High-Performance Automation and Centrifugation Preparation of Carbon Nanotubes for Analytical Ultracentrifugation

Uniquely effective technique for quantitative evaluation of single-walled and double-walled carbon nanotubes.

Abstract

This application note focuses on two challenging areas for nanoparticles: reliable, rapid scale-up of nanoparticle purification; and quantitative analysis of the concentration of species present. The Biomek 4000 Laboratory Automation Workstation helps overcome the human variable and gives a consistent, reproducible, high-throughput method for gradient setup, which provides a breakthrough for scale-up problems. Preparative Ultracentrifugation helps to maintain reliability between runs, making them highly reproducible. Analytical Ultracentrifugation is ideal for analyzing nanoparticles like nanotubes, quantum dots, and graphene, because it requires a low volume and low concentration—while providing statistically significant details about the composition of the nanoparticles in solution.

Introduction

Single-Walled Carbon Nanotubes (SWCNT) experienced increasing interest in the past 15 years for semiconductors,[1] fuel cells,[2] and biomedical applications.[3] All of these areas face two major bottlenecks, however. First, the synthesis of SWCNT results in numerous carbonaceous impurities as well as the possible presence of carbon nanotubes with multiple walls (MWCNT). Also, there is no reliable method to quantify the percentage of non-SWCNT material present.[4] Secondly, SWCNTs are synthesized in a heterogeneous variety of wrapping schemes [known as (n,m) chiralities] (Figure I)[5]; different chiralities have vastly different optical and electronic properties.[6,7] There exists a need for large-scale, post-synthesis separation of SWCNT into a single, homogeneous chirality, especially for semi-conductors (where metallic SWCNTs greatly reduce the on/off ratio)[8] and *in vivo* drug delivery and imaging applications.[9-11] Initial efforts to determine SWCNT impurity in solution have centered on electron microscopy, which lacks statistical significance, can be influenced by human bias, and has difficulty distinguishing between double-walled carbon nanotubes (DWCNT) with a 3 nm diameter, and SWCNT with a 1 nm diameter. UV-Vis absorption, Near-Infrared Fluorescence, and Raman spectroscopy all have been researched as solutions to the impurity problem; these techniques unfortunately contain inherent flaws that prevent them from being an acceptable solution for quantitatively analyzing SWCNTs impurities.[4]

As for chirality-enrichment of a single SWCNT species, one of the most successful techniques to date has been Density Gradient Ultracentrifugation (DGU).[12,13] DGU is able to achieve 99% purity of (6, 5) SWCNT, which is highly desirable. However, DGU has significant scalability issues due to the handmade gradient typically used.

In this application note, a work flow is presented that can quickly and reproducibly separate heterogeneous, bulk SWCNT into a single (6,5) chirality, confirmed by UV-Vis. The key features to this work flow are a rapid, two-minute ultracentrifuge treatment (Optima MAX-XP, Beckman Coulter, Inc.) to initially purify SWCNT by removing large aggregated species, followed by a density gradient setup by an automated liquid handler (Biomek 4000, Beckman Coulter Inc.) and use of an Optima X Series Preparative Ultracentrifuge to purify SWCNT and DWCNT using density gradient runs. The use of automation allows for better precision and reproducibility with the density gradient than can be achieved by hand. This level of precision is very relevant for separation of SWCNT, where the density steps only differ by roughly ±1% g/ml. An unsteady hand can easily disturb the gradient whereas the automation has no such concern. In the second part of this application note, we highlight how Analytical Ultracentrifugation can quantitatively distinguish between SWCNTs and length-fractionated DWCNTs. Analytical Ultracentrifuge (AUC) has traditionally been used primarily in protein analysis, but AUC has analytical abilities that are well-suited for characterizing nanoparticles. By determining sedimentation coefficient, diffusion coefficient, and frictional ratio of nanoparticles in various density solvents, AUC can 'fill in the gaps' of nanomaterial analysis where other techniques, such as electron microscopy and optical spectroscopy, are lacking.[14,15]

Experimental Procedures

Gradient Prep

The Density Gradient was made on the Biomek 4000 Workstation by using a P-1000SL Single-Tip Pipette Tool and P1000 wide bore tips. The method has flexibility to change volumes for each gradient as well as the number of tubes being prepared. A 14 mm 24-position tube rack was used to hold centrifuge tubes (Beckman Coulter P/N 331372) which were programmed in as new labware. A slow pipetting technique with liquid level sensing was used to minimize mixing of the gradients. The gradient was an overlaid gradient as shown in Table 1.

Table 1. Density Gradient Architecture Single-Chirality Separation

Layer in Gradient	Density (g/mL)	%OP	1st Iteration Volume (µL)	SC (% w/v)	SDS (% w/v)
1	1.160	30	900	0.75	0.175
2	1.147	27.5	756	0.75	0.175
3	1.133	25	972	0.75	0.175
4	1.120	22.5	1,188	0.75	0.175
5	1.107	20	1,188	0.75	0.175
6	1.093	17.5	1,305	0.75	0.175
SWCNT	1.133	25	1,800	2	0

Single-Walled Carbon Nanotube (SWCNT) Preparation[16]

20 mgs of SWCNTs were bath-sonicated in 2% sodium cholate (SC) in deionized water on a Branson M1800H ultrasonic cleaner for one hour in a 20 mL glass vial. Using a TLA 120.2 rotor in an Optima MAX-XP ultracentrifuge, SWCNT solution was centrifuged in open-top thick-wall polycarbonate centrifuge tubes (Beckman Coulter P/N 343778) at 22° C, 55,000 rpm (~131,000 × g) for two minutes, to crash out any large aggregates. 1100 µL of supernatant was collected with care, to avoid disturbing the pelleted aggregates, and used for density gradient run. 1.8 mL of SWCNT solution balanced to a density of 1.13 g/mL (25 %OP) using 2% SC+OP were inserted between 27.5% and 25% Optiprep layers (Sigma-Aldrich) in prefilled gradient tubes. The centrifuge tubes were balanced and filled within 2 to 3 mm of the top using DI water with the same surfactant ratio as Optiprep. Using a SW 41 Ti rotor in an Optima XPN, they were spun at 41,000 rpm (~288,000 × g) for 32 hours at 22° C using minimum acceleration and deceleration rates (Profile 9). After centrifugation, the top 2 mL were removed using a large syringe, taking care not to disturb the bands below. The region with (6,5) SWCNT was aliquoted in 150 µl fractions (Figure 5).

Double-Walled Carbon Nanotube (DWCNT) dispersion

20 mgs of DWCNTs were bath-sonicated in 2% SC in deionized water on a Branson M1800H ultrasonic cleaner for one hour in a 20 mL glass vial. Using a TLA 120.2 rotor in an Optima MAX-XP ultracentrifuge, DWCNT solution was centrifuged in open-top polycarbonate centrifuge tubes (Beckman Coulter P/N 343778) at 22° C, 30,000 × g for two minutes, to crash out any large aggregates. 1,300 µL of supernatant was collected with care, to avoid disturbing the pelleted aggregates, and used for density gradient run. A density gradient was prepped manually in polyallomer centrifuge tubes (Beckman Coulter P/N 331372) as shown in Table 2 in order to length-fractionate the DWCNT. 1.5 mL of DWCNT solution was overlaid on the gradient. The centrifuge tubes were balanced and filled within 2 to 3 mm of the top using DI water with 2% SC. They were spun using a SW 41 Ti rotor in an Optima XPN and a two-step program—first run at 15,000 rpm (~38,500 × g) for one hour, followed by a second run at 30,500 rpm (~159,500 × g) for one hour at 22° C with maximum acceleration and deceleration rates (Profile 0). After the centrifugation, 600 µL fractions were collected from top to bottom and fractions 4–6 were pooled (Figure 6).

Table 2. Density Gradient Architecture Double-Wall Separation

Layer in Gradient	Density (g/mL)	%OP	1st Iteration Volume (µL)	SC (% w/v)
1	1.320	60	1,500	2
2	1.160	30	1,500	2
3	1.133	25	1,500	2
4	1.107	20	1,500	2
5	1.08	15	1,500	2
6	1.053	10	1,500	2
DWCNT	1	0	1,500	2

Fraction Analysis

UV-Vis-NIR absorption plots (Paradigm, Molecular Devices) were taken of each SWCNT fraction from 400–1,000 nm; the fractions with the strongest absorption peaks at 570 and 990 nm, and minimal absorption peaks at other wavelengths, were pooled.

Dialysis
After fractionation, the separated, individual (6,5) chirality SWCNT were dialyzed with a 3.5 kDa MWCO cellulose membrane against 1% SC to remove iodixanol and sodium dodecyl sulfate from the SWCNT solution and to re-establish the surfactant coating of the SWCNT. Eight water changes were performed, with at least four hours between water changes. After dialysis, the resulting dispersion was concentrated using Amicon Ultra Centrifuge Filters (Millipore) on a Microfuge 20 microcentrifuge (Beckman Coulter, Inc.). The same procedure was performed with the pooled, length-fractionated DWCNT solution.

Sedimentation Range Determination
SWCNT and DWCNT solutions were run on a ProteomeLab XL-A Analytical Ultracentrifuge (Beckman Coulter, Inc.). (6,5) chirality-enriched SWCNT with an absorption of O.D. 0.85 at the 570 nm was loaded into a two sector 12 mm charcoal-filled EPON cell with quartz windows. 1% SC in DI water (taken from the last dialysis water change) was used as reference. Sample volume was 370 μL and reference volume was 380 μL. Another cell with the same specifications was loaded with length-fractionated DWCNT with an O.D. of 0.85 at 570 nm. A third cell was loaded with 50%/50% SWCNT/DWCNT dispersion by absorption at 570 nm. Initial run conditions were four hours at 27,000 rpm @ 22° C.[7] This experiment was repeated for absorption conditions with O.D. of 0.6 at 570 nm to check for concentration-dependent effects.

Sedimentation Analysis
Analysis was done in SEDFIT, fitting to models according to the Lamm equation. From the work of Arnold et al, a fit of a single-component Lamm equation considering diffusion should give the best fit for the data.[7] Sedimentation coefficients were compared for SWCNT and DWCNT; the ability of analytical ultracentrifugation to distinguish both species in solution was tested as well.

Size Distribution Analysis via Dynamic Light Scattering
The DelsaMax CORE Dynamic Light Scattering/Static Light Scattering instrument (Beckman Coulter, Inc.) was used to analyze a small volume of sample of the length-fractionated DWCNT and chirality-enriched (6,5) SWCNT. ~10 μL was placed in the quartz sizing cuvette and run at 25° C with 10 acquisitions, five seconds/acquisition. The representative curves were generated by analyzing the runs in Regularization (Multimodal) mode.

Results

The success of the DGU SWCNT separation can be easily visualized in Figure 5. Before ultracentrifugation (Figure 5a), the SWCNT appears as a black solution because of the heterogeneous mixture of chiralities that have absorption peaks throughout the visible range. After ultracentrifugation, individual chiralities emerge as colored bands (Figure 5b); the top, purple band contains (6,5) SWCNTs that are collected for AUC analysis. SWCNTs have Van Hoff singularity absorption peaks in the Near-Infrared and visible region; for (6,5) SWCNT coated with SC, the peaks theoretically occur at 570–580 nm and 980–990 nm.[6] The absorption plot in Figure 7 is taken after dialysis and concentration of the (6,5) SWCNT in 1% SC solution. The strong peaks at 571 nm and 990 nm, along with the lack of strong absorption peaks at other wavelengths, point to the purity of the chirality-enriched (6,5) SWCNT. DWCNT are length-fractionated following a similar procedure for SWCNT.[18] The top-most fraction, indicated in Figure 6b, should contain mostly unbundled DWCNT; dynamic light scattering data (Figure 8) confirms that the DWCNTs have an average length near 200 nm based on a diffusion coefficient of $2.1 * 10^{-8}$ cm^2/s.

Dynamic Light Scattering, taken on the DelsaMax CORE, also highlights the difficulty in discerning between single-walled and double-walled carbon nanotubes (Figure 8). While DWCNT and SWCNT have very different optical properties, including absorption (Figure 7) and fluorescence,[19] the physical diameters and density are very similar. Both have lengths between 100–1,000 nm and closely related diameters (~1 nm for SWCNT, ~2–3.5 nm for DWCNT[19]). This makes it difficult for ensemble techniques like light scattering to differentiate between the two species. Likewise, even electron microscopy has difficulty having small enough height sensitivity to distinguish SWCNT and DWCNT reliably, while counting of a few hundred nanotubes is not representative of a solution containing upwards of 10^{18} particles.

Analytical Ultracentrifuge is easily able to distinguish between SWCNT and DWCNT (Figure 9c). The DWCNT, due to poor surfactant coating of the sodium cholate molecules, sediment very rapidly during ultracentrifugation in SC buffer, with an average sedimentation coefficient of 80.4 ± 25.6 S (Figures 9b, 10b). In contrast, the SWCNT sediment slowly, as previously reported in literature,[17,20] with average sedimentation coefficient of 11.3 S

(Figures 9a, 10a). To truly demonstrate the power of AUC, a mixture was made of SWCNT and DWCNT. Both SWCNT and DWCNT solutions had absorptions of 0.894 O.D. at 570 nm; 175 µL of each solution was mixed and run in the AUC (Figure 9c). Looking at pure (6,5) SWCNT, it was shown that there are very few particles that have sedimentation coefficients greater than 30 S (Figure 10a), whereas the length-fractionated DWCNT contains very few particles that have sedimentation coefficient less than 30 S. By using 30 S as a cutoff point and integrating the sedimentation coefficient distribution plots (Figure 10c), the solution is quantitatively shown to contain 50.4% SWCNT and 49.6% DWCNT by absorption. This quantitative evaluation of SWCNT and DWCNT is unachievable by any other analytical technique. Two other SWCNT/DWCNT mixtures were tested to confirm the quantitative power of AUC. A 29% SWCNT/71% DWCNT by absorption was determined to have a ratio of 28.3%/71.7% by sedimentation coefficient distribution (Figure 10d). Similarly, a 71.4% SWCNT/28.6% DWCNT solution by absorption was shown to have a ratio of 64.7%/35.3% by sedimentation coefficient distribution (data not shown). Both tests used 30 S as the cutoff between the (6,5) SWCNT and DWCNT.
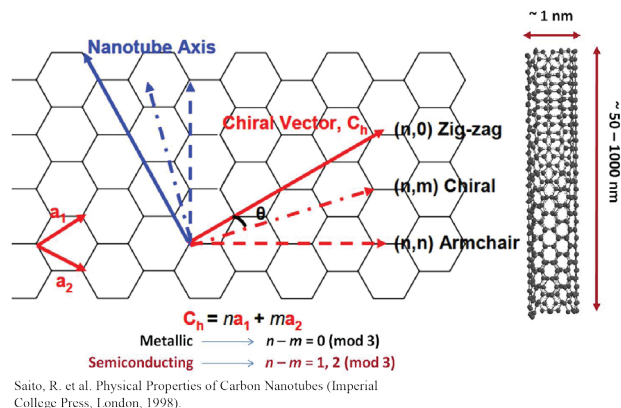

Saito, R. et al. Physical Properties of Carbon Nanotubes (Imperial College Press, London, 1998).

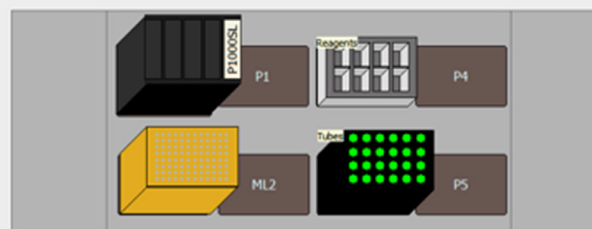**Figure 1.** Single-Walled Carbon Nanotube Schematic.

Figure 2. Deck Layout of the Biomek 4000 Workstation Showing the Basic Tools Required for Gradient Prep. (1) One 24-position tube rack for placing nanotubes: the centrifuge tubes fit the existing 24-position tube rack, but new labware type had to be created to accommodate the height of the tubes; (2) one P1000 tip box for P1000 Wide Bore tips; (3) one Biomek 4000 P1000SL Single-Tip Pipette Tool for liquid transfer; (4) one Modular Reservoir for gradient reagents.
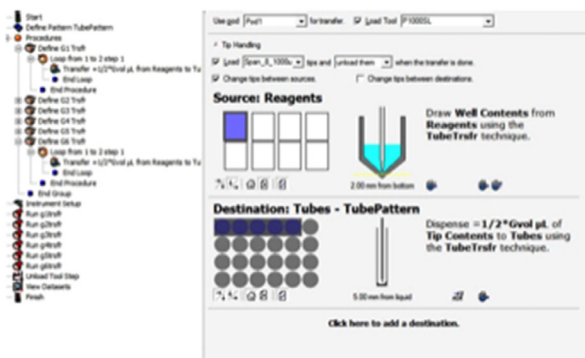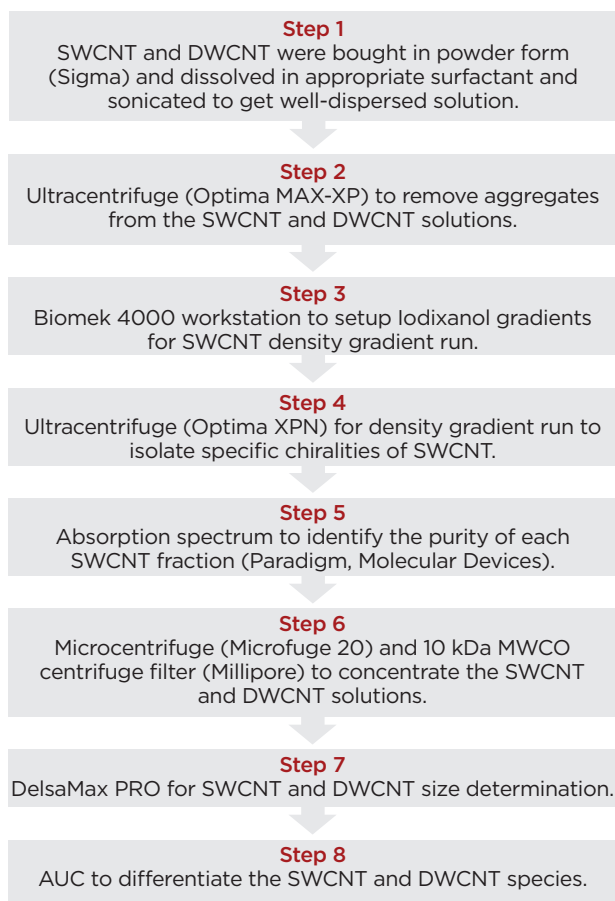

Figure 3. Nanotube Gradient Method. New tube transfer technique was created to minimize the mixing during gradient prep.

Step 1
SWCNT and DWCNT were bought in powder form (Sigma) and dissolved in appropriate surfactant and sonicated to get well-dispersed solution.

↓

Step 2
Ultracentrifuge (Optima MAX-XP) to remove aggregates from the SWCNT and DWCNT solutions.

↓

Step 3
Biomek 4000 workstation to setup Iodixanol gradients for SWCNT density gradient run.

↓

Step 4
Ultracentrifuge (Optima XPN) for density gradient run to isolate specific chiralities of SWCNT.

↓

Step 5
Absorption spectrum to identify the purity of each SWCNT fraction (Paradigm, Molecular Devices).

↓

Step 6
Microcentrifuge (Microfuge 20) and 10 kDa MWCO centrifuge filter (Millipore) to concentrate the SWCNT and DWCNT solutions.

↓

Step 7
DelsaMax PRO for SWCNT and DWCNT size determination.

↓

Step 8
AUC to differentiate the SWCNT and DWCNT species.
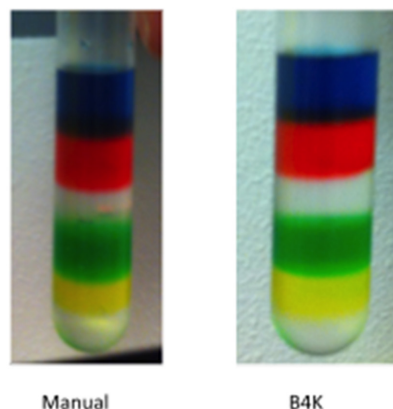

Manual   B4K

Figure 4. Test Gradient Preparation using Iodixanol with food coloring to show the distinct layering of each gradient, and comparison of manual versus Biomek 4000 workstation.
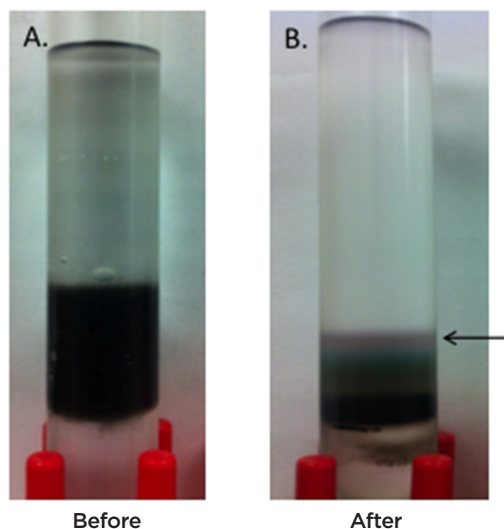
Before — **After**

Figure 5. (6,5) SWCNT Separation Based on Chirality. Pictures of centrifuge tube with SWCNT before (**5a**) and after (**5b**) Density Gradient Ultracentrifugation. 0.2 mL fractions were collected from the region with the purple tint (indicated with the arrow) and were subjected to absorption analysis and pooled based on absorbance peak at 575 nm.
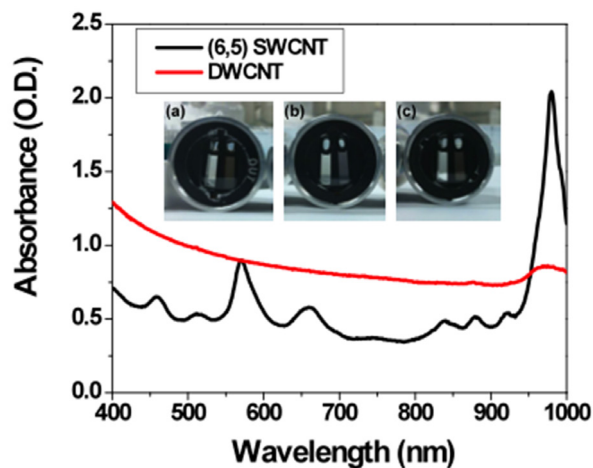

Figure 6. DWCNT Length Separation. Optical image of centrifuge tube with DWCNT (**6a**) before Density Gradient Ultracentrifugation and (**6b**) after Density Gradient Ultracentrifugation. 0.6 mL fractions were aliquoted and fractions 4–6 were collected for further analysis. Approximate location of fractions 4–6 is indicated with a brace (}).

Figure 7. Absorption Plot of Concentrated Length-Separated Double-Walled Carbon Nanotubes (DWCNT, Red Curve) and Chirality-Enriched (6,5) Single-Walled Carbon Nanotubes (SWCNT, Black Curve). Inset are images of the AUC cells with reference buffer in the left chamber and sample solution in the right chamber. (a) contains DWCNT only; (b) contains primarily (6,5) SWCNT, indicated by the strong peak at 570 and 980 nm.
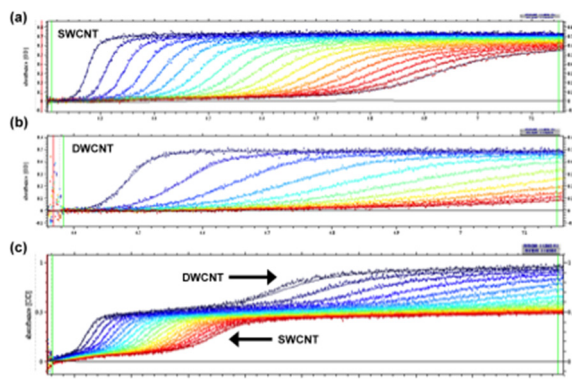

Figure 8. Representative Dynamic Light Scattering Data on the DelsaMax CORE. The carbon nanotube species generate the peaks above 100 nm in diameter while the surfactant micelles are represented by the peaks near 10 nm in diameter. Note that it would be impossible to distinguish between SWCNT and DWCNT based on dynamic light scattering.

Figure 9. AUC curves from SEDFIT. (**9a**) The raw absorbance data with fitting of a solution containing only (6,5) SWCNT. (**9b**) The raw absorbance data with fitting of a solution containing only length-fractionated DWCNT (**9c**) The raw absorbance data with fitting of a solution containing both (6,5) SWCNT and DWCNT.
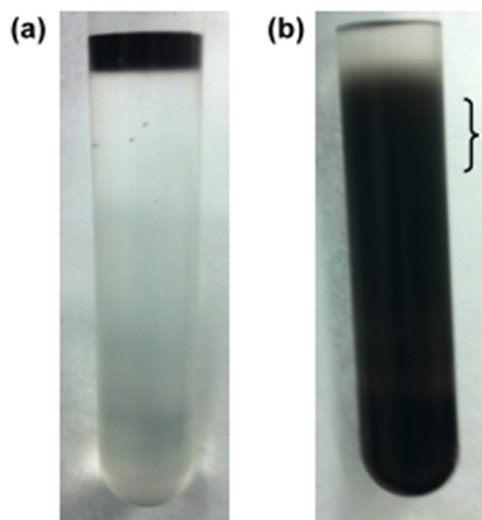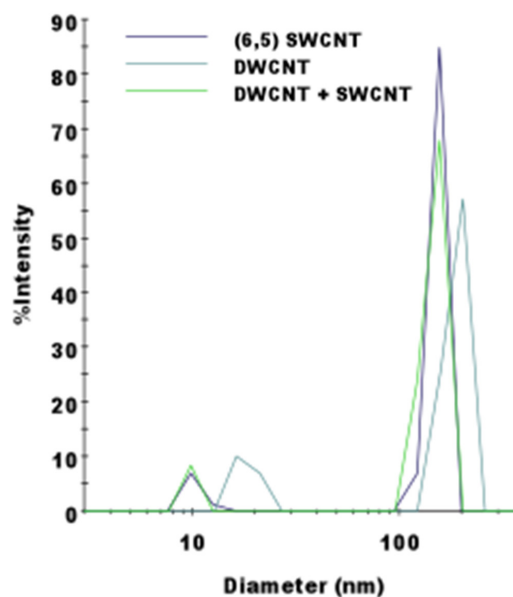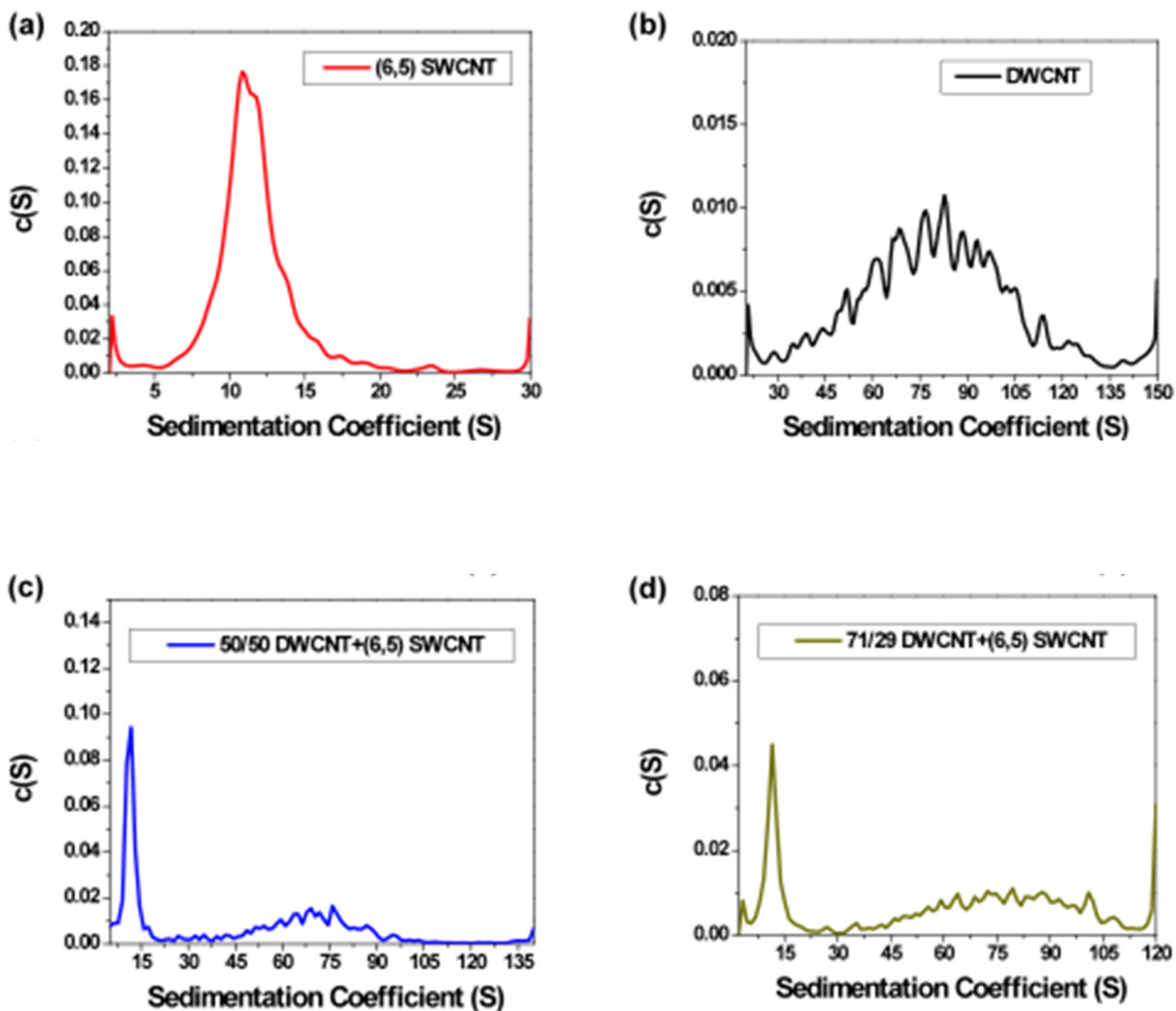
Figure 10. Sedimentation Coefficient Distribution Plots. (**10a**) Chirality-enriched (6,5) SWCNT. The average sedimentation coefficient for chirality-enriched (6,5) SWCNT is 11.3 S, which agrees well with literature. Note that essentially all particles in the (6,5) SWCNT solution have sedimentation coefficient less than 30 S. (**10b**) Length-Fractionated DWCNT. The average sedimentation coefficient for length-fractionated DWCNT is 80.4 ± 25.6 S; the large spread indicates that some DWCNT may exist as a bundled pair. Note that nearly all sedimenting particles in the DWCNT sample have sedimentation coefficient above 30 S. (**10c**) DWCNT and (6,5) SWCNT 50/50 mixture. DWCNT and (6,5) SWCNT solutions each had identical absorption at 570 nm and were mixed in equivolume amounts. Integrating the sedimentation distribution, 50.4% of the total signal has sedimentation coefficient between 5–30 S, with an average value of 11.2 ± 5.2 S, while 49.6% of the total signal has sedimentation coefficient between 30–140, with an average value of 70.2 ± 21.3 S. (**10d**) DWCNT and (6,5) SWCNT 71/29 mixture. DWCNT and (6,5) SWCNT solutions each had identical absorption at 570 nm and were mixed at a ratio of 71:29 DWCNT:(6,5) SWCNT. Integrating the sedimentation distribution, 28.3% of the total signal has sedimentation coefficient between 2–30 S, with an average value of 12.5 ± 3.9 S, while 71.7% of the total signal has sedimentation coefficient between 30–120, with an average value of 80.0 ± 21.0 S.

References

1. Baughman R H, Zakhidov A A, and de Heer W A. Carbon nanotubes—the route toward applications. *Science*. 297.5582; 787–792: (2002).

2. Che G et al. Carbon nanotubule membranes for electrochemical energy storage and production. *Nature*. 393.6683; 346–349: (1998).

3. Liu Z et al. Carbon nanotubes in biology and medicine: *in vitro* and *in vivo* detection, imaging and drug delivery. *Nano research*. 2.2; 85–120: (2009).

4. Lopez-Lorente A I, Simonet B M and Valcárcel M. Qualitative detection and quantitative determination of single-walled carbon nanotubes in mixtures of carbon nanotubes with a portable Raman spectrometer. *Analyst*. 138.8; 2378–2385: (2013).

5. Saito R. *Physical properties of carbon nanotubes*: (1998).

6. O'Connell M J et al. Band gap fluorescence from individual single-walled carbon nanotubes. *Science*. 297.5581; 593–596: (2002).

7. Liu H et al. Large-scale single-chirality separation of single-wall carbon nanotubes by simple gel chromatography. *Nature Communications*. 2; 309: (2011).

8. Zhang L et al. Assessment of chemically separated carbon nanotubes for nanoelectronics. *Journal of the American Chemical Society*. 130.8; 2686–2691: (2008).

9. Welsher K et al. A route to brightly fluorescent carbon nanotubes for near-infrared imaging in mice. *Nature nanotechnology*. 4.11; 773–780: (2009).

10. Liu Z et al. Supramolecular stacking of doxorubicin on carbon nanotubes for in vivo cancer therapy. *Angewandte Chemie International Edition*. 48.41; 7668–7672: (2009).

11. Bhirde A A et al. Targeted killing of cancer cells in vivo and in vitro with EGF-directed carbon nanotube-based drug delivery. *ACS nano*. 3.2; 307–316: (2009).

12. Green A A, Duch M C and Hersam M C. Isolation of single-walled carbon nanotube enantiomers by density differentiation. *Nano Research*. 2.1; 69–77: (2009).

13. Komatsu N and Wang F. A comprehensive review on separation methods and techniques for single-walled carbon nanotubes. *Materials*. 3.7; 3818–3844: (2010).

14. Carney R P et al. Determination of nanoparticle size distribution together with density or molecular weight by 2D analytical ultracentrifugation. *Nature Communications*. 2; 335: (2011).

15. Falabella J B et al. Characterization of gold nanoparticles modified with single-stranded DNA using analytical ultracentrifugation and dynamic light scattering. *Langmuir*. 26.15; 12740–12747: (2010).

16. Antaris A L et al. Ultra-Low Doses of Chirality Sorted (6, 5) Carbon Nanotubes for Simultaneous Tumor Imaging and Photothermal Therapy. *ACS nano*. 7.4; 3644–3652: (2013).

17. Arnold M S et al. Hydrodynamic characterization of surfactant encapsulated carbon nanotubes using an analytical ultracentrifuge. *ACS nano*. 2.11; 2291–2300: (2008).

18. Tabakman S M et al. Optical properties of single-walled carbon nanotubes separated in a density gradient: length, bundling, and aromatic stacking effects. *The Journal of Physical Chemistry C*. 114.46; 19569–19575: (2010).

19. Hertel T et al. Spectroscopy of single-and double-wall carbon nanotubes in different environments. *Nano letters*. 5.3; 511–514: (2005).

20. Fagan J A et al. Analyzing Surfactant Structures on Length and Chirality Resolved (6, 5) Single-Wall Carbon Nanotubes by Analytical Ultracentrifugation. *ACS nano*. 7.4; 3373–3387: (2013).

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at www.beckmancoulter.com