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Research Article

# Destabilization of Aqueous Colloidal $C_{60}$ Nanoparticles in the Presence of Various Organic Matter

The extractable fraction of aqueous colloidal $C_{60}$ nanoparticles ($nC_{60}$) was quantified using a liquid–liquid extraction method in the presence of five types of dissolved organic matter (DOM): Aldrich humic acid (AHA), Suwannee River fulvic acid (SFA), sodium dodecyl sulfate (SDS) micelle, liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and bovine serum albumin (BSA). The changes in toluene extractable fraction highly depended on the type and dose of DOM. Whereas an environmentally relevant concentration of AHA, 2–20 mg $L^{-1}$, was sufficient to reduce the $nC_{60}$ fraction easily destabilized, much higher dose of fulvic acid was needed to result in the similar degree of stabilization. A big contrast between two types of self-organized DOM, SDS micelle and POPC liposomes, was observed. Although SDS micelle significantly decreased the toluene extractable fraction of $nC_{60}$ at the dose greater than its critical micelle concentration, no apparent decrease in toluene extractable fraction was found in the presence of POPC liposomes up to 3000 mg $L^{-1}$. The toluene extractable fraction of $nC_{60}$ in the presence of BSA rapidly decreases at lower doses then gradually decreased at higher doses. An equilibrium complexation model was proposed to quantitatively describe the decrease in the extractability of $nC_{60}$ in the presence of DOM. The observed decrease in the extractability of $nC_{60}$ was well explained by the model and the complexation of $nC_{60}$ with DOM was thought to occur close to 1:1 molar ratio except for BSA. The association constants of $nC_{60}$ with DOM were in the order of BSA, AHA, SFA, and SDS micelle, showing the differences in the affinity to $nC_{60}$.

**Keywords:** Bioavailability; Complexation; Environmental fate; Extraction; Nanomaterial

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## 1 Introduction

Fullerenes and their derivatives are thought to be very promising nanomaterials due to their unique physico-chemical properties. With the increasing production of fullerenes, however, concerns have arisen in regard to their potential persistence in the environment and adverse effects on human and ecological receptors [1–7]. Although fullerenes themselves have extremely low water solubility [8, 9], it is suspected that they may be persistent and mobile in the environment, as highly stable colloidal aggregates of $C_{60}$ fullerene ($C_{60}$ nanoparticles, $nC_{60}$) have been formed under laboratory conditions [10–13]. The potential adverse effects of laboratory prepared $nC_{60}$ were assessed both in vivo and in vitro assays, including antibacterial activities [2–5], lethality to a human skin cell line [14], oxidative stress [1], and DNA damage in human lymphocytes [5].

Because fullerene nanoparticles present in an aggregated form under environmentally relevant conditions, their physico-chemical properties and potential toxic effects depend on the preparation methods [4, 15, 16] as well as the association with organic matter [17–21]. The rate of aggregation of $nC_{60}$ in the presence of $MgCl_2$ was significantly decreased in the presence of humic acid [18]. Similarly, the solubilization of $nC_{60}$ in water was dramatically enhanced in the presence of humic or fulvic acid [20] and the colloidal stability of $nC_{60}$ was increased in the presence of abundant human blood proteins [17, 21]. Although the mechanism of the stabilization of $nC_{60}$ by humic acids and blood proteins is not understood very well, interactions between $nC_{60}$ surfaces and the hydrophobic backbone of humic acid or blood albumin may be responsible for the increased colloidal stability and enhanced solubility of $C_{60}$ in water [20, 22]. Because dissolved organic matter (DOM) is likely to modify the surface properties of $nC_{60}$, interactions between $nC_{60}$ and DOM may inhibit the destabilization and further aggregation of $nC_{60}$ under high electrolyte medium.

For the quantification of $nC_{60}$ in water, both spectroscopic absorption methods [3–5, 10, 23–25] and chromatographic methods [2, 5, 26, 27] were used. Although spectroscopic methods were quick and

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**Abbreviations**: **AHA**, Aldrich humic acid; **BSA**, bovine serum albumin; **DLS**, dynamic light scattering; **DOM**, dissolved organic matter; **$nC_{60}$**, $C_{60}$ nanoparticles; **POPC**, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; **SDS**, sodium dodecyl sulfate; **TEM**, transmission electron microscopy; **TOC**, total organic carbon

 

efficient for measuring the concentration of $nC_{60}$ prepared in a laboratory condition, chromatographic methods are preferred for quantifying residual $nC_{60}$ in a complicated matrix [28]. Because chromatographic determination of aqueous $nC_{60}$ requires extraction steps, researchers evaluated efficient procedures extracting $nC_{60}$ from water. Earlier studies found that hydrophilic $nC_{60}$ aggregates cannot be directly extracted using organic solvents, such as toluene, but the extraction efficiency is enhanced by the addition of a oxidizing salt such as $Mg(ClO_4)_2$ [2, 27, 29, 30], or by increasing the ionic strength of the medium [12, 26, 27, 31]. A mild oxidant in the aqueous $nC_{60}$ solution is likely to reduce the negative surface charge of the $nC_{60}$ and thus, make it more hydrophobic and extractable by an organic solvent [2]. Similarly, increasing the ionic strength destabilizes the negatively charged $nC_{60}$ by reducing the thickness of the diffuse double layer [12].

Although the association of $nC_{60}$ with DOM should be very important not only for evaluating their environmental fate and toxicity but also for the development of extraction protocols for $nC_{60}$ in a complicated matrix, studies have focused on mostly humic acids and blood proteins and the contribution of other types of organic matter has been rarely reported. The primary objective of this study was to evaluate the increased stability of $nC_{60}$ in the presence of various DOM. A colloidal suspension of $nC_{60}$ was prepared by sonication with solvent exchange. The toluene extractable fraction of $nC_{60}$, which is easily destabilized after the addition of $Mg(ClO_4)_2$ or $MgCl_2$, was measured in the presence of five types of model organic matter: Aldrich humic acid (AHA), Suwannee River fulvic acid (SFA)), sodium dodecyl sulfate (SDS) micelle, and liposomes composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and bovine serum albumin (BSA). A simple equilibrium complexation model was also proposed to explain the observed changes in the extractability in the presence of DOM. The association constants and stoichiometric coefficients for the association of $nC_{60}$ with DOM were also determined.

## 2 Materials and methods

### 2.1 Materials

High purity (>99.8%) $C_{60}$ fullerene was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All solvents used for the extraction and HPLC analysis were of high purity. Technical grade AHA (sodium salt), SDS (>99%), and BSA (99%) were purchased from Sigma–Aldrich. Fulvic acid isolated from Suwannee River water was purchased from International Humic Substances Society (St. Paul, MN, USA). Chloroform solution, containing POPC (C16:0, C18:0), was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Technical grade AHA was further purified before use by adjusting the pH. The undissolved residue was removed after increasing the pH (11.0) of a solution containing 10 g $L^{-1}$ AHA by adding 1 N NaOH. One normal HCl was added to the supernatant to decrease the solution pH (2.0). The solution was stored for 24 h at room temperature. After centrifugation at 3000×g, purified AHA was obtained. For the dissolution of acidic AHA in distilled water, a small volume of 10 N NaOH was added to adjust the pH to ≈7. All other organic matters were used as obtained. BSA was dissolved in 20 mM Tris buffer solution at pH 7.4. The suspension of POPC liposomes was prepared via thin film hydration followed by rapid extrusion as described earlier [32]. The concentration of POPC liposome suspension was quantified by measuring total organic carbon content.

### 2.2 Preparation of aqueous $nC_{60}$ suspension

All aqueous $nC_{60}$ suspensions used in this study were obtained using solvent exchange under sonication [15, 24, 25, 31]. Briefly, $C_{60}$ powder was initially dissolved in toluene to 200 mg $L^{-1}$. Forty milliliters of the toluene solution containing $C_{60}$ was transferred to an amber glass bottle containing 200 mL de-ionized water. The toluene was evaporated over 2–4 days using a sonicator (28 kHz, 50 W). The residual toluene in the yellow-brownish suspension was further removed by sparging the solution with nitrogen gas. The resulting suspension of aqueous $nC_{60}$ was filtered through a 0.7-μm glass fiber filter (Whatman, UK) to remove any large aggregated particles and reduce the poly-diversity of the suspension. Although the suspension was highly stable, it was used in all the liquid–liquid extraction experiments within 3 wk after preparation.

### 2.3 Extraction of aqueous $nC_{60}$ suspension

#### 2.3.1 Solvent extraction after destabilization

A 1-mL aqueous $nC_{60}$ suspension sample was mixed with 0.40 mL of an aqueous solution, containing $Mg(ClO_4)_2$ or $MgCl_2$, to make desired concentrations of electrolyte solution in a 4-mL glass vial. One mL of toluene was then added to the solution. The vial was shaken at 150 rpm for 30 min to extract all the extractable $nC_{60}$ from the aqueous suspension. The solution was frozen at −20°C and the overlying toluene layer was taken for HPLC-UV analysis. Preliminary experiments showed that the destabilization and shaking times for the solvent extraction were sufficiently long and no further extractable $nC_{60}$ was found in the aqueous solution at sufficiently high doses of destabilizing agent.

#### 2.3.2 Evaluation of destabilization in the presence of dissolved organic matter

Aqueous solution containing organic matter was mixed with aqueous $nC_{60}$ suspension to prepare desired concentrations of $nC_{60}$ and organic matter. The final concentration of SDS micelle was greater than 3000 mg $L^{-1}$ because the reported critical micelle concentration was approximately 1000 mg $L^{-1}$ [33]. In order to ensure association/dissociation equilibrium between the free $nC_{60}$ and $nC_{60}$ associated with organic matter, the suspension was stored in the dark for 24 h, although the preliminary experiment with AHA showed that the association/dissociation equilibrium seemed to be almost instantaneous. The toluene extractable $nC_{60}$ in the presence of DOM was then quantified using the method described above.

### 2.4 Instrumental analyses

The $C_{60}$ fullerene concentrations in the toluene were analyzed using an HPLC-UV system equipped with a Waters 515 pump, a 717+ autosampler, and a Waters 996 photodiode array detector (Waters Corp., Milford, MA, USA). Ten μL of toluene solution was injected and separated on a Fortis C18 column (4.6 × 150 $mm^2$, 5-μm particle size; Fortis Technologies Ltd., Neston, Cheshire, UK) in an isocratic mode, using *n*-hexane/2-propanol (60:40) at a flow rate of 1 mL $min^{-1}$. The $C_{60}$ fullerene was quantified at 327 nm, with the retention time found to be 4.9 min at room temperature.

The light scattering intensity-weighted average diameter of $nC_{60}$ particles was measured by dynamic light scattering (DLS, FPAR-1000

 

Fiber-optics particle analyzer; Photal Otsuka Electronics, Osaka, Japan). The aggregate size was also visualized using a transmission electron microscopy (TEM) image via a Tecnai G2 F30 S-TWIN (FEI Company, Hillsboro, OR, USA), operating at 300 kV. The samples for the TEM imaging were prepared by depositing a drop of the aqueous suspension on a 200-mesh Cu Formvar carbon grid (Daedok Science, Daejon, Korea) and drying overnight at room temperature prior to the measurement. The total organic carbon content of the POPC liposome suspension was measured using a Sievers 5310C TOC analyzer (GE Analytical Instruments, Boulder, CO, USA).

## 3 Results and discussion

### 3.1 Characterization of aqueous $nC_{60}$ suspension

The final concentration of the stable $nC_{60}$ suspension prepared in this study was approximately 28 mg of extractable $nC_{60}$/L and it was diluted using de-ionized water to prepare desired concentrations of $nC_{60}$ suspension. The light scattering intensity-weighted average diameter of the aggregates via DLS was $115 \pm 18$ nm. The particle size distribution and extractable concentration of $nC_{60}$ did not significantly change after 7 months, showing that colloidal $nC_{60}$ were highly stable in water.

The size of an aggregated $C_{60}$ fullerene particle after filtration was almost uniformly spherical, unlike the big, polydiverse particles in the unfiltered suspension (Fig. 1). The hydrodynamic diameter was measured using DLS and found to be larger than the unit size of spheres in the TEM image, indicating that the spherical units in the TEM image may aggregate in the aqueous solution.

### 3.2 Destabilization by $Mg(ClO_4)_2$ and $MgCl_2$

Figure 2 shows the dramatic increase in the amount of $nC_{60}$ extracted on the addition of $Mg(ClO_4)_2$ and $MgCl_2$. The $nC_{60}$ recovered from the aqueous suspension was only $0.03 \pm 0.02$ mg $L^{-1}$, without the addition of any salts when the total extractable $nC_{60}$ was 1.2 mg $L^{-1}$. However, the extractable fraction of $nC_{60}$ rapidly increased with increasing dose of both salts. Almost all extractable $nC_{60}$ was extracted with only 3 mM of $Mg(ClO_4)_2$ or $MgCl_2$. Further addition did not show any changes in the amount of $nC_{60}$ extracted, which agrees well with earlier studies [27, 30]. At very low doses, 0.1 and 0.3 mM, the extraction efficiency was higher in the sample with $Mg(ClO_4)_2$. However, the variation was very large and the extractable fractions were not statistically different. Thus, the additional effects of $Mg(ClO_4)_2$ as a mild oxidant did not seem to be significant in destabilizing the aqueous $nC_{60}$ suspension. An increased ionic strength was likely to be the main cause of the destabilization. Preliminary tests also showed that the toluene extractable fraction did not vary significantly using AHA as a model DOM (Fig. 3). Thus, 30 mM of either magnesium salt was regarded as sufficient for destabilization. $Mg(ClO_4)_2$ was used for the destabilization of $nC_{60}$ in the presence of selected organic matters except for BSA. For BSA suspension, $MgCl_2$ was used to prevent potential denaturation of protein via oxidation.

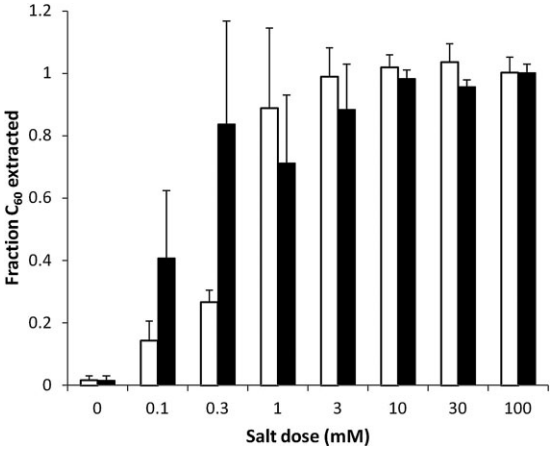


**Figure 2.** Effects of $MgCl_2$ (filled white bars) and $Mg(ClO_4)_2$ (filled black bars) and their dosage on the extraction of aqueous $nC_{60}$ suspensions. The quantified concentration of $nC_{60}$ was 1.63 mg $L^{-1}$. Error bars denote the standard deviation of triplicate samples.

### 3.3 Effects of dissolved organic matter

Figure 4 shows the $nC_{60}$ extracted using 30 mM $Mg(ClO_4)_2$ or 30 mM $MgCl_2$ in the presence of various concentrations of (a) AHA, (b) SFA, (c) POPC liposomes, (d) SDS micelle, and (e) BSA. Although the decrease in the toluene extractable fraction is somewhat greater at the higher initial $nC_{60}$ concentration, the values were not significantly different from each other for most doses tested. Thus, the stabilization of $nC_{60}$ with DOM does not seem to depend on $nC_{60}$ concentration in water. From the decreased toluene extractable fraction, stabilization of $nC_{60}$ by AHA was most significant. The extractable fraction decreased by a factor of 2 in a solution containing only 10 mg $L^{-1}$ AHA. This is consistent with recent studies

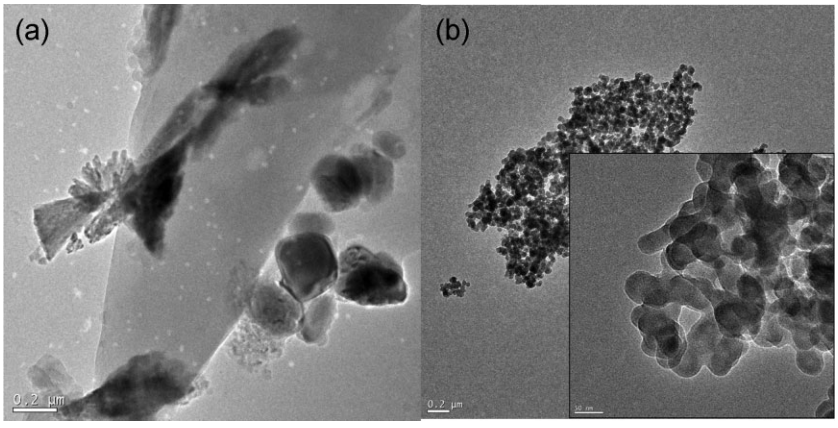


**Figure 1.** Transmission electron microscopy images of (a) an unfiltered aqueous $nC_{60}$ suspension and (b) a filtered $nC_{60}$ suspension. The image in the box is a magnified image.

 

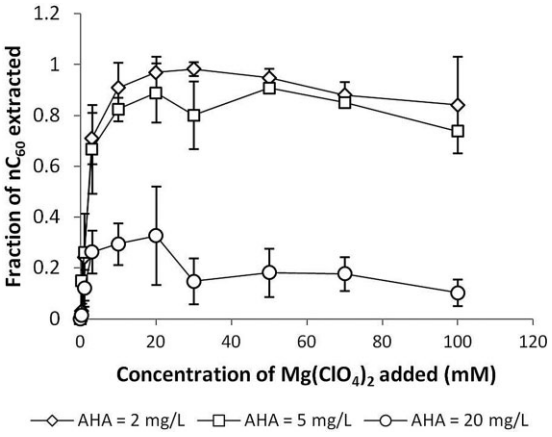


**Figure 3.** Effects of the $Mg(ClO_4)_2$ dose on the extraction of $nC_{60}$ in the presence of 2, 5, and 20 mg AHA/L. The total $nC_{60}$ concentration was 2.3 mg $L^{-1}$. Error bars denote the standard deviation of at least three samples.

showing that natural organic matter may stabilize carbonaceous nanoparticles, such as carbon nanotubes [34, 35] and fullerene aggregates [18–20, 24]. Rate of aggregation of the fullerene nanoparticles decreased with increasing humic acid concentration potentially due to steric hindrance and the reduced surface hydrophobicity after the adsorption of humic acid onto the surface of the $nC_{60}$ [18, 24], similar to that onto mineral surfaces [36].

Unlike the case with AHA, the extractable fraction did not significantly decrease in the presence of environmentally relevant doses of SFA, with an approximate 50% decrease observed at 500 mg $L^{-1}$. This is consistent with the earlier finding that humic acid enhanced the solubility of $nC_{60}$ to a greater extent than fulvic acid by a factor of 47–280 [20]. Humic acid contains a relatively low charge density and large hydrophobic aromatic backbone [37, 38]. Thus, it is likely that the adsorption of humic acid onto the $nC_{60}$ surface was more favored due to the stronger affinity of $nC_{60}$ to aromatic surfaces. The mechanisms for the disaggregation of $nC_{60}$ or the enhancement of aqueous solubility in the presence of DOM may differ from those for the extraction of $nC_{60}$ after destabilization. However, the initial stage of extraction is the loss of surface charge, and this process is likely to be blocked by the adsorption of humic acid.

The stabilization of $nC_{60}$ in the presence of two self-organized organic matter was significantly different from each other. While the extractable fraction was noticeably decreased at SDS concentration above its critical micelle concentration, no changes in the extractable fraction were observed in the presence of POPC liposomes up to 3000 mg $L^{-1}$. As discussed earlier, hydrophobic adsorption of DOM onto $nC_{60}$ surface is proposed to be the major mechanism of the further stabilization of $nC_{60}$. It is likely that $nC_{60}$ surface can be coated by hydrophobic tails of SDS micelle but not by POPC lipid bilayers. Although recent simulation study has shown that molecular $C_{60}$ may be located in the center of the lipid bilayer [39, 40], colloidal aggregates are not likely to be embedded in the center of thin lipid bilayers at least during the incubation time (24 h) in this study. This suggests that the direct transport of $nC_{60}$ across lipid bilayer via passive diffusion is not likely to occur in a short time, although it still remains the possibility for $nC_{60}$ to penetrate the lipid membranes via conversion to molecular $C_{60}$ form.

Stabilization of $nC_{60}$ with BSA was different from humic substances, AHA, and SFA. The extractable fraction rapidly decreased at lower BSA doses up to 30 mg $L^{-1}$ and then gradually decreased with increasing BSA concentration. Because albumin is highly abundant in blood, the stabilization of $nC_{60}$ in the presence of BSA should be significant to evaluate and assess the fate and potential adverse effects of $nC_{60}$ in the body. The stabilization of $nC_{60}$ in the presence of BSA is consistent with earlier findings in literature [17, 21, 22, 41]. Stable protein–BSA complex was identified when native BSA was incubated with γ-cyclodextrine-bicapped molecular $C_{60}$ fullerene [41] and $nC_{60}$ was reported to be stabilized in the presence of high human serum albumin concentration [21]. More recently, Maoyong et al. [22] showed that 10–100 mg $L^{-1}$ BSA significantly decreased the inhibition of polymerase activity by $nC_{60}$ and proposed a competitive sorption of BSA onto $nC_{60}$ with DNA polymerase. The results in this study confirm the earlier finding using toluene extractable fraction at large range of BSA doses as well as suggest the extraction efficiency of $nC_{60}$ included in biological samples.

## 3.4 Complexation model for the association of $nC_{60}$ with dissolved organic matter

The stabilization of aqueous $nC_{60}$ in the presence of DOM may be simplified, as follows:

$$nC_{60}(\text{extractable}) + m\text{DOM} \leftrightarrow nC_{60}\text{-}(\text{DOM})_m(\text{unextractable}) \quad (1)$$

where $nC_{60}$-$(DOM)_m$ denotes $nC_{60}$ coated by DOM, which is not extractable under the experimental conditions. Rearranging Eq. (1) by assuming an association constant, $K$, an expression for the toluene extractable fraction ($f$) can be obtained as follows:

$$\frac{1}{f} = 1 + K[\text{DOM}]^m \quad (2)$$

Although [DOM] represents free DOM in Eq. (1), the range of DOM concentrations in this study was higher than the estimated molar concentration of $nC_{60}$ because $nC_{60}$ is thought to have higher molecular weight than those of DOM studied. Thus, [DOM] was assumed to be equal to the total DOM concentration spiked in the solution for fitting experimental data. Although the Eq. (1) is based on molar concentration, mass concentrations were used instead because the molar concentration of $nC_{60}$ could not be calculated and the form of the Eq. (2) does not change with unit conversions. Table 1 represents the equilibrium association constants and the exponent, $m$, for the $nC_{60}$ with model DOM studied using Eq. (2). The strong correlation coefficients and $F$ values indicate that the equilibrium model may be used to estimate the stability of $nC_{60}$ in the presence of organic matter. The stoichiometric coefficient ($m$) was close to unity for all DOMs except for BSA, indicating that this stabilization would occur at 1:1 molar ratio. However, the stabilization behavior by BSA is quite different from other DOM. As shown in Fig. 2, the rapid decrease in the extractable fraction was observed at the lower dose of BSA, followed by the gradual decrease at higher dose, resulting the exponent $m$ much smaller than unity. This may represent that one BSA can stabilize more than one $nC_{60}$ aggregate. However, potential denaturation of BSA during vigorous mixing with toluene may break the interaction between BSA and $nC_{60}$ and enhance the toluene extractability in higher BSA doses.

 

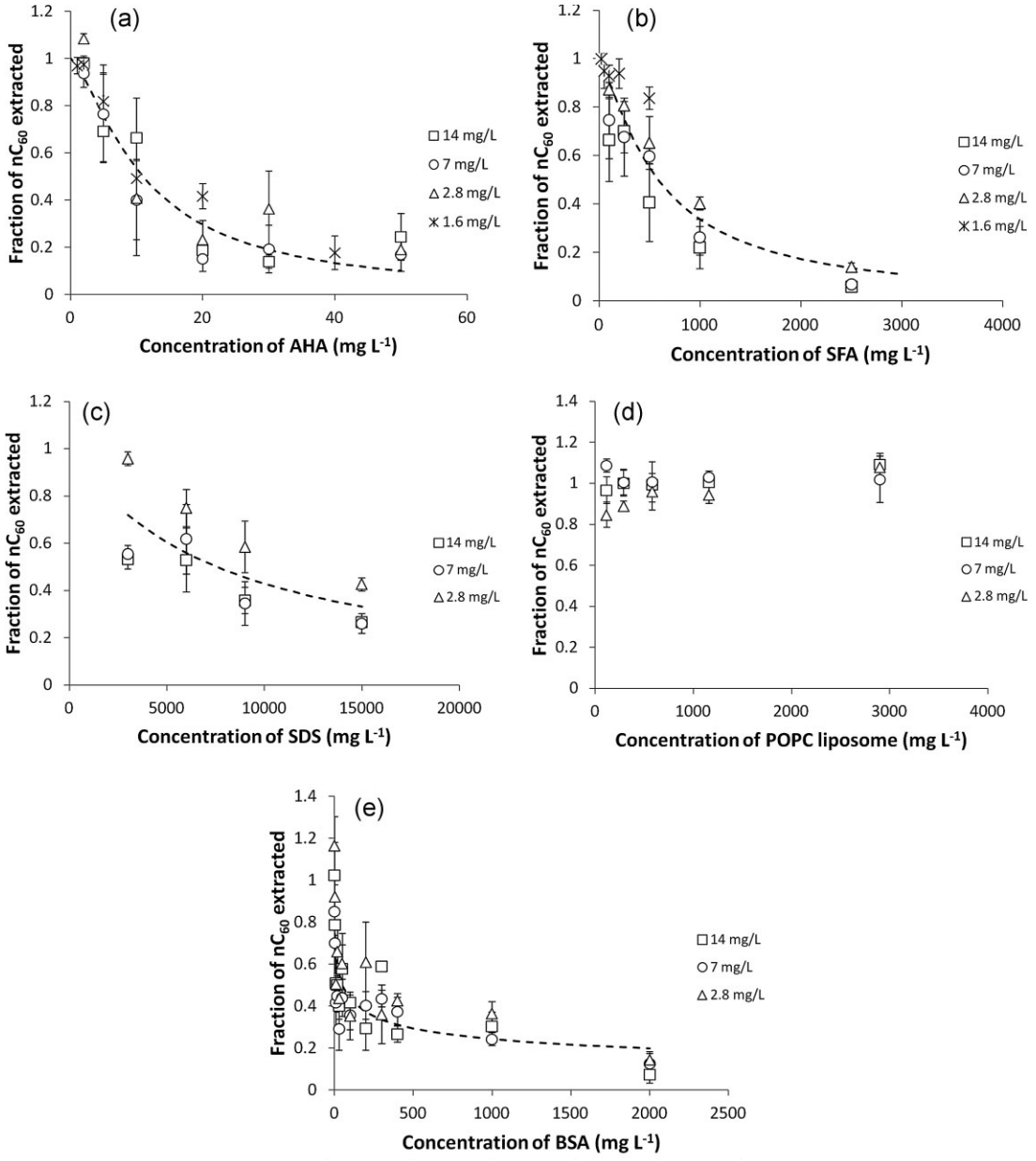


**Figure 4.** The fraction of $nC_{60}$ extracted with increasing dose of (a) Aldrich humic acid, (b) Suwannee River fulvic acid, (c) sodium dodecyl sulfate micelle, (d) POPC liposomes, and (e) bovine serum albumin. The total extractable $nC_{60}$ concentrations were 1.63 (*), 2.8 (△), 7.0 (○), and 14 mg $L^{-1}$ (□) and the dose of destabilizing agent ($Mg(ClO_4)_2$ or $MgCl_2$) was 30 mM. Each data point represents an average of at least three replicates and error bars denote the standard deviation of at least three samples. Dashed lines represent best-fit using Eq. (2).

**Table 1.** Association constants and the exponents for $nC_{60}$ association with DOM

| DOM | $K$ | $m$ | $r^2$ | $F$ | $n$ |
|---|---|---|---|---|---|
| Aldrich humic acid (AHA) | $0.0314 \pm 0.0136$ | $1.443 \pm 0.167$ | 0.91 | 443 | 24 |
| Suwannee River fulvic acid (SFA) | $0.000293 \pm 0.000409$ | $1.276 \pm 0.371$ | 0.85 | 295 | 20 |
| Sodium dodecyl sulfate (SDS) micelle | $0.000107 \pm 0.000354$ | $1.024 \pm 0.371$ | 0.48 | 78.9 | 12 |
| Bovine serum albumin (BSA) | $0.235 \pm 0.061$ | $0.374 \pm 0.057$ | 0.67 | 252 | 36 |

 

## 4 Concluding remarks

The results from this study suggest that aqueous $nC_{60}$ particles can be further stabilized in the presence of DOM, thus the toluene extractable fraction after adding destabilizing salts was reduced. This stabilization was highly dependent on the type of DOM. AHA containing long hydrophobic carbon backbone and BSA containing hydrophobic domain were likely to readily adsorb onto $nC_{60}$ surface via van der Waals interaction and to increase the colloidal stability. SFAs and SDS micelles also stabilized $nC_{60}$ although the association constants were smaller than those for BSA and AHA. On the contrary, POPC liposomes showed almost no effects on the stability of $nC_{60}$, indicating that coating of $nC_{60}$ surface by liposomes would not likely to occur. Although comparative stabilization of $nC_{60}$ by various DOM was discussed, further research will be required to link the observed changes in the stability of $nC_{60}$ to its bioavailability and potential toxic effects on aquatic organisms.

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