

高速液体クロマトグラフィー-タンデム質量分析法による生物試料中フラーレンの分析法の開発

久保田領志[#], 田原麻衣子, 清水久美子, 杉本直樹, 広瀬明彦, 西村哲治Development of a liquid chromatography-tandem mass spectrometry method for the determination of fullerenes C₆₀ and C₇₀ in biological samplesReiji Kubota[#], Maiko Tahara, Kumiko Shimizu, Naoki Sugimoto, Akihiko Hirose, and Tetsuji Nishimura

Wide application of fullerenes in various areas would increase the risk of occupational and environmental exposure to human. However, information about toxicity and biological behavior of fullerenes is not sufficient for the risk assessment at present. For the determination of fullerene C₆₀ in biological samples, an analytical method using high performance liquid chromatography – tandem mass spectrometry (LC-MS/MS) and extraction procedure from tissues of experimental animals was established in this study. Using LC-MS/MS with an atmospheric pressure chemical ionization in negative mode, C₆₀ were identified and quantified. After optimization of mobile phase and separation column, good separation of peak of fullerene and sensitivity were obtained in case of using toluene and acetonitrile as the mobile phases and Develosil RPFULLERENE as the separation column. For method validation, rat brain, kidney, liver, lung, spleen tissues and blood were used for recovery tests. Good results were obtained and the recovery percentages were found to be between 98.1% and 106.5%.

Keywords: fullerene, analytical method, LC-MS/MS, biological Sample, extraction

1. はじめに

近年, ナノテクノロジー素材である人工ナノ粒子 (Engineered nanoparticles, ENPs) は新機能や優れた物理化学的特性を持つことから, 情報・通信, 医療, 環境, エネルギー等の様々な分野において次世代を担うキーテクノロジーとして期待され, 急速に種類や生産量が増加しつつある. 代表的なENPsとしては, カーボンナノ粒子 (フラーレンやカーボンナノチューブ等), 金属・合金・金属酸化物のナノ粒子, ポリマーナノ粒子, 量子ドット等があり, 賦形剤, 乳白剤, 触媒, 半導体等の工業分野だけでなく, ヒトに直接関係する製薬, 食品, 化粧品等の分野においても用いられている¹⁾. 一方で, それらENPsのヒトへの健康影響や環境への放出, 生態系への影響については多くの点で未解明のままであり, 特にENPsによるヒトに対する健康影響評価への関心が高まっているのが現状である^{2,3)}.

カーボンナノ粒子のフラーレンは, グラファイト, ダイヤモンドに次ぐ第三の炭素同素体であり, 他の炭素同素体とは異なる多数の五角面と六角面を持つ閉多面体か

ご型構造の分子である⁴⁾. その独特な構造と特性のためにフラーレンと内包フラーレンや化学修飾フラーレンなどの誘導体は, 他の炭素同素体とは異なりフリーラジカルスカベンジャー⁵⁾, 光誘起DNA切断⁶⁾, HIV-1プロテアーゼ活性阻害⁷⁾, ヒト細胞 (ヒト皮膚線維芽細胞 (HDF細胞), ヒト肝ガン由来細胞 (HepG2細胞), およびヒト星状細胞 (NHA細胞) における細胞毒性⁸⁾を示すことが報告されている. フラーレンとその誘導体の急速な商用化により, ヒトが職業や環境から経口, 経皮, 経気道曝露するリスクが増大することが推測されている. しかしながら, 曝露によるヒト健康への影響は殆ど明らかにされておらず, 有害影響評価や体内動態に関する研究は十分ではない. *in vivo*⁹⁾や*in vitro*¹⁰⁾によるフラーレンやその誘導体の有害影響を正しく評価するためには, フラーレンの曝露評価が不可欠である. そのためには, フラーレンの高感度, 高精度および選択性が高い分析化学的手法が必要である. しかしながら, 分析化学的手法を用いた生物試料中のフラーレンやその誘導体の測定に関する研究は数例あるだけで¹¹⁻¹³⁾, これらの研究を行う上でも, 分析法の開発が急務となっている.

そこで本研究では, 高速液体クロマトグラフィー-タンデム質量分析法 (LC-MS/MS) を用いたフラーレン (C₆₀) の高感度測定法の確立の目的とし, 投与した実験動物の

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組織からのフラーレン類の抽出等の前処理法を検討した。

2. 実験方法

2.1 試薬

C₆₀はMaterials Technologies Research社製 (Cleveland, USA) (純度99.98% (メーカー成績値)) またはフロンティアカーボン (株) (東京, 日本) 製ナノムパープルSUH (純度>99.9% (メーカー成績値)) を、C₇₀はMaterials Technologies Research社製 (Cleveland, USA) (純度99.5% (メーカー成績値)) を用いた。高速液体クロマトグラフィー用2-プロパノール、アセトニトリル、およびトルエンは和光純薬工業 (株) (大阪, 日本) 製を用いた。また、生化学用のドデシル硫酸ナトリウムも和光純薬工業 (株) (大阪, 日本) 製を用いた。標準原液はC₆₀またはC₇₀を10mg精密に量り取り、それぞれトルエン10mLに加え超音波処理および攪拌して溶解したものとし、分析まで-20℃で保存した。標準液は標準原液からトルエンで段階的に希釈したものを用時調製した。

2.2 装置

LC-MS/MSには、高速液体クロマトグラフとしてWaters社 (Milford, USA) 製Alliance 2695 HPLCシステムを、タンデム質量分析装置として同社製Micromass Quattro micro APIトリプル四重極質量分析計を連結したものをを用いた。

2.3 LC-MS/MSの分析条件

移動相の最適化は、以下の2組の条件 (条件1: A1-A2, 条件2: B1-B2) で比較検討した。条件1では、移動相A1=トルエンおよびA2=2-プロパノールを、A1/A2の割合を27%/73%, 36%/64%および45%/55%に設定した。条件2では、移動相B1=トルエンおよびB2=アセトニトリルを、B1/B2の割合を50%/50%, 60%/40%および70%/30%に設定した。条件1, 2共にアイソクラテックモードで送液し、流速は0.2および1.0 mL/minとした。カラムオープンの温度は40℃ (一部30℃も用いた) を用いた。オートサンプラーは10℃で維持し、注入量は5μLもしくは20μLで行った。

イオン化法は、大気圧化学イオン化法 (APCI) のネガティブイオンモードを用い、Multiple Reaction Monitoring (MRM) モードにより高感度定量分析を行った。対象物質の分離カラムによる分離条件を比較検討するためにWaters社製 Atlantis dC18 (3 μm, 2.1 × 150 mm) (極性化合物の保持にすぐれ、100%水系移動相でも使用できるC18系逆相カラム) および野村化学 (株) (瀬戸, 日本) 製Develosil RPFULLERENE (5 μm, 4.6 × 250 mm) (C18

系) に比べ、保持が大きく、高い耐久性を示すC30系逆相カラム) を用いた。LC-MS/MSのシステム制御、データ収集および解析にはWaters MassLynx version 4.0を用いた。

MRM測定条件は、標準液を直接注入し、イオン検出強度が最大になるように最適化した。以下に条件を示す。APCIのコロナ電流は15μA, ソース温度, APCIプローブ温度はそれぞれ120℃, 400℃を用いた。デゾルベーションガス流量およびコーンガス流量はそれぞれ600L/hr, 50L/hrとした。インタースキャンディレイは0.1secs, インターチャンネルディレイは0.05secsに設定し、ドウェルタイムは0.5secsを用いた。フラーレンのプレカーサイオン, プロダクトイオンについては、C₆₀がm/z = Q1 720 → Q3 720, サロゲート物質として回収率補正に用いるC₇₀ではm/z = Q1 840 → Q3 840とし、コリジョンエネルギーはC₆₀, C₇₀ともに60eV, コーン電圧は120Vを採用した。

2.4 試料の前処理法

生物試料からのC₆₀の抽出はMoussaら (1997)¹⁴⁾ の方法を簡便化して行った。分析まで冷凍保存していた臓器の重量を測定し、ホモジナイズし、50~200mgをガラス製のホモジナイズ管に量り取った。これに0.1Mのドデシル硫酸ナトリウム0.5mL, および内部標準物質溶液として1.0mg/L C70トルエン溶液0.5mLを添加し、穏やかにホモジナイズした。ホモジネートをガラス製の遠沈管に移し、ホモジナイズ管を1.0mLのトルエンで5回洗浄し、その洗液をホモジネートに合わせた。次にホモジネートを合わせた遠沈管に0.5mLの酢酸を加え遮光し、室温で5時間295/minで振とう後、30分間3500rpmで遠心した。遠心後、上澄みを分取し、LC/MS/MS分析用試料とした。

3. 結果と考察

3.1 移動相の最適条件の検討

フラーレンの分離における移動相の最適条件を検討した。まず、C18系逆相カラムのAtlantis dC18カラムを用い、2組の移動相の条件でそれぞれ100μg/LのC₆₀とC₇₀のトルエン混合標準液を測定し、それぞれのピークの分離状況を比較した。Fig.1に移動相の条件1: A1-A2を用いた場合のMRMクロマトグラムを示す。A1およびA2の比率を変動させてC₆₀とC₇₀の分離状況を比較した結果、A1/A2=45%/55%の条件では、C₆₀とC₇₀のピークが完全に重なり、両者のピークの分離は得られなかったが、A1/A2=36%/64%ではC₆₀のピークにC₇₀のショルダーピークが観察されるようになり、さらにA1/A2=27%/73%ではC₆₀とC₇₀のピークは完全に分離した。

Fig.2に移動相の条件2: B1-B2を用いた場合のMRMク

ロマトグラムを示す。B1 / B2の比率を変動させてC₆₀とC₇₀の分離状況を比較した結果、B1 / B2=70% / 30%の条件ではC₆₀のピークにC₇₀のショルダーピークが観察された。一方、B1 / B2=60% / 40%の条件ではC₆₀とC₇₀のピークは完全に分離し、B1 / B2=50% / 50%の条件ではC₆₀とC₇₀のピークの間隔がさらに広がった。

C₆₀とC₇₀のピークは条件1に比べ、条件2の移動相により良好なピーク分離が観察された。また、条件1に比べ条件2においてC₇₀のピーク形状がシャープであり、検出感度も良好であった。以上の結果から、移動相としてトルエンとアセトニトリルを用いることとした。

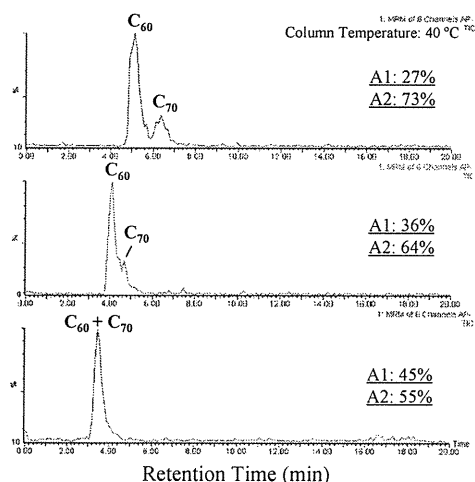


Fig. 1 Comparison of separation of fullerenes C₆₀ and C₇₀ by Atlantis dC18 separation column, A1: 2-Propanol/Toluene (1:9 v/v); A2: 2-Propanol, flow rate: 0.2mL/min, injection volume: 5 μ L

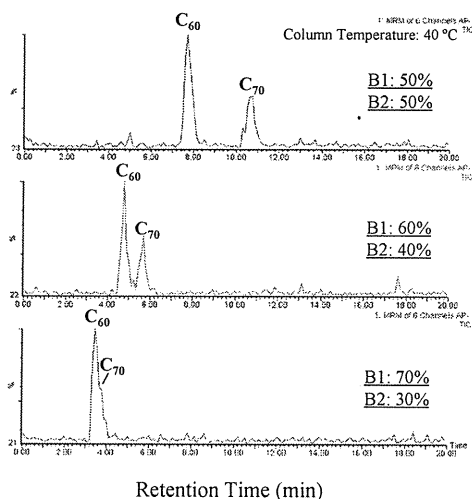


Fig. 2 Comparison of separation of fullerenes C₆₀ and C₇₀ by Atlantis dC18 separation column, B1: Toluene; B2: Acetonitrile, flow rate: 0.2mL/min, injection volume: 5 μ L

3.2 分離カラムの検討

分離カラムの検討を行った。Atlantis dC18カラムおよびC30逆相カラムのDevelosil RPFULLERENEカラムを用いてそれぞれ100μg/LのC₆₀とC₇₀のトルエン混合標準液を測定し、ピーク分離状況を比較した。Develosil RPFULLERENEカラムで、移動相がB1 / B2=50% / 50%ではC₆₀とC₇₀のピークは20分以内に溶出せず、B1 / B2=60% / 40%ではC₆₀のみ20分以内に溶出した（データ示さず）。一方、B1 / B2=70% / 30%、カラムオープン温度が40°Cの条件において、20分以内に両ピークとも溶出され、シャープなピークが観察された。また、C₆₀とC₇₀のピークはそれぞれのピークトップの間隔が3分程度というように十分な分離能が確認され、検出感度も良好であった (Fig. 3)。一方、同じ条件において、Atlantis dC18カラムではC₆₀とC₇₀のピーク分離は不十分であり、ピーク形状も良くなかった (Fig.2)。さらに、Develosil RPFULLERENEカラムにおいてカラムオープン温度を30°Cにして分離状況を比較したところ、40°Cに比べC₆₀とC₇₀のピークはそれぞれのピークトップの間隔が4分程度というようにより良好に分離することが確認された (Fig.3)。以上の結果から、LC-MS/MSの諸条件を下記にまとめた。

装置 : Waters Alliance 2695,

Waters Micromass Quattro micro API

分離カラム : Develosil RPFULLERENE (5 μm, 4.6 ×

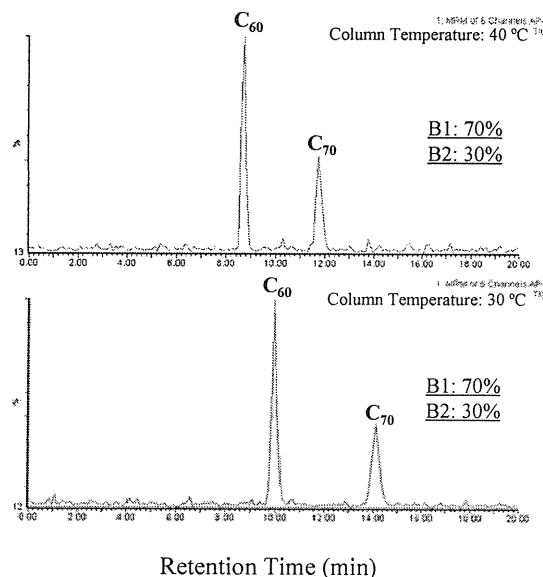


Fig. 3 Comparison of separation of fullerenes C₆₀ and C₇₀ by Develosil RPFULLERENE separation column, B1: Toluene; B2: Acetonitrile, flow rate: 1mL/min, injection volume: 20 μ L

250 mm)

移動相：トルエン/アセトニトリル=70%/30%

アイソクラティック法

流速：1mL/min

カラムオープン温度：30℃

試料注入量：20 μ L

イオン化法：APCIネガティブイオンモード

この条件では定量下限値は10 μ g/Lであった。すなわち組織重量に換算すると1.1 μ g/g wet wt.であった。

3.3 生物試料を対象としたフラーレンの抽出方法の検討

実験動物の臓器からのフラーレンの抽出方法を確立するため、実験動物の臓器組織を用いたフラーレンの添加回収試験を行った。検討には日本SLC (株) から購入したSlc:Wistar(SPF)ラット雄6週齢から得た肝臓、腎臓、脾臓、肺、脳、および血液を用いた。血液および全ての臓器においてC₆₀の回収率は100%前後(平均値 \pm 標準偏差: 102.7 \pm 3.9%)と良好な結果が得られた (Fig.4)。また、MRMクロマトグラムにおいても、全ての臓器から抽出した試料において良好なピーク形状が得られ、また、妨害ピークや干渉等は認められなかった。C₇₀の回収率についても100%前後と良好な結果(平均値 \pm 標準偏差: 98.6 \pm 4.5%)が得られたことから、C₇₀をサロゲートとして用いることが妥当であることが示された。C₇₀の回収率を用いC₆₀の回収率補正を行った結果、補正前(102.7 \pm 3.9%)と補正後(104.4 \pm 6.6%)でC₆₀の回収率はほぼ同程度であった。以上の結果より、曝露評価のために本研究で示したトルエンによる振とう抽出法によって組織中のC₆₀の分析を行うことができ、生物試料を対象と

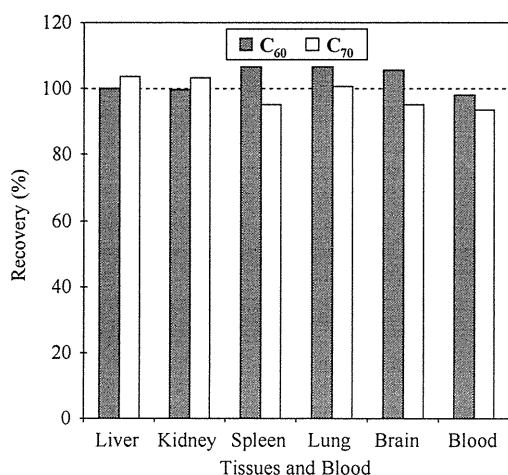


Fig. 4 Comparison of recovery of fullerenes C₆₀ and C₇₀ from tissues and blood of Slc:Wistar(SPF) rat.

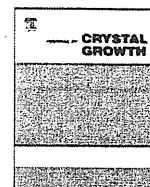
する際にC₇₀により抽出効率の補正を行うことでより正確な分析が可能であることが示された。

4. おわりに

フラーレンのヒトにおける健康影響評価を最終目標に、LC-MS/MSによりC₇₀を抽出操作の補正として用いるC₆₀の測定法、さらには生物試料を対象としたフラーレンの抽出方法を検討した。本報で示した方法により、生物試料を対象としたフラーレンの分析が可能となり、曝露評価を実施する有効な方法であると言える。

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The effect of solvent ratio and water on the growth of C₆₀ nanowhiskers

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ARTICLE INFO

Article history:

Received 23 April 2010

Received in revised form

12 June 2010

Accepted 14 June 2010

Available online 17 June 2010

Keywords:

A1. Liquid–liquid interfacial precipitation method (LLIP)

B1. Fullerene nanowhiskers

B1. C₆₀ nanowhiskers

B1. Fullerene

ABSTRACT

The growth of C₆₀ nanowhiskers (C₆₀NWs) prepared by a modified liquid–liquid interfacial precipitation method is investigated, focusing on the effect of solvent ratio and water content in the C₆₀–toluene–isopropyl alcohol (IPA) solution system. The precipitation of C₆₀NWs was markedly influenced by the solvent ratio of toluene to IPA, and the C₆₀NWs were found to grow longer above a critical diameter (*D_c*), which depends on the solvent ratio. The addition of a small amount of H₂O to the C₆₀–toluene–IPA solution promoted the growth of C₆₀NWs. This catalytic effect of water on the growth of C₆₀NWs was confirmed also by the experiment using heavy water (D₂O) and by the decrease of growth activation energy of C₆₀NWs with increase of H₂O content in the C₆₀–toluene–IPA solution.

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1. Introduction

C₆₀ nanowhiskers (C₆₀NWs) are thin single crystal nanofibers composed of C₆₀ molecules [1]. In 2001, C₆₀NWs were found to precipitate in a colloidal solution of lead zirconate titanate (PZT) containing a small amount of C₆₀ [2]. In addition to the C₆₀NWs, various kinds of fullerene nanowhiskers (FNWs) that are composed of C₇₀, C₆₀[C(COOC₂H₅)₂], La@C₈₂(Ad) (Ad = adamantylidene) and so forth have been synthesized using the liquid–liquid interfacial precipitation method (LLIP method) [3–5]. Single crystal tubular nanofibers composed of C₇₀, “C₇₀ nanotubes” [6], were also synthesized by the LLIP method as well as C₆₀ nanotubes [7,8].

The LLIP method uses the interdiffusion process between a good solvent solution of fullerene and a poor solvent of fullerene after placing the good solvent solution of fullerene on the poor solvent to precipitate the FNWs [3,9]. The interdiffusion occurs by self-diffusion [3] or can be assisted by manual mixing or ultrasonication [10–12]. The LLIP method without the process of manual mixing or ultrasonication is called the “static LLIP method” in this paper.

Not only long fibrous C₆₀NWs with lengths greater than several millimeters [13] but also short C₆₀NWs with lengths from several micrometers to several tens of micrometers can be prepared by the LLIP method [10,14–16].

Cha et al. [17] fabricated vertically grown C₆₀ microtubes on a porous alumina membrane substrate by slowly injecting IPA

through the membrane pores into a C₆₀-saturated toluene solution in order to induce supersaturation of C₆₀ on the membrane surface. Further, Cha et al. [18] fabricated tree-shaped bundled C₆₀NWs by injecting IPA into a toluene solution of C₆₀ containing the ferrocene derivative of C₆₀ molecules through a porous alumina membrane. Recently Sathish et al. [19] and Satish and Miyazawa [20] fabricated various morphologies of fine C₆₀ crystals containing C₆₀ nanosheets, short C₆₀NWs with lengths between a few micrometers and several micrometers and nanorhombuses of C₆₀ by the LLIP method. Slightly later Masuhara et al. [21] also reported the production of various morphologies of spherical particles, whiskers, hexagonal plates, nanoballs and nanobipyramids of C₆₀, where good solvent solutions of C₆₀ were injected into poor solvents of C₆₀.

C₆₀NWs exhibit semiconductor properties [22,23], and were first applied to field-effect transistors [24]. Various other applications of C₆₀NWs are expected in catalysts [1,25–28], fuel cells [29,30], solar cells [31], and so forth.

As shown in our earlier paper [4], C₆₀NWs with diameters less than 100 nm can be synthesized by the static LLIP method. Fibrous C₆₀NWs with a small diameter of 87 nm and a length of more than 5 μm were produced. Afterward, Chong et al. [32] studied the fabrication of C₆₀NWs by dropping a toluene solution of C₆₀ into IPA to precipitate C₆₀ crystals and reported a needle-like crystal of C₆₀ with a diameter of about 80 nm and a length of about 500 nm for an example of “true nanoscale single crystal of C₆₀”. The fabrication of nanoscale C₆₀ single crystal was also demonstrated by the formation of a needle-like crystal with a small diameter of 45 nm and a length of 108 nm by dropping a C₆₀-saturated toluene solution into IPA under ultrasonication at 10 °C [11].

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As shown above, a variety of methods derived from the static LLIP method have been used to synthesize C_{60} NWs of various lengths and diameters. The size control of C_{60} NWs is important in their practical application. For example, short C_{60} NWs with lengths of several micrometers are appropriate for solar cell application [31], while long C_{60} NWs are appropriate when they are used to fabricate non-woven sheets of C_{60} [33]. C_{60} NWs with small sizes will be required for their application to transistors.

In order to control the size of C_{60} NWs, the growth mechanism of C_{60} NWs must be clear. Up to now, the following items have been reported:

- (1) Growth of C_{60} NWs is promoted by illumination of visible light and depends on the wavelength of light [34,35].
- (2) Growth of C_{60} NWs depends on temperature. The growth activation energy of C_{60} NWs was measured to be 52.8 kJ/mol in the system of C_{60} -saturated toluene solution and IPA [15].
- (3) Growth of C_{60} NWs is promoted by addition of a small amount of water [36].
- (4) Morphology of precipitated C_{60} crystals depends on the solvent ratio of toluene to IPA and the amount of added water [36].

We partly reported the effects of water and solvent ratio on morphology and growth of C_{60} NWs in our preliminary experiments [36]. Hence in the present paper, the above items (2)–(4) are more minutely investigated and the effects of solvent ratio and water on growth of C_{60} NWs are discussed in detail.

The results shown below must be useful for growth control of C_{60} NWs and will also be informative in the synthesis of fullerene nanowhiskers using the solvent combinations different from toluene and IPA as well.

2. Experimental

2.1. Synthesis of C_{60} NWs using different solvent ratios

In preparing C_{60} NWs, toluene (99.5% purity, as-received non-dehydrated grade, Wako Pure Chemical Industries, Ltd., Japan) was used as a good solvent of C_{60} and IPA (as-received non-dehydrated grade, Wako Pure Chemical Industries, Ltd., Japan) was used as a poor solvent of C_{60} . The water content of the as-received IPA was measured to be 0.02% and that of the as-received toluene was measured to be 0.01% using a Karl Fischer titrator (Coulometer 831 KF). Ground C_{60} powder (99.5% purity, MTR Ltd., USA) was dissolved in toluene by ultrasonication for 30 min and filtered to remove the undissolved C_{60} powder.

C_{60} NWs were prepared by a modified LLIP method as follows. The C_{60} -saturated toluene solution was poured into a 10 ml transparent glass bottle and IPA was slowly added to the C_{60} -saturated toluene solution to form a liquid–liquid interface in a water bath at 20 °C. Next, the glass bottles were manually shaken 30 times in order to obtain homogeneous precipitation of embryo crystals of C_{60} and kept in an incubator (SANYO MIR-153) at a growth temperature of 20 °C. The above procedure is different from that of our initial static LLIP method without the manual mixing process [3]. C_{60} NWs were synthesized for various volume ratios of the C_{60} -saturated toluene solution to IPA between 1:7 and 7:1. Toluene to IPA ratio in the volumetric proportion ($x:y$) is designated as $xTyl$.

The length and diameter of C_{60} NWs were measured by optical microscopy (Nikon ECLIPSE ME600) and scanning electron microscopy (SEM, JEOL JSM-6700F) for exactly 100 C_{60} NWs in each sample.

2.2. Synthesis of C_{60} NWs using isopropyl alcohol added with water

The addition of water to the C_{60} -toluene-IPA solution was performed by use of IPA added with distilled water (Wako Pure Chemical Industries, Ltd., Japan). The volume ratio of the C_{60} -saturated toluene solution to the IPA containing water was set to be 1:1. The content of water in IPA was set to be less than 2.5 mass % H_2O .

The formation of liquid–liquid interface and the mixing of solutions were similarly performed as shown above. Instead of H_2O , heavy water (D_2O , ISOTEC, 99.96 at%) was also used to study the effect of water on the growth of C_{60} NWs for comparison. The growth experiments were performed between 5 and 20 °C. In this paper, “water” normally means H_2O as long as no special notice is indicated.

3. Results and discussion

3.1. Effect of solvent ratio on growth of C_{60} NWs

Fig. 1 shows the glass bottles observed immediately after the manual mixing and those 24 h after the manual mixing. The opaque glass bottles 1T3I, 1T1I and 3T1I of Fig. 1(a) became transparent 24 h after the manual mixing and the highest yield of C_{60} precipitates is observed in the bottle of 1T1I. The purple color observed in the glass bottles of 1T1I–7T1I shows the excess C_{60} molecules dissolved in the solutions, which shows that an appropriate amount of IPA is necessary to attain complete precipitation of C_{60} crystals.

On the other hand, as shown in Fig. 2, granular precipitates other than C_{60} NWs were observed in the samples prepared by use of the solutions of 1T7I, 1T5I and 3T1I. Examples of the granular crystals are indicated by the white arrows. Although those granular crystals became less with increasing the toluene content as shown in the SEM images of 1T5I, 1T3I and 1T1I, only granular precipitates like those in Fig. 2(e) were formed in the solutions of 3T1I, 5T1I and 7T1I. This fact shows that an optimum solvent ratio exists for the growth of C_{60} NWs. Especially, the C_{60} NWs of 1T1I show the best developed linear morphology and no granular precipitates are found.

The formation of granular particles means isotropic crystal growth of C_{60} . The modes of isotropic crystal growth and anisotropic crystal growth of C_{60} are strongly related with the solvent ratio as discussed later.

Fig. 3 shows the relationship between length and diameter of C_{60} NWs for the four solvent ratios of 1T7I, 1T5I, 1T3I and 1T1I.

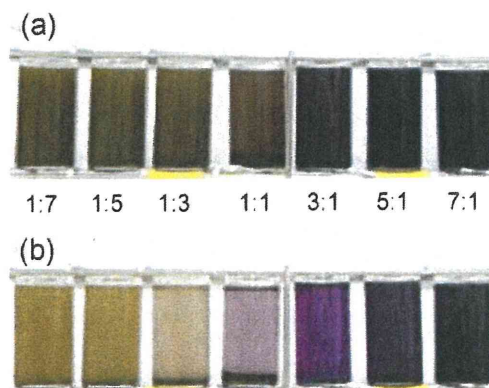


Fig. 1. (a) Glass bottles observed immediately after the manual mixing and (b) those observed 24 h after the manual mixing at the growth temperature of 20 °C.

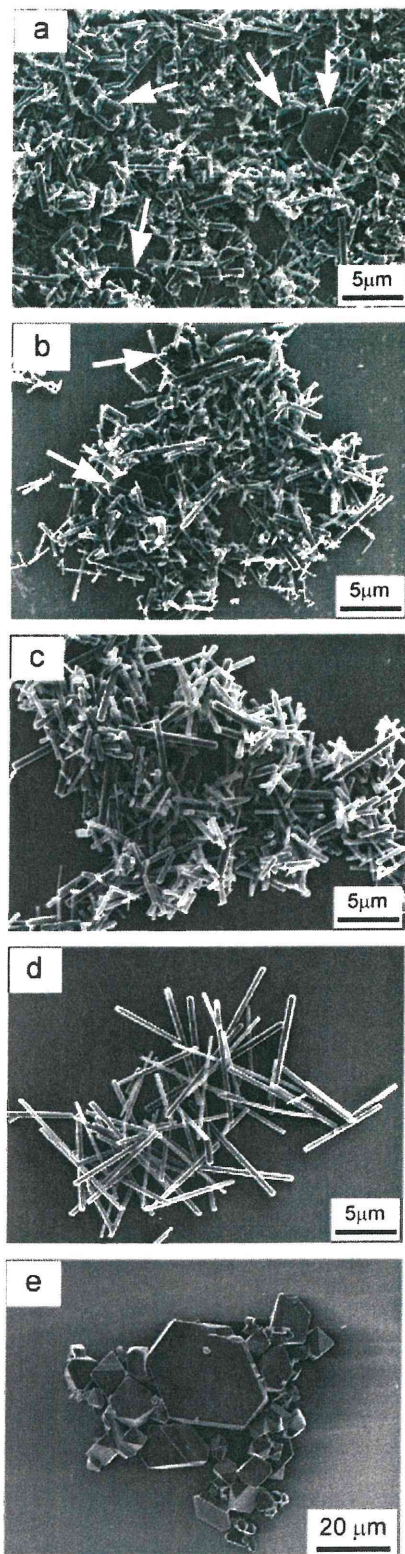


Fig. 2. SEM micrographs of the C_{60} NWs grown for 24 h by use of the solutions with different volume ratios of C_{60} -saturated toluene and IPA at 20 °C. Toluene:IPA (v/v)= (a) 1:7, (b) 1:5, (c) 1:3, (d) 1:1 and (e) 3:1.

The best grown C_{60} NWs are shown in Fig. 3(d) of 1T11, corresponding to the image of Fig. 2(d). On the other hand, diameter of the C_{60} NWs shows large scatter about length of the

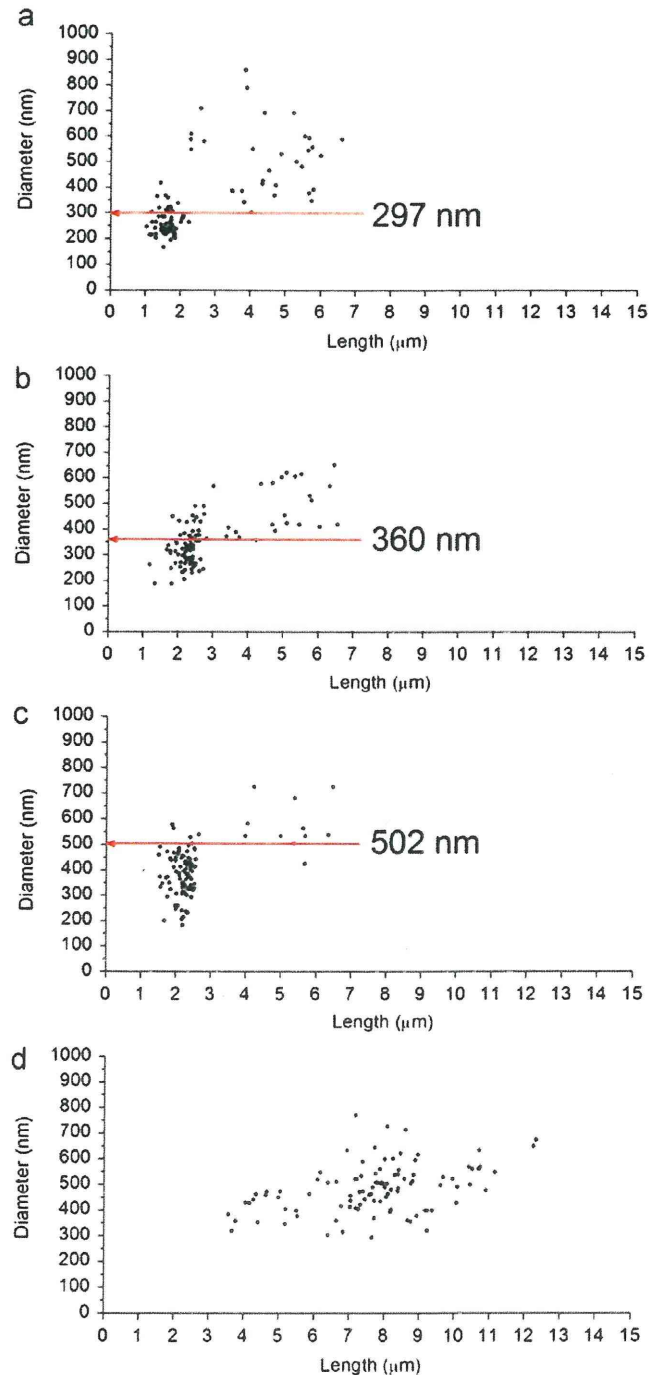


Fig. 3. Relationship between length and diameter of C_{60} NWs synthesized at different solvent volume ratios of toluene and IPA for the growth time of 24 h at 20 °C. Toluene:IPA (v/v)= (a) 1:7, (b) 1:5, (c) 1:3 and (d) 1:1.

C_{60} NWs. Moreover, it is noted that there is a critical diameter (D_c) above which the length of the C_{60} NWs increases, i.e., the whisker length becomes significantly larger above the critical diameters hypothetically set at 297 nm (b), 360 nm (c) and 502 nm (d). This fact shows that the crystal nuclei of C_{60} grow isotropically for the diameters less than D_c and grow anisotropically for diameters greater than D_c . In the solution-grown crystals, it is generally accepted that the crystals can grow above a critical radius R_c that is determined by supersaturation of the solution at a given temperature [37].

The relationship between toluene content of the solutions (1T7I, 1T5I, 1T3I) and D_c value was plotted and is shown in Fig. 4, where the D_c value increases linearly with increasing content of toluene. This fact implies that the D_c value depends on the supersaturation of C_{60} .

The linear curve fit is expressed as $D_c = 13.5x + 83$ (nm), where x is the concentration of toluene in the C_{60} -toluene-IPA solution. Although the fitted curve does not possess the rigorous quantitative accuracy due to the above hypothetically set D_c values, it is qualitatively suggested that the D_c value increases with decrease in supersaturation caused by the intermixing of toluene and IPA. If the above fitted curve is assumed to hold for the concentration of 100% toluene, the D_c value in the solution without IPA will become 1432 nm. This diameter of 1432 nm may be regarded as the critical diameter of C_{60} needle-like crystals that can be grown in a C_{60} -saturated toluene solution at 20 °C. The D_c value approaches 83 nm with decreasing the toluene concentration, which suggests that the diameter of C_{60} NWs can be smaller than 100 nm as described in the introductory section. Hence the optimization of solvent ratio is a crucial factor for the controlled growth of C_{60} NWs.

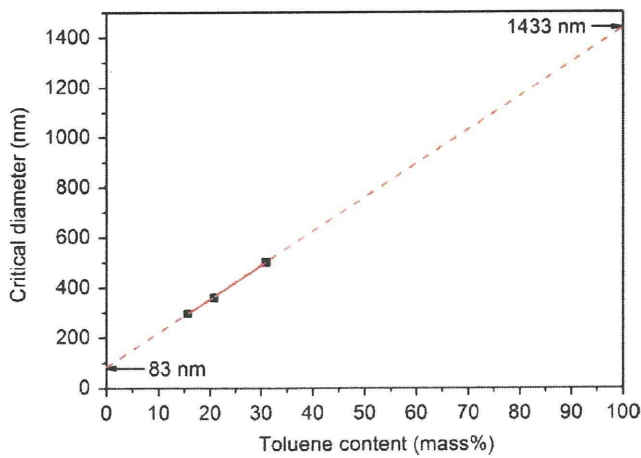


Fig. 4. Critical diameter (D_c) of C_{60} NWs plotted as a function of toluene content (mass %) in the C_{60} -toluene-IPA solutions.

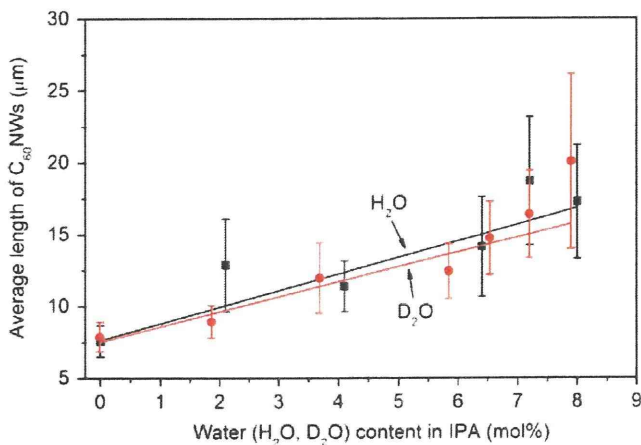


Fig. 5. Average length of C_{60} NWs plotted as a function of water (H_2O , D_2O) content in IPA for the growth period of 24 h at 20 °C.

3.2. Effect of water on the growth of C_{60} NWs

In our preliminary experiment, the addition of water was observed to promote growth of C_{60} NWs [36]. In order to confirm this effect of water, experiments have been performed by use of D_2O as well as H_2O and the result of length measurement of C_{60} NWs is shown in Fig. 5 for cases of both H_2O and D_2O additions.

The horizontal axis is expressed in mole percentage to compare the effect of water by the number of added water molecules. Since the addition of excess amount of water was shown to result in destabilization of C_{60} NWs by our previous experiment [36], the water content in solutions was set to be small. The length value for each data point is the mean value of

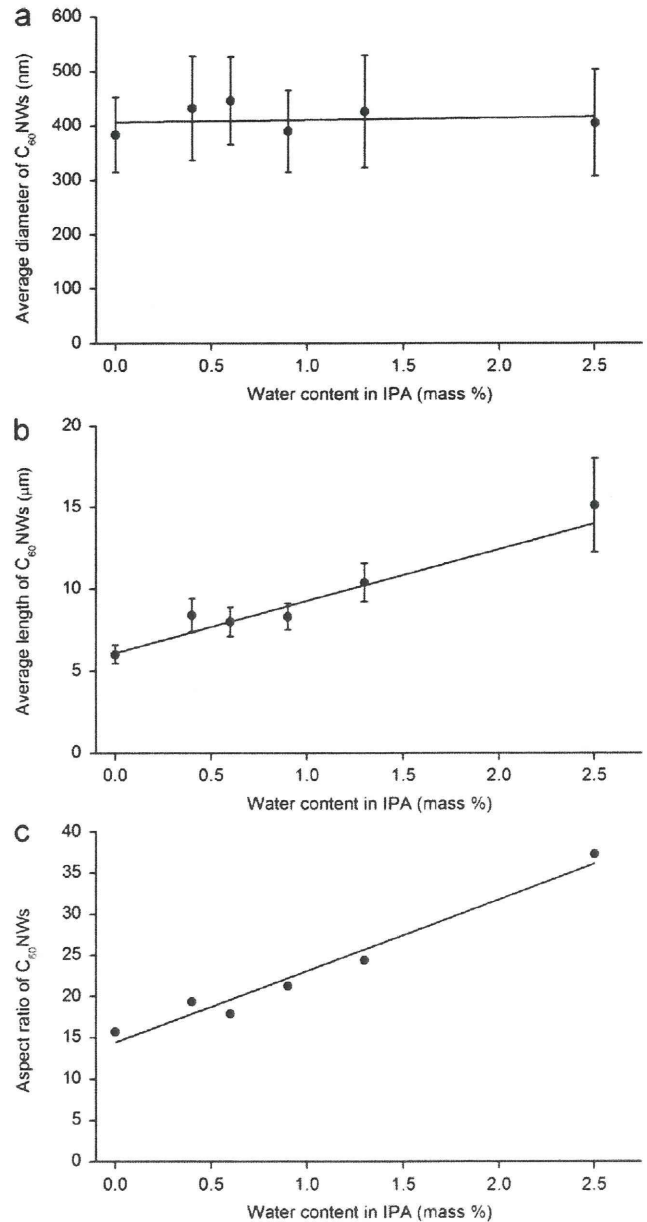


Fig. 6. (a) Average diameter, (b) average length and (c) aspect ratio of the C_{60} NWs plotted as a function of water content (mass %) in IPA. C_{60} NWs were grown at 20 °C for 24 h.

exactly 100 C₆₀NWs. It is clearly shown that the addition of small amount of H₂O and D₂O promotes growth of C₆₀NWs.

The positive effect of H₂O on growth of C₆₀NWs is confirmed by the experiment of D₂O addition, which can eliminate the effect of impurity H₂O contained in the used toluene and IPA reagents. Although the addition of D₂O promotes growth of C₆₀NWs, the effect of D₂O is shown to be a little weaker than the effect of H₂O addition. The small discrepancy between the two linear curves is considered to be caused by a deuterium isotope effect.

Although length of the C₆₀NWs increases with increasing content of water in the solution, diameter of the C₆₀NWs remains almost constant as shown in Fig. 6(a). Fig. 6(b) shows the average length of C₆₀NWs plotted as a function of water (H₂O) content for the same sample of C₆₀NWs of Fig. 6(a), where the whisker length is shown to increase with increasing water content as shown above. The aspect ratio (length/diameter) can be calculated using the data of Fig. 6(a) and 6(b), and is plotted as Fig. 6(c). It is found that the aspect ratio can be widely varied between 10 and 40 just

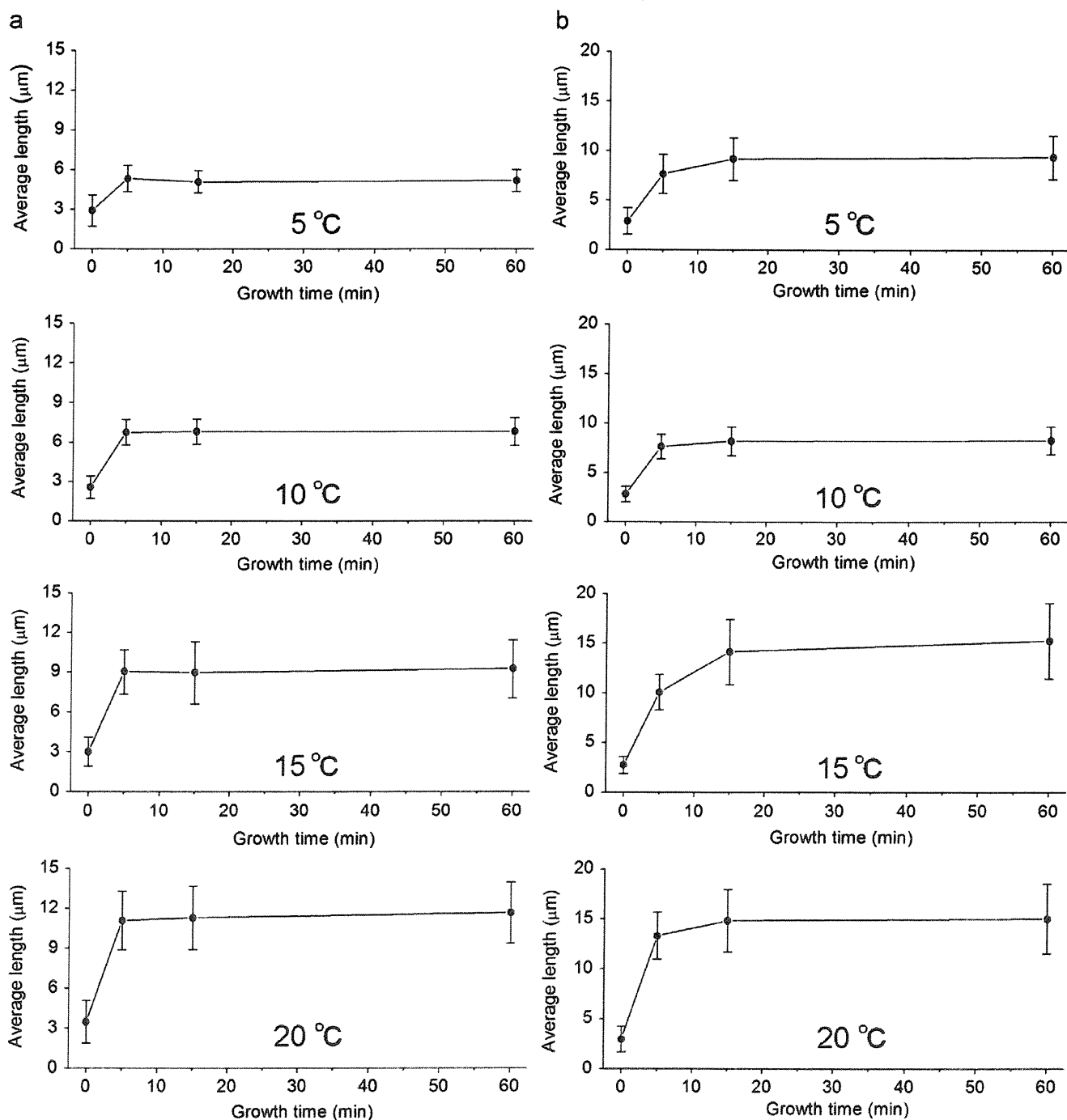


Fig. 7. Average lengths of the C₆₀NWs synthesized by use of IPA containing (a) 1.3 mass % H₂O and (b) 2.5 mass % H₂O, measured as a function of growth time at temperatures between 5 and 20 °C.

by controlling the small content of water. This fact shows that the control of water content in solvents is very important for the length control of C₆₀NWs.

Length of the C₆₀NWs was measured as a function of growth time at temperatures between 5 and 20 °C for each water content in IPA as shown in Fig. 7. Growth time was defined as the elapsed time from the end of the manual mixing. Hence, the C₆₀NWs show finite lengths of a few micrometers at the initial growth time of 0 min in each figure. The growth of C₆₀NWs becomes slower for longer growth time. This phenomenon shows that supersaturation of C₆₀ becomes smaller with the growth of C₆₀NWs that consume the dissolved C₆₀ molecules.

Measured length growth rates were plotted and are shown in the Arrhenius plots of Figs. 8(a) and (b) as a function of 1/T, where T is the absolute temperature (K). The length growth rates were calculated from the length increment of the initial 5 min as previously reported [15].

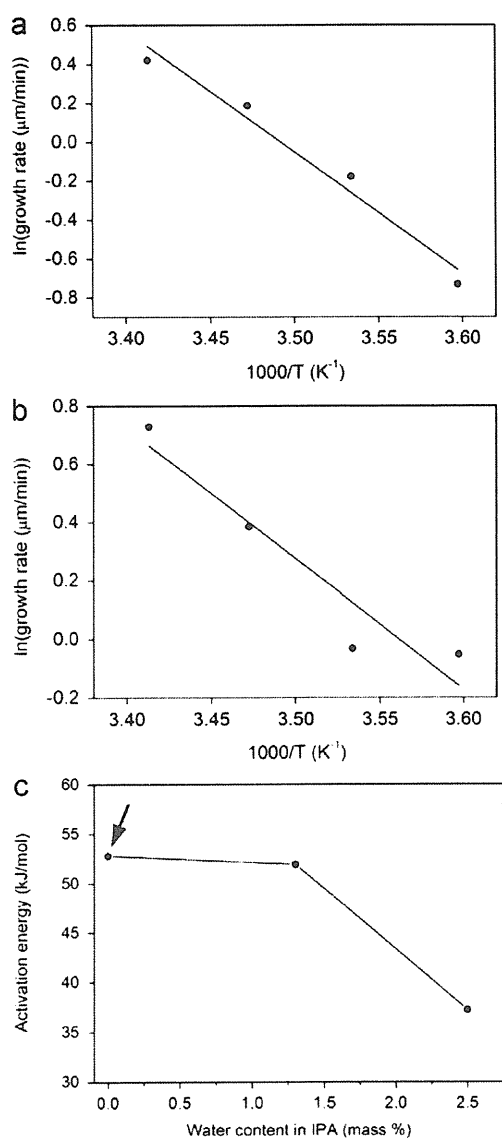


Fig. 8. Arrhenius plots of the length growth rate of C₆₀NWs prepared by use of IPA containing (a) 1.3 mass % H₂O, and (b) 2.5 mass % H₂O and (c) activation energy for the growth of C₆₀NWs plotted as a function of H₂O content in IPA. The data point of 0 mass % H₂O is the value previously reported [15].

The whisker growth activation energy can be calculated from the slope of the Arrhenius plots and is shown as Fig. 8(c). The point marked by arrow is the activation energy of 52.8 kJ/mol measured in our previous experiment using the solvents without water addition [15]. The growth activation energy is found to become smaller with increasing water content. Hence, it is suggested that water acts as a catalyst in the growth of C₆₀NWs.

4. Conclusions

The above results can be summarized as follows:

- (1) Morphology of C₆₀ precipitates synthesized by the modified LLIP method is strongly influenced by volume ratio of the C₆₀-saturated toluene solution to IPA. The sample prepared using the solution with a volume ratio of 1:1 for toluene and IPA showed the best growth of C₆₀NWs.
- (2) C₆₀NWs can grow longer above a critical diameter D_c . D_c value decreases with increasing IPA content in the C₆₀-toluene-IPA solutions.
- (3) Length growth of C₆₀NWs is promoted by the addition of small amounts of H₂O, while their diameter remains almost constant. This fact can be utilized to control the aspect ratio of C₆₀NWs.
- (4) Positive effect of water addition on growth of C₆₀NWs was also confirmed by the use of D₂O.
- (5) Growth activation energy of C₆₀NWs decreased with increasing water content. It is suggested that water catalyzes the growth of C₆₀NWs.

Acknowledgement

The authors are grateful to Dr. Takatsugu Wakahara (NIMS) for his valuable comments and discussions. Part of this research was supported by Health and Labour Sciences Research Grants (H21—Chemistry—Ippan-008) from the Ministry of Health, Labour and Welfare of Japan.

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The effect of water on the stability of C₆₀ fullerene nanowhiskers

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Received: 26 July 2010 / Accepted: 26 October 2010
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Abstract The morphology of C₆₀ precipitates synthesized by using isopropyl alcohol (IPA) added with water was investigated in order to know the effect of water on the growth of C₆₀ nanowhiskers (C₆₀NWs) in C₆₀-toluene-IPA solution systems. The stability of C₆₀NWs decreased and granular crystals of C₆₀ were formed in the solutions when IPA added with an excess amount of water was used in the liquid-liquid interfacial precipitation method. The C₆₀NWs were found to be destabilized with time in the solutions added with water. The C₆₀NWs dried in air showed similar Raman profiles irrespective of the use of IPA with and without water addition. The Raman profiles of granular C₆₀ single crystals showed the base lines much flatter than those of C₆₀NWs, indicating that C₆₀NWs possess a disordered crystal structure. By optimizing the growth condition, short C₆₀NWs with aspect ratios ranging from 3 to 10 and an average length of about 1.8 μm were successfully fabricated. The short C₆₀NWs are expected to be applicable for electrodes of organic thick film solar cells.

Keywords Fullerene nanowhisker · Liquid-liquid interfacial precipitation method · LLIP method · C₆₀ nanowhisker · Fullerene

Introduction

Since the discovery of C₆₀ fullerene nanowhiskers (C₆₀NWs) in 2001 (Miyazawa et al. 2001; Miyazawa 2009), C₆₀NWs have been variously applied for field-effect transistors (FETs) (Ogawa et al. 2006, 2008), fuel cells (Wang et al. 2009), solar cells (Somani et al. 2007), catalysts (Sathish et al. 2008), templates for chemical synthesis (Minato and Miyazawa 2006), and so forth.

C₆₀NWs can be synthesized by the liquid-liquid interfacial precipitation method (LLIP method), where a poor solvent of C₆₀ is placed on a C₆₀-saturated good solvent solution (Miyazawa et al. 2002) in order to form a liquid-liquid interface between the C₆₀-saturated good solvent solution and the poor solvent. C₆₀ embryo crystals nucleate at the liquid-liquid interface due to the formation of supersaturated C₆₀ solution that is caused by the interdiffusion between the good solvent solution and the poor solvent, and grow into long C₆₀NWs. The above LLIP method proposed in 2002 is called the “static LLIP method” which does not accompany such disturbances as caused by ultrasonication,

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manual mixing, and so forth. Various modified LLIP methods that utilize the mixing of good solvent solutions of fullerenes and poor solvents of fullerenes have been developed until today. It has been known that the growth of C₆₀NWs is influenced by light (Tachibana et al. 2003), temperature (Hotta and Miyazawa 2008), solvent ratio, and impurity water (Miyazawa and Hotta 2010). Although the impurity water contained in the solution markedly influences the growth rate of C₆₀NWs, the addition of excess amount of water was found to destabilize the growth of C₆₀NWs in our preliminary experiment (Hotta and Miyazawa 2009).

Hence, in this paper, the influence of water on the morphology of C₆₀ precipitates is minutely investigated using D₂O as well as H₂O and it will be shown that the addition of excess amount of water hinders the formation of C₆₀NWs.

On the other hand, C₆₀NWs can grow from several micrometers to the order of millimeters or more in length (Miyazawa and Ringor 2008; Hotta and Miyazawa 2009). Although long C₆₀NWs are suitable for the preparation of non-woven C₆₀ sheets (Miyazawa 2003), the size control of short C₆₀NWs is also important in their application. For example, short C₆₀NWs with lengths of several micrometers were successfully applied for the fabrication of organic thick film solar cells (Somani et al. 2007). Recently we showed the synthesis of short C₆₀NWs with lengths less than 5 μm by a modified LLIP method (Miyazawa and Hotta 2010) and have improved the method in order to synthesize much shorter C₆₀NWs with uniform lengths and diameters by optimizing the synthetic condition. Hence, the study to fabricate the short C₆₀NWs will also be presented in this paper.

Experiment

In the synthesis of C₆₀NWs by the LLIP method using solutions containing water, isopropyl alcohol (IPA) (as-received, Wako Pure Chemical Industries, Ltd., Japan) mixed with distilled water (Wako) was gently added to C₆₀-saturated toluene solutions in 10 mL transparent glass bottles at 20 °C. Next, the bottles were capped and manually shaken 30 times and stored in an incubator (SANYO MIR-153) at 20 °C. This modified LLIP method combined with the hand shaking process is named “hand shaking LLIP

method”, here. The volume ratio of the C₆₀-saturated toluene solutions to IPA containing water was fixed to be 1:1 and the content of water in IPA was set to be less than 4 mass% H₂O. Instead of H₂O, heavy water (D₂O, ISOTEC, 99.96 at%) was also used in order to reduce the effect of impurity H₂O contained in the as-received solvents IPA and toluene and to confirm the experimental reproducibility. The H₂O content of as-received IPA was measured to be 0.02% and that of as-received toluene (Wako) was measured to be 0.01% by a Karl Fischer titrator (Coulometer 831 KF). IPA and toluene were used without further purification. In this paper, “water” normally means H₂O as long as no special notice is indicated. Raman spectroscopy analyses (JASCO NRS-3100, Japan, laser excitation wavelength of 532 nm) were conducted for the C₆₀NWs in order to examine the effect of water addition on the structure of C₆₀NWs.

In preparing short C₆₀NWs, 2.25 mL of toluene solution saturated with C₆₀ was poured into a glass bottle (10 mL) and 6.75 mL of IPA was gently added into the bottle to form a liquid–liquid interface at 15 °C, where the solvent volume ratio is 1 (toluene):3 (IPA). Next, the glass bottle was capped and manually shaken 30 times in order to obtain homogeneous embryo crystals and kept in an incubator at a growth temperature of 15 °C for 1 day.

Results and discussion

Morphology change of C₆₀ precipitates by addition of water

Figures 1, 2, 3, and 4 show optical microscopy images of the C₆₀ precipitates that were formed by using IPA containing different amounts of water (H₂O, D₂O). Figure 1 shows the C₆₀ precipitates observed 1 day after the start of synthesis, while Fig. 2 shows those observed 7 days after the start of synthesis for different H₂O contents. Granular C₆₀ precipitates appeared for the H₂O contents greater than 2.3 mass% in IPA in the samples of 1 day aging, while in the case of 7 days aging, the granular C₆₀ precipitates appeared at a H₂O content of 2.0 mass% in IPA which was lower than the corresponding value of 2.3 mass% H₂O for 1 day aging. The above observation shows that the stability of C₆₀NWs in solution decreases with increasing the H₂O content

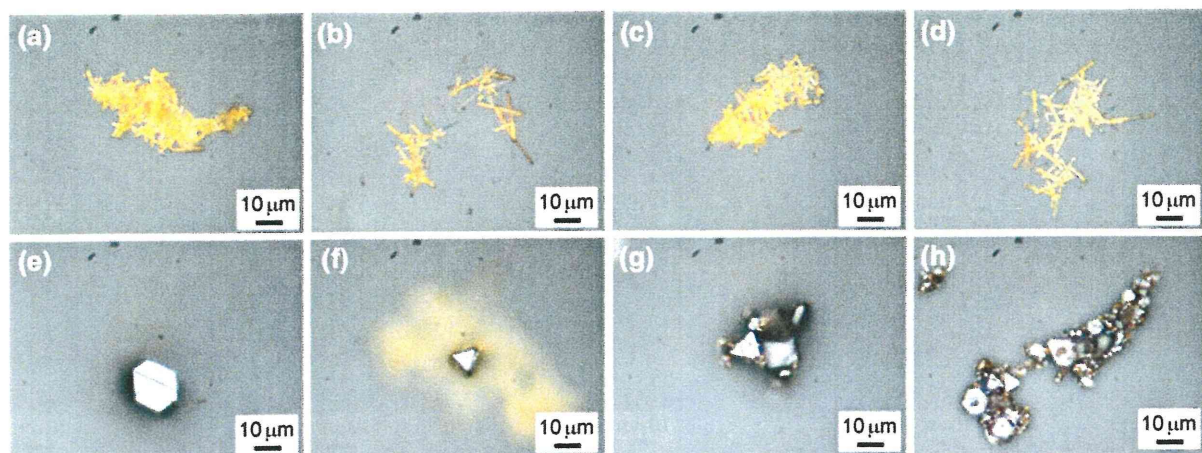


Fig. 1 Optical micrographs of the C_{60} precipitates formed by using IPA with different H_2O contents and aged for 1 day in solutions. **a** 0 mass% H_2O , **b** 0.6 mass% H_2O , **c** 1.3 mass%

d 2.0 mass% H_2O , **e** 2.3 mass% H_2O , **f** 2.5 mass% H_2O , **g** 2.8 mass% H_2O , and **h** 3.8 mass% H_2O

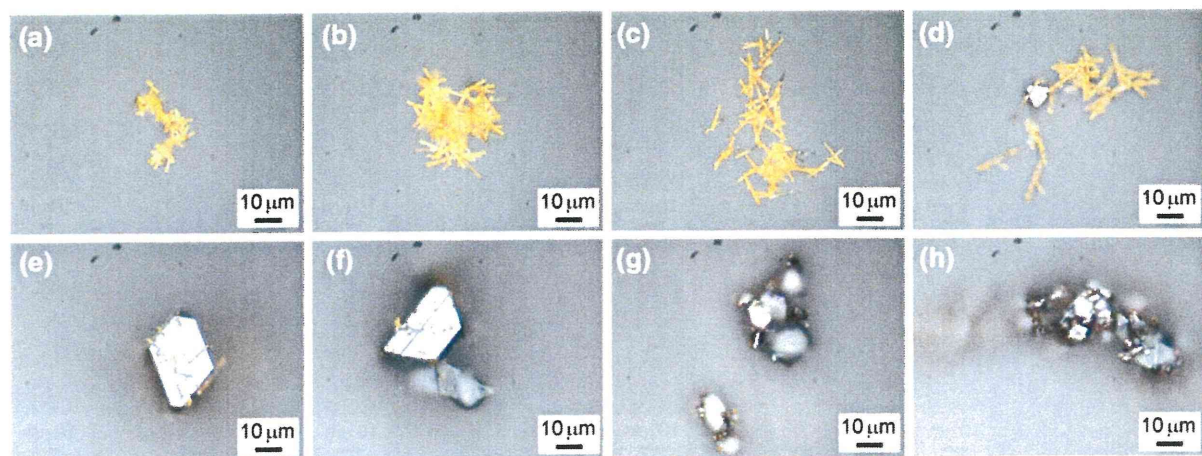


Fig. 2 Optical micrographs of the C_{60} precipitates formed by using IPA with different H_2O contents and aged for 7 days in solutions. **a** 0 mass% H_2O , **b** 0.6 mass% H_2O , **c** 1.3 mass%

d 2.0 mass% H_2O , **e** 2.3 mass% H_2O , **f** 2.5 mass% H_2O , **g** 2.8 mass% H_2O , and **h** 3.8 mass% H_2O

and with time. This experiment by use of the hand shaking LLIP method was repeated once again, and the same results as observed in Fig. 1 were obtained. This good reproducibility of the hand shaking LLIP method was also shown in our previous growth study of C_{60} NWs (Miyazawa and Hotta 2010).

Figure 3 shows the similar observations that were performed using D_2O instead of H_2O . The granular C_{60} crystals appeared for the D_2O contents greater than 2.8 mass% for 1 day aging, while in the case of 7 days aging (Fig. 4), the granular C_{60} crystals appeared at the D_2O contents greater than 1.3 mass% in IPA. Hence,

in the case of D_2O addition also, the stability of C_{60} NWs in solution is found to decrease with increasing the amount of added D_2O and with time. Since the experiment using D_2O can reduce relatively the effect of impurity H_2O contained in solution, this observation using D_2O also confirms that the water destabilizes C_{60} NWs in solution. Further it is conjectured that the water would destabilize C_{60} NWs in air for a long time. The above observations suggest that the destabilization of C_{60} NWs occurs in the solutions containing water with concentrations greater than a critical value.

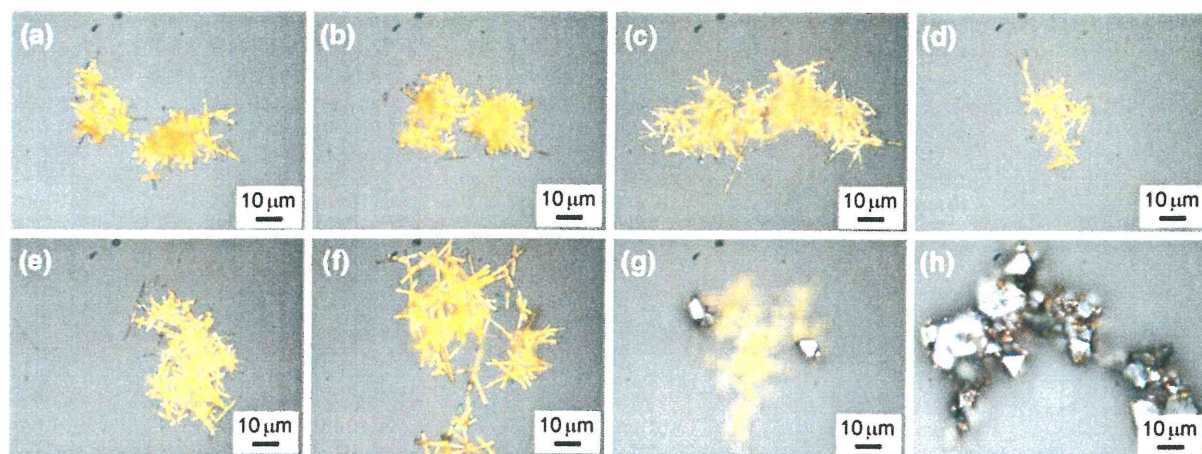


Fig. 3 Optical micrographs of the C_{60} precipitates formed by using IPA with different D_2O contents and aged for 1 day in solutions. **a** 0 mass% D_2O , **b** 0.6 mass% D_2O , **c** 1.3 mass%

D_2O , **d** 2.0 mass% D_2O , **e** 2.3 mass% D_2O , **f** 2.5 mass% D_2O , **g** 2.8 mass% D_2O , and **h** 3.8 mass% D_2O

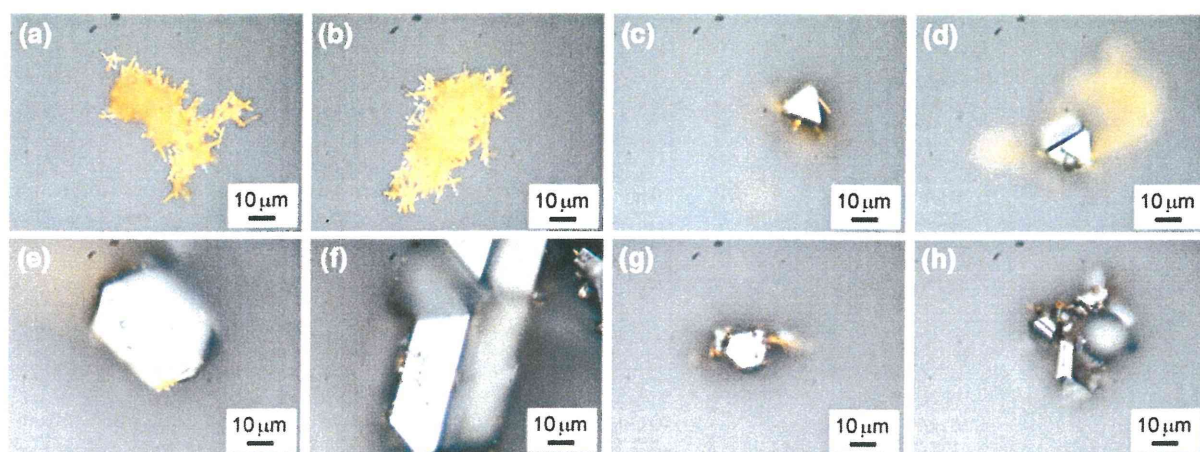


Fig. 4 Optical micrographs of the C_{60} precipitates formed by using IPA with different D_2O contents and aged for 7 days in solutions. **a** 0 mass% D_2O , **b** 0.6 mass% D_2O , **c** 1.3 mass%

D_2O , **d** 2.0 mass% D_2O , **e** 2.3 mass% D_2O , **f** 2.5 mass% D_2O , **g** 2.8 mass% D_2O , and **h** 3.8 mass% D_2O

The water molecules have a large dipole moment of 1.85 D (Kemp and Gordon 2008), while IPA molecules have a dipole moment of 1.6 D (Nakao et al. 1997). It is known that the addition of small amounts of water to IPA promotes the growth of C_{60} NWs by lowering the growth activation energy of C_{60} NWs (Miyazawa and Hotta 2010). This fact implies that a high desolvation energy of C_{60} can be lowered by the catalytic effect of water (Hotta and Miyazawa 2008). It is assumed that water molecules reduce the interaction forces between C_{60} molecules and toluene molecules in solution and that the accumulation process of C_{60} molecules on the surface of C_{60} NWs is enhanced. On the other hand, the

water molecules are expected to interact strongly with IPA molecules and to form the complicated water–IPA molecular clusters that hinder the interdiffusion between toluene and IPA as reported in a system of water–methyl alcohol (Guo et al. 2003). As a result, the supersaturation degree of C_{60} , the driving force of whisker formation, will be decreased by addition of water and lead to the formation of granular crystals. Actually, we showed that the reduction of supersaturation leads to the formation of granular crystals instead of C_{60} NWs (Miyazawa and Hotta 2010).

Although the reduction of growth activation energy by addition of water enhances the growth

of C₆₀NWs, it is also expected to accelerate the reverse redissolution reaction of C₆₀NWs which leads to the reprecipitation of granular C₆₀ crystals. Hence, the formation of granular C₆₀ precipitates by addition of excess amounts of water is conjectured to be induced by combination of the following two mechanisms.

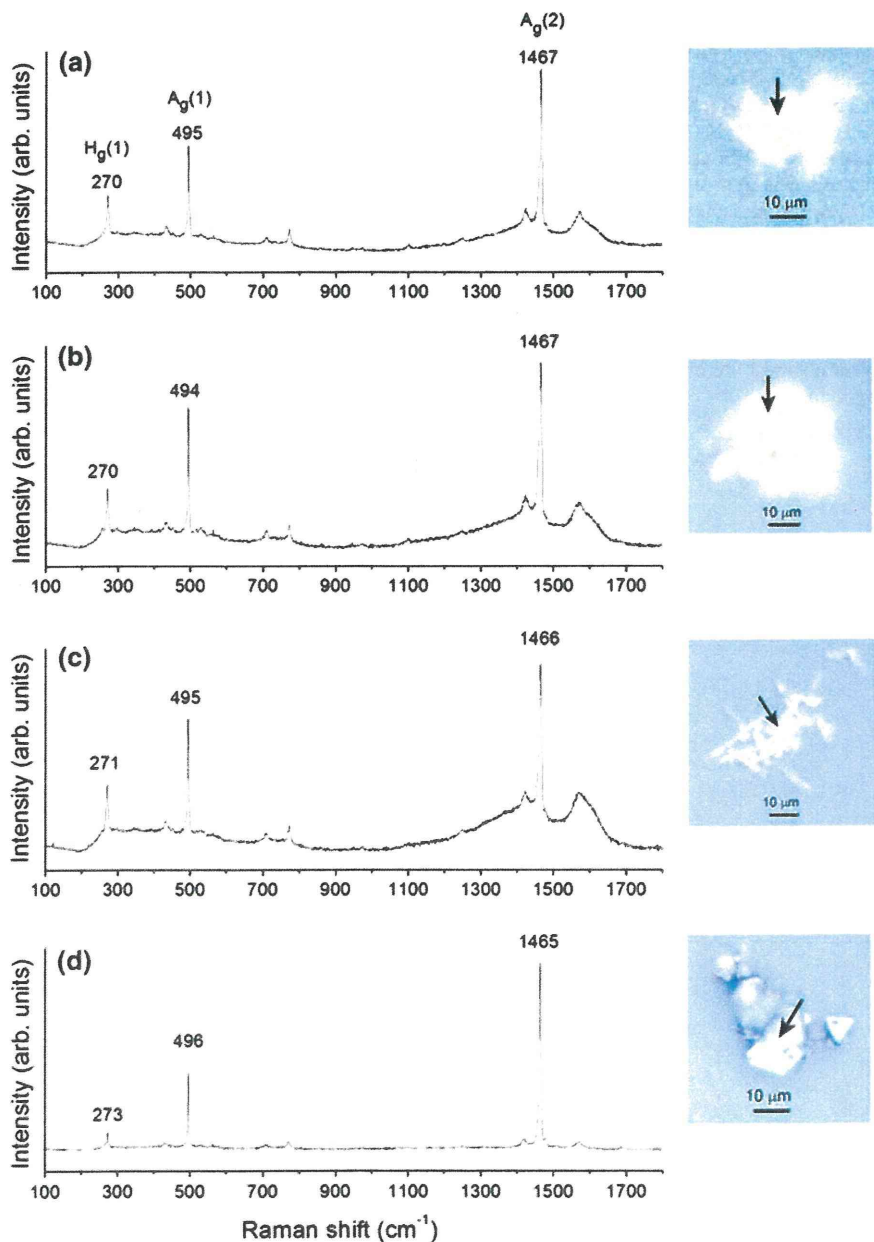
(1) Reduction of the supersaturation of C₆₀ with time which leads to the nucleation of granular crystals.

(2) Acceleration of the reverse redissolution reaction of C₆₀NWs by the reduction of growth activation energy, leading to the reprecipitation of granular C₆₀ crystals.

Raman spectroscopy of the C₆₀NWs synthesized by using IPA added with water

As shown above, the morphology of C₆₀ precipitates depends on the content of water added to IPA.

Fig. 5 Raman spectra of the C₆₀ precipitates prepared by using IPA containing. **a** 0 mass% H₂O, **b** 1.3 mass% H₂O, **c** 2.5 mass% H₂O, and **d** 2.8 mass% H₂O, respectively. The optical micrographs in **a**, **b**, and **c** show the measured C₆₀NWs and the micrograph in **d** shows the measured granular C₆₀ precipitate. The places where the Raman spectra were obtained are indicated by arrows

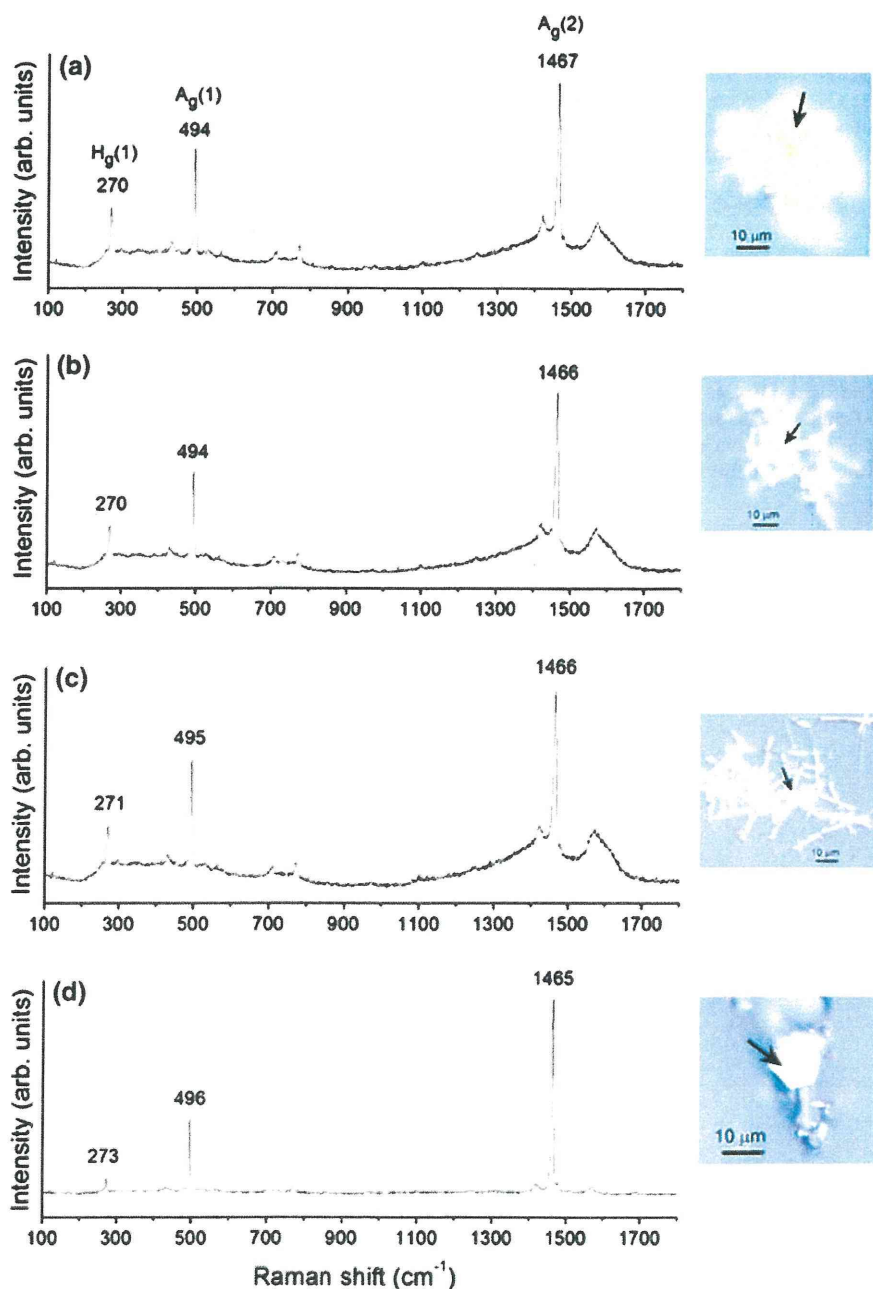


Figures 5 and 6 show the Raman profiles for the C_{60} NWs synthesized by using IPA with and without the water (H_2O , D_2O) addition. The Raman profiles were obtained for the C_{60} precipitates dried in air.

The Raman profiles of Fig. 5a–c were obtained for the C_{60} NWs shown in the optical micrographs, while the Raman profile (d) was measured for the arrowed granular C_{60} single-crystal precipitate. The three

strong peaks ($H_g(1)$, $A_g(1)$, $A_g(2)$) have almost similar wavenumbers irrespective of the H_2O content in IPA, showing that the H_2O addition does not influence the crystallographic structure of C_{60} NWs. It is noticed that the base line of profile (d) is much flatter than the other base lines of (a), (b) and (c) Raman profiles. This result indicates that the single-crystal C_{60} precipitates have a better crystallinity than the C_{60} NWs.

Fig. 6 Raman spectra of the C_{60} precipitates prepared by using IPA containing a 1.3 mass% D_2O , b 2.5 mass% D_2O , c 2.8 mass% D_2O , and d 3.8 mass% D_2O , respectively. The optical micrographs in a, b, and c show the measured C_{60} NWs and the micrograph in d shows the measured granular C_{60} precipitate. The places where the Raman spectra were obtained are indicated by arrows



The Raman spectroscopy measurement was also performed for the C₆₀NWs prepared by using IPA added with D₂O as shown in Fig. 6. Like the cases of Fig. 5, the H_g(1), A_g(1), and A_g(2) peaks have similar wavenumbers, showing that the addition of D₂O does not influence the crystallographic structure of C₆₀NWs. The granular single-crystal C₆₀ precipitate has the base line much flatter than those of C₆₀NWs like the case of Fig. 5, showing the better crystallinity than the C₆₀NWs.

Although both the H₂O and D₂O additions do not give clear influences on the crystallographic structure of C₆₀NWs, the addition of H₂O and D₂O was found to give the strong influence on the morphology of C₆₀ precipitates and their stability in solution.

Preparation of short C₆₀NWs

As described in the introductory part, the growth control of C₆₀NWs is of importance for their application to organic thick film solar cells. Somani et al. fabricated short C₆₀NWs with lengths between 2 and 5 μm and diameters between 500 and 1000 nm by a modified LLIP method, where a C₆₀-saturated toluene solution was added to IPA drop by drop. Hence, this method does not form a stable liquid–liquid interface and instantaneously produces short C₆₀NWs. This method is very similar to our method that produces short C₆₀NWs by adding droplets of C₆₀-saturated toluene solution into IPA under ultrasonication (Miyazawa et al. 2005).

However, in the modified LLIP method of this paper, as shown in the experimental part, a stable liquid–liquid interface is first produced and then the interface is forcibly disturbed by manual mixing in order to obtain homogeneously precipitated C₆₀NWs. Using this method, very short C₆₀NWs with an average length of 1.8 ± 0.8 μm and an average diameter of 387 ± 131 nm were successfully synthesized as shown in the scanning electron microscopy (SEM) image of Fig. 7. The inset image shows a magnified image of the arrowed C₆₀NW with a hexagonal cross-section. The hexagonal cross-sectional image reflects the hexagonal crystal structure of C₆₀NWs grown in solution (Minato and Miyazawa 2005).

Figure 8 shows the distributions of length, diameter, and aspect ratio (length/diameter) of the above C₆₀NWs. The C₆₀NWs have lengths less than 5.5 μm,

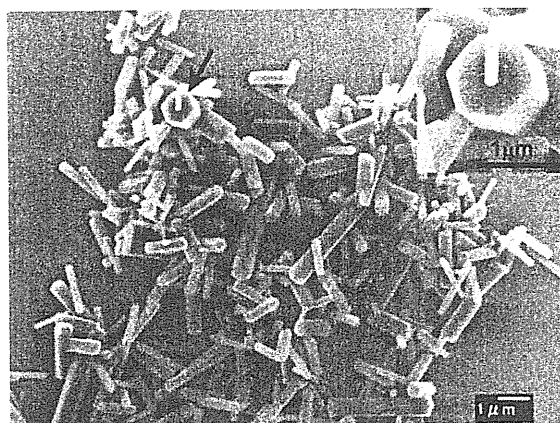


Fig. 7 SEM image of the short C₆₀NWs synthesized by a modified LLIP method. The inset shows a magnified image of the arrowed part

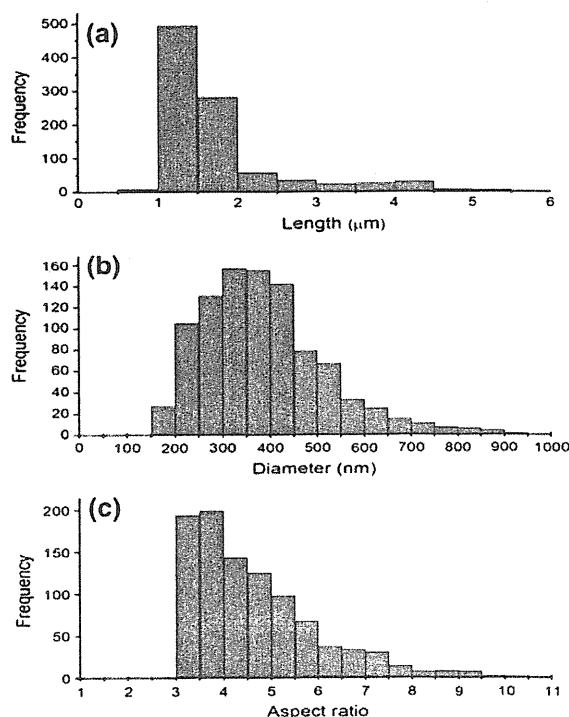


Fig. 8 a Length, b diameter, and c aspect ratio (length/diameter) distributions of the synthesized short C₆₀NWs

diameters less than 900 nm, and their aspect ratio ranges from 3 to 10. Since fibers are defined as particles with a length at least three times their diameter (The Royal Society and the Royal Academy of Engineering 2004), the synthesized short C₆₀NWs can be classified into the category of fibers with the smallest aspect ratio.

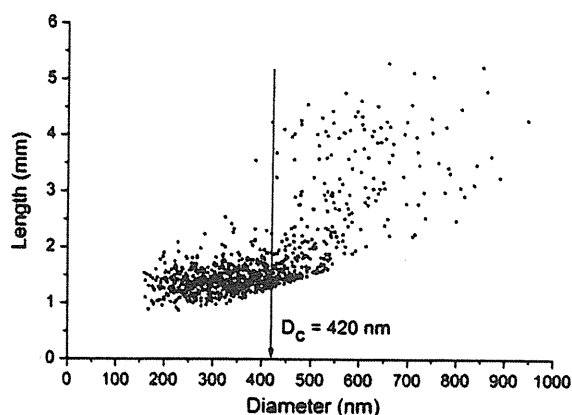


Fig. 9 Relationship between length and diameter of the C_{60} NWs synthesized at 15 °C using a C_{60} -saturated toluene solution and IPA at a volume ratio of 1:3

As shown above, the C_{60} NWs synthesized by us have the diameters and lengths smaller than those ever reported. The newly synthesized short C_{60} NWs would be more appropriate for the organic thick film solar cell application using C_{60} NWs. As shown in Fig. 9, it is observed that C_{60} NWs grow longer above the diameters greater than about 420 nm. This diameter (~ 420 nm) is regarded as a critical diameter D_c above which C_{60} NWs start to grow rapidly (Miyazawa and Hotta 2010). On the other hand, the D_c value for the growth temperature of 20 °C and the same solvent ratio of 1 (toluene):3 (IPA) with the case of Fig. 9, the D_c value was about 502 nm which is larger than the present value of 420 nm (Miyazawa and Hotta 2010). This fact is consistent with the hypothesis that the D_c value becomes smaller with increasing the supersaturation of C_{60} in solution with decreasing the growth temperature.

Conclusion

The above research can be summarized as follows.

- (1) The stability of C_{60} NWs decreased and granular crystals of C_{60} appeared in the solutions prepared by using IPA added with excess amounts of water. The C_{60} NWs were found to be destabilized with time in the solutions added with water. These phenomena were observed in both of the experiments using H_2O and D_2O .
- (2) The C_{60} NWs dried in air showed similar Raman profiles irrespective of the use of IPA with and

without the water addition. The Raman profiles of granular C_{60} single crystals showed the base lines much flatter than those of C_{60} NWs, indicating that C_{60} NWs possess a disordered crystal structure.

- (3) Short C_{60} NWs with aspect ratios ranging from 3 to 10 and an average length of about 1.8 μm were successfully fabricated. The short C_{60} NWs will be efficiently applied for the electrodes of organic thick film solar cells.

Acknowledgment Part of this research was supported by Health and Labour Sciences Research Grants (H21—Chemistry—Ippan-008) from the Ministry of Health, Labour and Welfare of Japan.

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