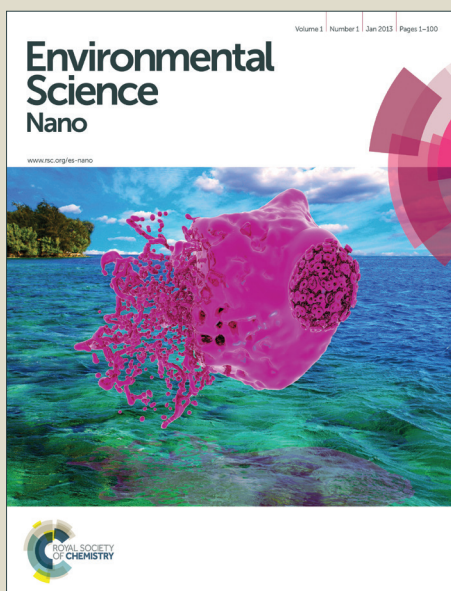


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ARTICLE

Colloidal stability of (functionalised) fullerenes in the presence of dissolved organic carbon and electrolytes

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Carbon-based nanoparticles such as fullerenes have been widely applied in personal care products, drug delivery systems, and solar cells. The properties of nanoparticles have been increasingly studied because of their applications and their potential risks to the environment and human health. Many studies have focused on the environmental fate and properties of C₆₀. However, there is currently limited information available on the environmental properties of functionalised fullerenes. This study focuses on the colloidal stability of two fullerenes (C₆₀ and [6,6]-diphenyl-C₆₂-bis(butyric acid methyl ester)) in water in the presence of dissolved organic carbon (DOC) and different electrolytes (NaCl and CaCl₂). Suspended fullerene concentrations were determined with high resolution Orbitrap mass spectrometry. The size was determined by multi-angle light scattering, dynamic light scattering and for the first time flow cytometry. The suspended concentrations of the fullerenes were stabilised by low concentrations of DOC (2 mg C/L) in the presence of NaCl. However, sedimentation of DOC occurred at low concentrations of CaCl₂ (>~ 2 mM) which caused removal of (functionalised) fullerenes. The results show that (functionalised) fullerenes can be rapidly removed in natural aqueous systems in the presence of low concentrations of DOC and multivalent inorganic electrolytes.

Nano Impact

Understanding of the fate and behaviour of nanoparticles is an important part of risk assessment. It is therefore imperative that the influence of various parameters on the stability and size of fullerene clusters in aqueous matrices is properly understood. To achieve this, the influence of humic acid, sodium and calcium on the size of three different fullerenes is investigated. The clusters were analysed using DLS, FFF-MALS and flow cytometry. These data will help to ease the estimation of fullerene behaviour in complex aqueous matrices.

Introduction

Fullerenes or buckyballs and their functionalised forms have been used in personal care products,¹ biomedical applications such as drugs delivery systems in cancer therapy² and in electronic applications as an *n*-type semiconductor in polymer composites in organic photovoltaic cells.³ Fullerenes also occur as a result of natural processes, see references cited in Isaacson *et al.*⁴ Bare fullerene molecules have their carbon atoms in a

symmetrical cage structure consisting of hexagons and pentagons, for C₆₀ with a total diameter of about 1 nm.⁵ Functionalised fullerenes such as [6,6]-diphenyl-C₆₂-bis(butyric acid methyl ester) (C₆₀BisPCBM) are derivatised with polar functional groups to increase their solubility and to improve their electronic properties for application in organic photovoltaic cells.

Fullerenes have shown toxic effects in bacteria,⁶ fish,⁷ and human cell lines.⁸ Fullerenes exhibit a very low solubility in

water (calculated aqueous solubilities of C_{60} are $< 8 \text{ ng/L}$)^{9, 10, 9, 10} However, upon entering water, fullerenes (as C_{60}) can form stable aqueous suspensions (denoted as nC_{60}) with physicochemical properties that are different from C_{60} in the condensed phase.⁴ Water-stable agglomerates with negative charge are formed over time with increasing pH,^{11, 12} decreasing ionic strength,^{11, 13} higher initial fullerene concentration,¹⁴ and longer storage time.¹³ These properties in aqueous suspension such as particle size and shape are also dependent on the way of preparing fullerene suspensions, i.e., by extended stirring, sonication, or solvent exchange. Sonication and solvent exchange result in more spherical shapes and a homodisperse size distribution, while extended stirring (for two to seven weeks) leads to more angular shapes and a polydisperse size distribution.^{11, 14} For this reason, sonication and solvent exchange were chosen in this study to prepare fullerene suspensions.

The colloidal stability of fullerenes in the environment is affected by a multitude of factors such as ionic strength of the solution and the nature of the ions present, humic acids, and the charge of the fullerenes (zeta potential). The relevant factors involved in the stabilization and agglomeration of fullerene suspensions in time are considered important for an improved understanding and assessment of the fate of (functionalised) fullerenes in the environment and in waste water and drinking water treatment.¹⁵ This study focuses on the colloidal stability of nC_{60} and a functionalised product of C_{60} (nC_{60} BisPCBM) in the presence of dissolved organic carbon (DOC) and different electrolytes. The functionalised product was selected to gain more insight into the influence of functional groups on the colloidal stability. Previously, it was shown by Bouchard *et al.* that functionalised fullerenes exhibit a higher critical coagulation concentration (CCC) – and thus a higher colloidal stability – compared to bare fullerene molecules.²³ This was probably caused by either steric hindrance of the side-group or different particle-particle interaction energies. The present study complements the results of Bouchard *et al.* by investigating the effect of DOC on functionalised fullerenes.

The ionic strength of fullerene suspensions with and without DOC was varied by increasing monovalent and divalent inorganic cation concentrations (NaCl and CaCl_2). Fullerene concentrations were measured in time with liquid chromatography coupled to high resolution mass spectrometry. Particle size analysis of the suspensions was performed with dynamic light scattering (DLS) and asymmetric flow-field-flow fractionation coupled to multi-angle light scattering (AF4-MALS). In addition, the influence of NaCl on the fullerene clusters was measured using flow cytometry, a technique commonly used in medical science and biotechnology for cell analysis. To the best of our knowledge this is the first time that fullerene aggregates were characterised using this technique. Flow cytometry excels in being a fast analytical tool and can be used for characterisation of organic and inorganic suspensions.¹⁶ Although the focus of technical innovation of flow cytometry naturally lies in cell analysis, it has been used to analyse non-organic particles using basic Side-/Forward Scatter

properties with promising results. Importantly, environmental samples have been measured directly by using FCM without extensive sample pre-treatment steps and the reduction of measuring time.¹⁷ Therefore, this technique can be a valuable tool for analysing environmental samples in the near future.

Materials and methods

Chemicals and Reagents

C_{60} ($>99.9\%$; CAS 99685-96-8; $MW = 720.660 \text{ g/mol}$) was obtained from Materials and Electrochemical Research Corporation. C_{60} PCBM ([6,6]-phenyl- C_{61} -butyric acid methyl ester; $>99\%$; CAS 160848-22-6; $MW = 910.901 \text{ g/mol}$), and C_{60} BisPCBM ([6,6]-diphenyl- C_{62} -bis(butyric acid methyl ester); $>99.5\%$; CAS 1048679-01-1; $MW = 1101.143 \text{ g/mol}$) were obtained from Solenne. C_{60} PCBM was used as a surrogate standard in the LC-MS analysis for the calibration and quantification of C_{60} and C_{60} BisPCBM. This compound was selected as surrogate standard because C_{60} PCBM eluted between C_{60} and C_{60} BisPCBM in the chromatographic analysis and mimics these compounds best in the absence of isotopically labelled standards. The molecular structures of C_{60} and C_{60} BisPCBM are shown in Figure 1. Inorganic salts (NaCl , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, NaN_3 , and NaOH) were obtained from J.T. Baker. The organic buffer 3-(N-morpholino)propanesulfonic acid (MOPS) was obtained from Sigma-Aldrich. Toluene and ethanol (HPLC grade) were obtained from J.T. Baker. Leonardite humic acid containing 63.81% organic carbon was obtained from the International Humic Substances Society. Water (resistivity $>18 \text{ M}\Omega/\text{cm}$) was obtained from a Milli-Q water purification system (Millipore).

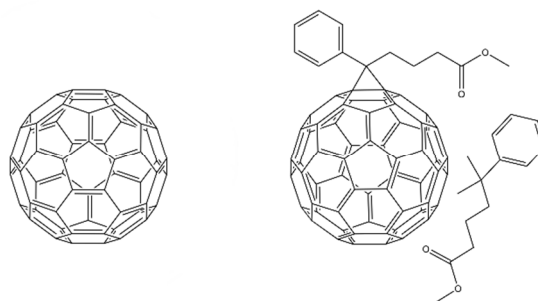


Fig. 1 Molecular structures of C_{60} and C_{60} BisPCBM ($C_{84}H_{28}O_4$). Note that C_{60} BisPCBM is a mixture of different isomers due to several possibilities to substitute the side-chain(s).

Preparation of fullerene suspensions

Aqueous suspensions of (individual) fullerenes were made by solvent exchange with toluene. Glass vials of 40 mL were filled with 30 mL of water and 3 mL of ethanol was added to the vials. The vials were placed in an ultrasonic bath (Branson 3210) that was held at a constant temperature of 30 °C. A volume of 0.5 mL (60.32 mg/L C_{60}) or 0.6 mL (61.91 mg/L C_{60} BisPCBM) fullerene solution dissolved in toluene was infused at 25 $\mu\text{L}/\text{min}$. into the vials. This produced a white emulsion that slowly converted into a transparent solution after

3–4 hours of degassing in the ultrasonic bath. The suspensions were pooled in an Erlenmeyer flask of 500 mL. The final weight of both suspensions increased by 4% (w/w) of the initial water volume. This increase was attributed to the presence of residual ethanol in the suspension(s). The solution was filtered through a Whatman GF/C glass fibre membrane filter (nominal pore size 1.2 μm) and stored at room temperature in the dark. The amounts recovered from vials, filter, and suspension were 87% for C_{60} and 85% for $\text{C}_{60}\text{BisPCBM}$. The amounts adsorbed to the glass wall of the vials, retained on the filter and suspended in solution were 32%/32%/23% for C_{60} and 40%/8%/37% for $\text{C}_{60}\text{BisPCBM}$, respectively.

Preparation of dissolved organic carbon

A stock solution of DOC was prepared by dissolved 20 mg of Leonardite humic acid in 100 mL of water adjusted to pH 11 with 1 N NaOH. The solution was stirred overnight and filtered through Pall Gelman polyethersulphone membrane filters (VWR; pore size 0.45 μm). Filters were rinsed with about 200 mL of Milli-Q water prior to filtration of the DOC solution. After filtration of 25 mL of DOC, the filter was replaced to prevent clogging of the filter. The concentration of non-purgeable organic carbon was measured with a TOC-VCPH Shimadzu combustion analyser. Treatments containing DOC were prepared by adding 0.2 to 4 mL of this stock solution to 20 mL of water (TOC concentrations ranged from 1 to 20 mg/L organic carbon).

Static equilibration of fullerene treatments

In separate experiments for C_{60} and $\text{C}_{60}\text{BisPCBM}$, a volume of 5 mL of fullerene suspension was added with a glass pipette to 20 mL clear glass vials (VWR) containing 10 mL of DOC or water with final concentrations of 1 mM NaN_3 and 10 mM MOPS (duplicate samples). The biocide was added to prevent biodegradation of DOC. A constant protonation state of DOC was ensured by addition of an organic buffer (MOPS). This buffer does not precipitate when divalent electrolytes are added to solution in contrast to e.g., inorganic phosphate buffers. The pH of the final solution was 6.94 and did not change during the course of the experiments. After 3 h of equilibration of fullerenes and DOC, the NaCl or CaCl_2 solutions were added and the vials were subsequently shaken by hand. The final electrolyte concentrations with and without DOC (2 mg C/L) were 25, 38, 59, 90, 138, 212, 326, and 500 mM for the NaCl treatments and 0.25, 0.48, 0.93, 1.80, 3.47, 6.71, 12.95, and 25 mM for the CaCl_2 treatments. Treatments containing no added electrolytes, 25 mM NaCl, and 0.93 mM CaCl_2 , respectively, were sampled in time (7 sampling times within 33 d for nC_{60} and 5 sampling times within 23 d for $\text{nC}_{60}\text{BisPCBM}$) to study the time dependence of the colloidal stability of both fullerenes in the presence or absence of DOC.

The treatments with increasing electrolyte concentrations were sampled after a static equilibration time of 3 d. This equilibration time was chosen as incubation time for the measurement of fullerene concentrations in all electrolyte and electrolyte+DOC treatments because this period is considered a

relevant environmental timeframe as residence times of water in rivers often range from several days to one month while timeframes in waste water and drinking water treatment systems range from hours to day(s) for the full treatment process. After static equilibration at room temperature in the dark, samples of 2 mL were withdrawn from the upper layer for all treatments. Liquid-liquid extraction (LLE) was performed by adding 3 mL of toluene and 1 mL of 30 g/L NaCl solution. NaCl was added to the solutions to aid in the recovery of the fullerene(s). A surrogate standard of 50 μL of C_{60}PCBM dissolved in toluene was added to the toluene phase (final concentration of 8.93 $\mu\text{g/L}$) for quantification of the fullerenes. The vials were shaken horizontally at 200 rpm for 30 min. A volume of 1 mL of the toluene phase was subsequently sampled and the samples were stored at 4 $^\circ\text{C}$ prior to LC-Orbitrap MS analysis.

Liquid chromatography coupled to mass spectrometry

A detailed description of the analysis and detection of several functionalised fullerenes (also including C_{70}) can be found in Kolkman et al.¹⁸ Quantification was based on the sum of the peak areas of the accurate masses (within a 5 ppm mass window) of the fullerene compound and all its related adducts.¹⁹ Calibration curves of C_{60} and $\text{C}_{60}\text{BisPCBM}$ were linear in the range of 0 to 32 $\mu\text{g/L}$. The limits of detection were 0.33 $\mu\text{g/L}$ for C_{60} and 0.22 $\mu\text{g/L}$ for $\text{C}_{60}\text{BisPCBM}$.

Flow-field-flow fractionation

The diameter of gyration of the fullerene aggregates was measured with a Postnova AF2000 system (Postnova Analytics GmbH) consisting of an AF4 module coupled to a UV detector (Shimadzu) and a MALS detector (Postnova). The obtained size distributions were analysed with Postnova software program version 1.1.0.31. A volume of 100 μL of sample was injected into a thin channel of water flowing over a membrane (10 kDa regenerated cellulose, Postnova) with Milli-Q as eluent. At the moment of injection, the sample was focused into a small laminar band by opposing flows. After the focus flow of 1 mL/min. stopped after 5 min, the cross-flow (1 mL/min) exponentially decreased to 0.1 mL/min at 46 min. resulting in a higher retardation of large size components. In the MALS technique, the intensity of scattered light is averaged over time in the order of 0.01 to 0.1 s and is related to the absolute molar mass that gives a root mean square radius (or radius of gyration) of the particles.

Dynamic light scattering and electrophoretic mobility

The hydrodynamic diameter was measured with DLS using a NanoZS Zetasizer from Malvern instruments equipped with a He-Ne laser operating at 633 nm. Scattered light intensities were measured at an angle of 173 $^\circ$ and a refractive index of 2.20²⁰ was used for nC_{60} and $\text{nC}_{60}\text{BisPCBM}$. The size distributions were analysed with Malvern Dispersion Technology Software version 5.10. In the DLS technique, diffusion coefficients are measured from fluctuations of scattered light in the order of micro- to milliseconds due to

Brownian motion of the particles. The hydrodynamic diameter derived from these diffusion coefficients includes solvent molecules that move with the particle. The zeta potential or electrophoretic mobility was also determined with a NanoZS Zetasizer in folded capillary cells (Malvern).

The particle size and electrophoretic mobility measurements were all made at the start of the sedimentation experiments for the stock suspensions of nC₆₀ (441 µg/L) and nC₆₀BisPCBM (718 µg/L). The stock suspensions were used because both methods required relatively high fullerene concentrations for a reliable measurement.

Flow cytometry

Fullerene suspensions in NaCl and CaCl₂ solutions were measured on a BC Gallios™ flow cytometer. The cytometer was optimized for the measurement of nanoparticles, using fluorescent dye-labeled polystyrene microspheres of 100, 300, 500 and 900 nm in size, which were obtained from BioCytex as a ‘Megamix-Plus’ kit. W² collection angle was used with forward and side scatter channels for best resolution, gain (linear amplification) values were set at 75 and 20, respectively, and both voltages were set at 700 volt. Low flow speed was used to avoid particles ‘swarming’ artifact. Integral logarithmic scale was used for data interpretation. When measuring on a nanometer scale, the ‘noise’ that is picked up by the flow cytometer overlaps with actual matter that passes the laser. Generally, this noise is blocked by setting a Forward Scatter threshold when measuring cells. However, the threshold has to be set to zero to measure small particles. To overcome this issue, noise was defined by measuring deionized water and setting a gate around the artificial events before commencing with the measurement of fullerene suspensions. During the fullerene suspension analysis all events outside of this gate were regarded as actual matter passing the laser.

Statistical and data analyses

All statistical tests were performed with Graphpad Prism 5.00. Replicate values (from different particle size methods and fullerene concentrations in different treatments) were compared with an unpaired t-test at a statistical significance of $p < 0.05$ assuming normally distributed values. Variances of compared treatments were not significantly different ($p < 0.05$).

The decrease in fullerene concentrations in the sedimentation experiments was fitted with first-order kinetics, $c_0 = c_t \times \exp(-k \times t)$, where c_0 and c_t are initial and final fullerene concentrations, respectively, and k is the sedimentation rate constant.

Results and discussion

Particle size analysis

The particle size distribution of the fullerene suspensions prepared via solvent exchange in the absence of electrolytes was measured with DLS and AF4-MALS for nC₆₀ (Figure 2A)

and nC₆₀BisPCBM (Figure 2B). The particle size distribution of nC₆₀ with DLS (measured as hydrodynamic diameter) showed a broader distribution compared to that of AF4-MALS (measured as diameter of gyration). The maximum scattering intensities measured with both techniques were similar for nC₆₀, see Figure 2A. The hydrodynamic diameter at maximum intensity of nC₆₀ was close to the diameter based on the Z-average (intensity-weighted average) calculated with the DLS software, see Table 1.

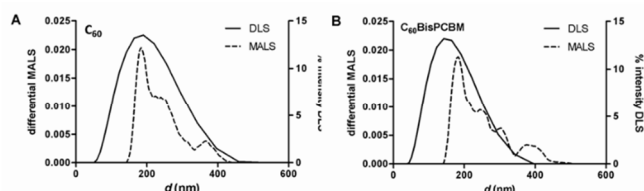


Fig. 2 Particle size distribution of nC₆₀ (A) and nC₆₀BisPCBM (B) measured with dynamic light scattering (DLS) and multi-angle light scattering (MALS).

The hydrodynamic diameter at maximum intensity of nC₆₀BisPCBM measured with DLS was significantly smaller compared to the particle diameter measured with AF4-MALS, see Figure 2B. The particle diameters determined for the nC₆₀ suspension were in agreement with intensity-weighted averages from the literature that were determined with DLS for suspensions of nC₆₀ prepared by either solvent exchange ($d = 90$ to 219 nm),^{6, 11, 14, 21-23} sonication ($d = 166$ nm)²⁴ or extended stirring methods ($d = 171$ to 193 nm).^{11, 14, 23, 25, 26} A literature value for the particle diameter of a functionalised fullerene ($d = 186$ nm for nC₆₀PCBM) prepared by extended stirring for 5 months²⁵ is also in close agreement with the values for nC₆₀BisPCBM determined in this study. The measured zeta potentials of nC₆₀ and nC₆₀BisPCBM were negative (see Table 1) and in a similar range as literature values (-47 to -30 mV) for nC₆₀ prepared by solvent exchange and sonication.^{11, 21, 23}

Table 1 Particle diameters ($d \pm$ SD) based on maximum or Z-averaged intensity measured with dynamic light scattering (DLS) and multi-angle light scattering (MALS). The zeta potential ($\zeta \pm$ SD) is measured with phase analysis light scattering (PALS). Numbers in parentheses indicate the number of measurements.

Parameter	Technique	nC ₆₀	nC ₆₀ BisPCBM
d_{\max} (in nm)	DLS	185 ± 4 (4)	154 ± 2 (4)
d_z (in nm)	DLS	174 ± 5 (4)	151 ± 5 (4)
d_{\max} (in nm)	MALS	178 ± 13 (3)	182 ± 3 (3)
ζ (in mV)	PALS	-41 ± 1 (3)	-53 ± 1 (3)

Flow cytometry

To understand the effects of salt on the size of the fullerenes aggregates, C₆₀ suspensions were further analysed using flow cytometry. This technique can detect particles and can show changes in particle size (forward scatter) and type/granularity (side scatter). The data depicted in Figure 3 shows the scatter blots for sodium solution with and without fullerenes. The blue dots are noise. Red dots are events (particles) that are measured

outside the noise gate. These particles are larger than 100 nm. It can be seen that fullerene complexes are present in the 26 mM NaCl solution that exceed the ~100 nm measurement threshold of the Gallios flow cytometer. There is already a change in the fullerene clusters being picked up by the flow cytometer between 26 mM and 86 mM NaCl in which a minor shift to larger particles can be observed (shift to larger forward scatter intensity). However, increasing the NaCl concentration by a factor of 7 to 598 mM resulted in the clear formation of larger C_{60} clusters. These signals cannot be seen in the control measurements. The same experiments were done with $CaCl_2$ (results not shown). An increase in particle size was observed. However, already in the fullerene-free solutions of $CaCl_2$ these particles were found, meaning that no information on the fullerene clusters can be gained from these experiments. These particles in the $CaCl_2$ solution are probably a result of impurities in the $CaCl_2$ salt. This observed sensitivity of the flow cytometer for the $CaCl_2$ solution suggests that in order to use FCM to directly measure fullerenes in for example environmental samples, more research is needed.

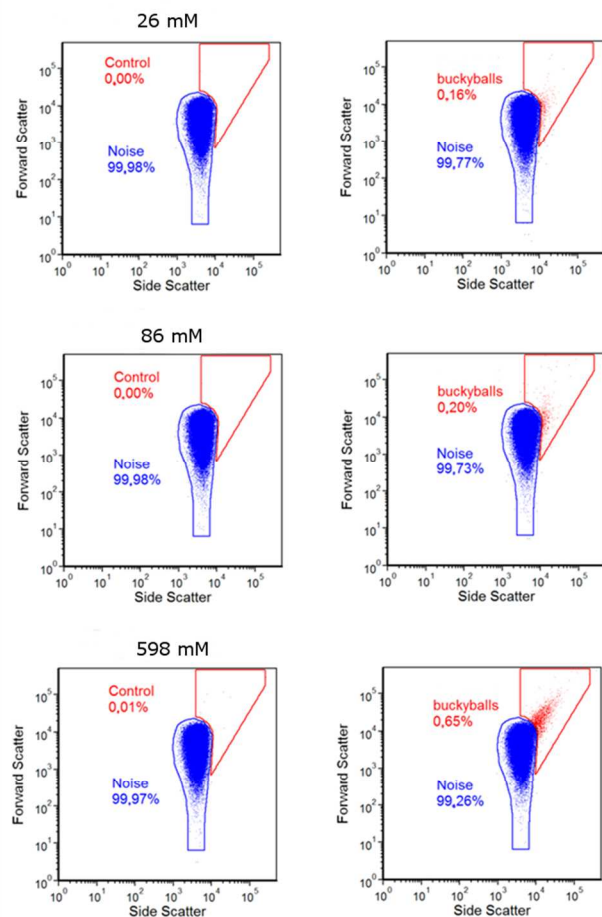


Fig. 3 Flow cytometry scatter blots: Left side shows the scatter blots for the sodium chloride solutions (blanks). Right side shows the sodium chloride solution with fullerene (buckyball) clusters. Each dot in the scatter plots represents an event (meaning the detector gives a signal) consisting of the forward scatter and side scatter information. The forward scatter signals relates to the particle size and side scatter intensity to the granularity or shape of the particle.

Extraction recovery of fullerenes

The effect of DOC on the extraction recovery of both fullerenes with LLE was studied with the following DOC concentrations: 1, 2, 5, 10, and 20 mg C/L. Sequential extraction of nC_{60} was tested for samples without DOC and with 2 and 20 mg C/L. Only 1% of C_{60} was found in the second extraction step for the samples without DOC and with 2 mg C/L, whereas 2 to 3% was found in the second extraction of the sample containing 20 mg C/L. No C_{60} could be recovered in the third extraction step with toluene of the sample with 2 mg C/L, while the sample with 20 mg C/L could not be extracted for a third time because of emulsification of the toluene-water interface. All subsequent LC-Orbitrap MS measurements of LLE samples were therefore performed by taking 1 mL from the first extraction with toluene. The extraction recoveries of nC_{60} and $nC_{60}BisPCBM$ for samples with DOC concentrations ranging from 1 to 20 mg C/L are shown in Figure 4. Recoveries of samples at relatively low concentrations of DOC were close to 100% and no corrections for recovery were therefore applied in subsequent experiments. $C_{60}BisPCBM$ showed a lower recovery of about 90% at the highest DOC concentration tested (no corrections were applied here). This was probably due to stronger interactions of the side-chains of $C_{60}BisPCBM$ with DOC.

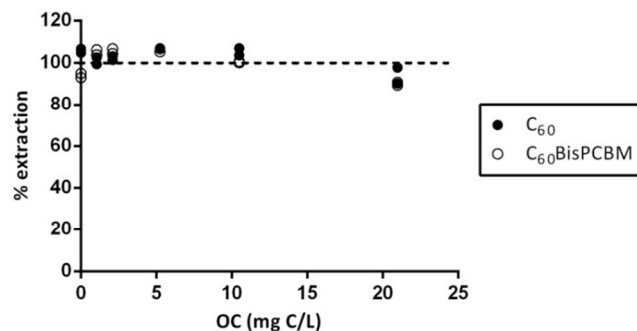


Fig. 4 Extraction recoveries of nC_{60} and $nC_{60}BisPCBM$ from an aqueous solution versus organic concentrations of Leonardite humic acid.

Time dependence of colloidal stability

The concentrations of nC_{60} were followed in time for the treatments containing 25 mM NaCl and 0.9 mM $CaCl_2$, see Figure 5A (conditions typical for freshwater). The concentrations of nC_{60} measured after 33 d in 25 mM NaCl and 0.9 mM $CaCl_2$ treatments in the absence of DOC significantly decreased to 80 ± 1 and 88 ± 1 %, respectively, compared to the treatment without electrolytes (control). Half-lives of nC_{60} due to settling, calculated via first-order kinetics ($t_{1/2} = \ln 2/k$), amounted to 61 ± 4 d for the NaCl treatment and 93 ± 9 d for the $CaCl_2$ treatment. Settling occurred because monovalent and divalent electrolytes increased the aggregate size of nC_{60} colloids (see flow cytometry data above). However, concentrations of $nC_{60}BisPCBM$ in treatments containing the same electrolyte concentrations without DOC were not affected over a period of 23 days, see Figure 5B.

Colloids of nC_{60} BisPCBM seemed to be more stable compared to nC_{60} colloids. The relative stability of nC_{60} and nC_{60} BisPCBM suspensions without DOC could be explained by the negative charges attained on the surface of the fullerene(s)¹². The treatments of nC_{60} and nC_{60} BisPCBM containing both electrolytes and DOC did not show any significant decrease (data not shown). The stability of the fullerene suspensions in the DOC treatments shows the ability of a relatively low DOC concentration (2 mg C/L) to stabilize nC_{60} colloids in aqueous solutions for prolonged periods of time.

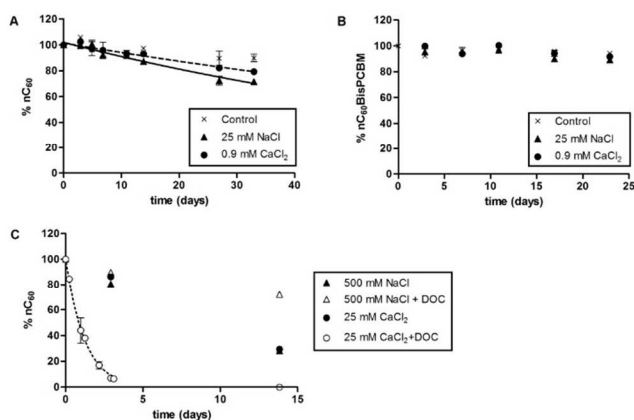


Fig. 5 Percentage of original nC_{60} (A and C) and nC_{60} BisPCBM (B) as a function of time.

Previously, agglomerate sizes of nC_{60} were observed to be smaller in the presence of Suwannee river humic acid suggesting that humic acid sterically stabilized suspensions of nC_{60} .^{26, 27} Concentrations of nC_{60} in treatments containing 500 mM NaCl (typical for seawater) in the absence of DOC were measured after 3 and 14 days. This treatment showed a strong reduction of the nC_{60} concentrations to $\sim 30\%$ from 3 to 14 days (see Figure 5C). Agglomeration and subsequent removal of nC_{60} presumably takes weeks to even months at natural freshwater DOC and electrolyte concentrations. Sedimentation of fullerenes in the absence of DOC seemed to be an on-going process, see Figure 4C for treatments without DOC. Removal of nC_{60} with DOC in treatments containing 25 mM $CaCl_2$ (typical for seawater) was however a very fast process (half-life of 0.85 ± 0.04 days, see Figure 5C) with only 6 to 8% residual nC_{60} suspended in solution after 3 days and an absence of nC_{60} in solution after 14 days. This process occurred because of formation of DOC aggregates by intermolecular bridging via calcium complexation.²⁸ Subsequent sorption of nC_{60} to the formed DOC aggregates resulted in rapid removal of nC_{60} from the aqueous phase suggesting that fullerenes and DOC strongly interact. Complexation of Ca^{2+} ions with DOC reduces the charge on DOC hence promoting aggregation and sedimentation. Alternatively, Ca^{2+} bridges the nC_{60} which are associated with DOC to form larger agglomerates. Which process dominates cannot be distinguished here. In contrast,

monovalent electrolytes such as Na^+ ions only function as charge screening counter ions.²⁹ The colloidal stability of fullerene suspensions is usually quantified by determination of the CCC with time-resolved DLS. Faster agglomeration rates of fullerenes will influence sedimentation rates and their subsequent removal from water.³⁰ Divalent electrolytes obviously result in lower CCC values for nC_{60} colloids compared to monovalent electrolytes.¹¹ Enhanced agglomeration of fullerenes was observed earlier in the presence of both $CaCl_2$ and dissolved organic carbon (DOC = 1 mg C/L of Suwannee river humic acid).²⁶

Ionic strength dependence on colloidal stability

The effect of NaCl (25-500 mM) on the concentrations of nC_{60} and nC_{60} BisPCBM after a static equilibration time of 3 d is shown in Figure 6A. The NaCl concentrations covered the range of concentrations of monovalent electrolytes found in Dutch river water ($c_{Na+K} = 0.95-4.07$ mM; river Meuse, averaged over 2001-2011)³¹ and in seawater ($c_{Na+K} = 479$ mM).

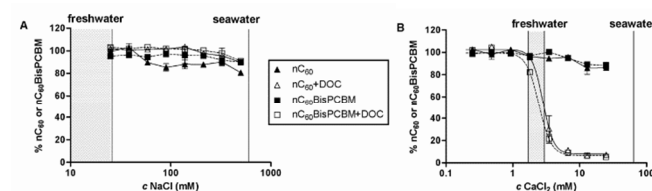


Fig. 6 Effect of the presence of (A) NaCl and (B) $CaCl_2$ on the concentrations of nC_{60} (solid lines) and nC_{60} BisPCBM (dashed lines) in the presence or absence of 2 mg C/L DOC. Note that the x-axis has a logarithmic scale.

In the treatments containing 500 mM NaCl, nC_{60} concentrations in treatments without and with DOC significantly decreased to $76 \pm 1\%$ and $85 \pm 1\%$ compared to the treatments without electrolytes, see Figure 6A. However, the decrease in nC_{60} BisPCBM concentrations for both treatments was not significant. The stabilization of nC_{60} by DOC is explained by sorption of nC_{60} to DOC in solution,³² whereas more extensive sedimentation and/or possible glass wall sorption results in lower fullerene concentrations in the treatments without DOC. The DOC source used was a commercial humic acid which is one of the most hydrophobic humic acids that is currently available. Recently, measurements of fullerene settling over a 1-year period with 4 natural DOC samples (hardness, $[Ca^{2+} + Mg^{2+}] = 0.10-0.33$ mM) indicated that a higher colloidal stability of nC_{60} correlated with more hydrophobic DOC.³³ This suggests that molecular properties of DOC such as aromaticity are important in the interactions between DOC and fullerenes. The relatively high hydrophobicity of the DOC source used in this study is probably responsible for the sorption and subsequent stabilization of fullerenes at already relatively low DOC concentrations (2 mg C/L). The effect of $CaCl_2$ (0.25-25 mM) on the concentrations of nC_{60} and nC_{60} BisPCBM after a static equilibration time of 3 days is shown in Figure 6B. The highest $CaCl_2$ concentrations used were about 12 times higher than the concentrations of divalent electrolytes in the river

Meuse ($c_{\text{Ca+Mg}} = 1.66\text{-}3.01$ mM; averaged over 2001-2011),²⁹ but were lower than the concentration present in seawater ($c_{\text{Ca+Mg}} = 63$ mM). The concentration of nC₆₀ in the CaCl₂ treatment without DOC significantly decreased to $81\pm 1\%$ of the initial concentration, whereas the decrease in the concentration of nC₆₀BisPCBM was not significant. However, the concentrations of nC₆₀ and nC₆₀BisPCBM at the higher CaCl₂ concentrations in the presence of DOC plummeted to 7 ± 1 and $6\pm 1\%$ of the stock concentration, respectively. This decrease apparently occurred because of sedimentation of DOC by complexation with Ca²⁺ ions.

Environmental implications

This study has shown that different size characterization methods, i.e., dynamic light scattering and multi-angle light scattering, resulted in observed particle diameters of nC₆₀ and nC₆₀BisPCBM suspensions in a similar range of about 150-190 nm. Furthermore, flow cytometry was successfully used to see the effect of NaCl on fullerene clusters. Analysis of fullerene concentrations showed that stable aggregates of (functionalised) fullerenes were formed in the presence of relatively low concentrations of DOC. However, the simultaneous presence of DOC and divalent electrolytes lead to fast sedimentation of DOC-fullerene complexes. In the presence of only monovalent electrolytes this is not observed. Although numerous works have already been published on the colloidal stability of fullerenes, more data is obviously needed to better predict the stability of functionalised fullerenes in the aquatic environment. The colloidal stability will determine the distribution, persistence, and bioavailability of (functionalised) fullerenes. Both heteroaggregation with natural colloids (such as inorganic metal oxides, organic matter and clay minerals) and solution chemistry (e.g., pH, ionic strength) will ultimately determine the fate and transport of (functionalised) fullerenes in the environment.^{11, 30} Knowledge of the colloidal stability is therefore important to assess the environmental risks of these compounds in environmental waters as well as for adopting appropriate removal strategies in waste and drinking water treatment. For example, literature data show that nC₆₀ was efficiently removed from water with coagulation/ flocculation, but removal of the far more hydrophilic and soluble fullerene derivative fullerol (C₆₀(OH)₂₄) was less efficient.⁵

Currently, mathematical modelling of the exposure of nanoparticles in the environment depends on the direct availability of both concentrations and aggregation state of nanomaterials.^{34, 35} Reliable information on environmental exposure data of functionalised fullerenes is therefore required. An accurate time-dependent model of fullerene sedimentation should include DOC and other environmental sorbent concentrations as well as information about temperature, pH, ionic strength, and type of ions present in solution. Measurement of the critical coagulation concentration of functionalised fullerenes in the presence of monovalent to trivalent inorganic cations and DOC sources provides more mechanistic understanding of the relevant molecular properties involved in heteroaggregation of both nanoparticles and organic

colloids.³⁶ In addition, measurement of sedimentation rates of functionalised fullerenes in settling experiments will give more information on the removal kinetics and exposure concentrations of nanoparticles in the aquatic environment.^{37, 38}

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