dropwise addition with a syringe. The solution turned white with the formation of nPS when synthesis was successful.

Radiolabelled 250 nm nPS (14C-nPS250) was prepared (Exp 3.2) by a surfactant-free emulsion polymerisation. The initiator solution was prepared separately with KPS 3 wt% (14.66 mM in the final water phase), NaOH 2 wt% (56.7 mM in the final water phase), and Milli-Q water 17 wt% were mixed in a glass vial. Styrene 1 wt% and Milli-Q water 59 wt% were mixed at room temperature in a 40 mL vial. The vial was sealed with a rubber septum. The headspace inside the flask was then purged with nitrogen for 30 s. The vial was immersed in a silicone oil bath at 70 °C for 3 min, with stirring. In the meanwhile, the vial medium reached 70 °C. The initiator solution was injected through the rubber septum, using a syringe.

Purification of PS latexes. All chemicals unreacted were systematically removed by ultrafiltration (exclusion size of membrane: 30 000 g mol−1) with a dialysis membrane. Water was frequently changed for 48 h, until the conductivity of water remained constant at a value of <1.5 µS cm−1.

DLS, surface charge (ζ-potential) (Supplementary Table 1). After completion of the polymerisation reaction, the I hydrodynamic diameters of the latices were characterised in water at 20 °C. The average sizes and ζ-potentials of the polymer particles were measured using a multi-angle Nicomp DLS Z3000 (Particle Sizing System, Port Richey, FL). DLS measurements give a value called ζ-average size (or cumulative mean), which is an intensity mean, and the polydispersity index. The latex dispersions are optimised for the DLS measurements by diluting the crude, concentrated, reaction product with an excess of Milli-Q water until the count rate of the resulting mixture is within the optimal range. The final concentrations were typically about 1 mg L−1 and can be estimated from the concentration of the primary emulsion. Each sample was analysed at least five times.
Transmission and scanning electronic microscopy (TEM, STEM) (Supplementary Figs. 1 and 2 and Table 2). All particle morphology and size measurements were recorded with a JEM-TEM-14000 transmission electron microscope at Plymouth Electron Microscopy Centre (PlyEM, University of Plymouth, United Kingdom, operated at an acceleration voltage of 80 kV) and with a scanning transmission electron microscope (STEM, HD-2700-Cs, Hitachi, Japan, ETH Zurich, operated at an acceleration voltage of 200 kV). Samples were prepared and dried at ambient temperature under a laminar flow fume hood. Each latex sample was diluted to about 0.01% solid content and a drop was placed onto a carbon-coated copper grid 200 mesh. Grids were then rinsed five times in five different drops of Milli-Q water. Grids were kept in a desiccator cabinet to remove all humidity overnight. Additional contrasting agents were not applied. At least 10 TEM images were obtained from at least 10 different areas on each grid. The diameters of at least 100 particles in each sample were determined on TEM images recorded using ImageJ software (open source, https://image.nih.gov/ij/).

Fourier transform infrared (FTIR) spectroscopy (Supplementary Fig. 3). The polymers were characterised by FTIR spectroscopy (Alpha FTIR, Bruker) working in Attenuated Total Reflectance (ATR) mode with a DTGS detector. Spectra were recorded with 16 scans at 4 cm\(^{-1}\) resolution, covering the spectral range between 4000 and 600 cm\(^{-1}\). FTIR measurements were performed to confirm polymerisation success and library spectral matching was used to identify each product.

Yield determinations. To assess synthesis yields, all masses were measured before and after synthesis for the cold synthesis. Comparison with the yields of the radiolabelled synthesis were made by measuring radioactivity before synthesis and after the purification step.

Scallop exposure design. In a preliminary experiment aimed at verifying the usefulness of radiolabelled nPS for bioaccumulation studies, a group of 12 king scallops was exposed to phosphor screens sensitive to beta radiation for 1 week and scanned using Transmission and Scanning Electron Microscopy (TEM, STEM) (Supplementary Fig. 3).

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Author contributions
M.A.-S.-C., S.J.R., R.C.T. and T.B.H. planned and designed the research. M.A.-S.-C. performed the radiolabelling, histological preparations and analytical measurements. M.A.-S.-C. and M.-A.C. performed exposure experiments and sample preparation. M.A.-S.-C. analysed data. M.A.-S.-C. and R.K. performed T.E.M. and S.E.M. and analysed images. M.A.-S.-C., S.J.R., R.C.T., T.B.H., R.K. and M.-A.C. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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