Spiral Countercurrent Chromatography Enrichment, Characterization, and Assays of Carbon Nanotube Chiralities for Use in Biosensors

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ABSTRACT: Single-walled carbon nanotubes (SWCNTs) are synthetic materials that hold great promise for electronics that are smaller and more versatile than the current silica-based technologies. But as-produced SWCNTs are generally a mixture of nanotubes with different structures that have vastly different properties. Separating these SWCNTs from multiwalled and metallic carbon nanotubes is vital to explore their individual properties and commercial utility ranging from optics to semiconductors. Compounding the problem of commercial investigation is that the semiconducting SWCNTs are also a mixture of different diameters and/or chiralities with different properties. Analyzing properties of enriched semiconducting SWCNT chiralities has only recently been possible through separation techniques such as aqueous two-phase solvent systems. Our study illustrates a semipreparative spiral countercurrent chromatography (CCC) separation of a commercial mixture of SWCNTs into distinct enriched fractions. A new mixer–settler spiral disk rotor was applied in this study, in which we compare the enriched SWCNTs for their effectiveness in biosensors with a high-throughput model assay, followed by antibody-mediated detection of Escherichia coli. Our results demonstrate that CCC-enriched responsive SWCNTs for biosensors can be used in our model assay, as well as for the detection of E. coli. To date, we believe that this is the first study along with Liu et al. [Chirality-controlled synthesis of single-wall carbon nanotubes using vapour-phase epitaxy. Nat. Commun. 2012, 3, 1199] to demonstrate a specific utility of separated SWCNT species.

INTRODUCTION

Natural and man-made pure carbon compounds including graphene, fullerenes, and carbon nanotubes (CNTs) show great potential for a wide variety of applications ranging from solid-state physics to medicine. In particular, CNTs have received a great deal of attention due to their mechanical strength and electronic properties. These carbon compounds are poised to replace silicon in the inexorable march toward smaller, more efficient, and more compact electronic instruments.

CNTs are carbon–carbon extended polymers in two dimensions with fused sp² orbitals that have aromatic properties.¹² The sheets form long nanotubes of various diameters from less than 1 nm to a few hundred nm and have lengths of 500 nm to several hundreds of μm. Nanotubes are generally distinguished as being single-walled CNTs (SWCNTs) or multiwalled carbon nanotubes (MWCNTs), but both can have anisotropic structures and properties. The hexagonal array of the carbon atoms can have a left-handed or a right-handed spiral pattern. The dimensions and directionality of the patterns of the hexagonal honeycomb-like lattice are described by vectors, with certain values of n and m dictating their chirality and properties.³⁴ Over 12 distinct SWCNT chiral types or chiralities have been reported to date,⁵⁻⁶ but the commercial utility of these chiralities has not been explored.

SWCNTs can exhibit conductive or semiconductor properties depending on their chirality. These semiconductor properties can provide very fast and sensitive electronic detection in disparate applications, such as field-effect transistors (FETs), nanoscale sensors, conducting films, optimized near-IR fluorophores, and drug conjugates.⁶⁻⁹ While these specific properties of SWCNTs are still being envisioned for commercial applications, there is a dearth of chiral SWCNTs in the research marketplace.

Although methods to seed and grow homogeneous SWCNT chiralities are still being developed, they have not yet reached the stage of mass production.¹⁰ Therefore, the capability to isolate chiral SWCNT dispersions for uses such as electronic circuits will be greatly aided by a scalable and cost-effective chromatographic separation. Chiral SWCNTs have been separated by differential gradient ultracentrifugation.³⁻⁵ It was reported that column chromatography using agarose, Sephacryl,
and other dextran (DEX)-related solid supports achieved separation of semiconducting CNTs and metallic species, but these have yet to be scaled up for commercial applications and were not tested for their potential in specific commercial applications.

Aqueous two-phase extraction methods using DEX can separate CNTs by diameter, and their ability to bind detergents has been previously demonstrated. The use of DEX-based solvent systems and detergents as agents for selective extraction can be readily adapted to the countercurrent chromatography (CCC) liquid–liquid partitioning system, which is carried out in a long tubing or flow path to separate molecules in the two-phase solvent systems configured for maximum separation. CCC with the use of spiral-design rotors, introduced by Ito in the last few years, is very optimal for the preparative separation of proteins, the large biomolecules. The primary advantage of these rotors, including the mixer–settler spiral disk rotor, is that the retention of the high-viscosity aqueous two-phase solvent (ATPS) system is high, that is, 60%–80% of the stationary phase at moderate flow rates, which is not possible in the other types of CCC instruments. This has made possible its application to large molecules and now the CNTs. This indicates that CCC has significant capabilities to increase the resolution and achieve high mass recovery of semiconducting SWCNTs out of commercial preparations.

Here, we investigate a new improved spiral-design rotor with more clarified CCC procedures to purify a commercial preparation of SWCNTs. An application that is readily testable is their use in CNT-FET biodetectors.

A variety of protein-detection methods have been developed for real-time analysis, which eliminates the need for labeled detector molecules such as secondary antibodies. Such label-free detection methods can dramatically cut the time and cost associated with many assays by removing reagents and instrumentation needed for labeling and imaging. Fast, real-time, and quantitative information on biomarkers is believed to be a critical step toward efficient personalized medicine. Label-free detection of proteins can be achieved through FETs, including CNT-FET.

Using previously characterized circuitry templates to add SWCNTs, we developed a model assay using an organic protonated polymer (polybren) to mimic the changes in resistance due to protein binding to the nanotubes. This model assay is fast, easy to perform, and saturable. Our model assay was performed on the SWCNT fractions isolated by CCC and showed a clear difference in the response of enriched SWCNT chiralities. The enriched light-blue SWCNTs showed a greater response in our model assay. To verify that the model assay was reflecting real-world conditions, we coated antibodies to an infectious Escherichia coli strain onto circuits treated with the same enriched SWCNTs and measured the resistance. Our results show that the model assay directly correlates to the real-world E. coli detection.

Taken together, these data show that SWCNT functional assays can be performed directly on enriched SWCNT fractions. Simple model assays like the one described here will undoubtedly speed the characterization and properties of individual SWCNT chiralities.

**RESULTS AND DISCUSSION**

The need for separating as-produced semiconducting SWCNTs according to their chiralities is a bottleneck that slows their development as next-generation nanoelectronics because different SWCNT chiralities have different properties. Many SWCNT separation methods based on classical methods have not translated to commercially available collections of SWCNT chiralities. Existing methods for obtaining single-chirality SWCNT purification are cumbersome, often requiring large-volume extraction steps. In addition, the effect of the SWCNT purification media on direct applications of SWCNTs has, in many cases, not been fully explored.

In contrast to column chromatography that uses a solid adsorptive support, CCC uses a pair of immiscible solvents, one used as the stationary phase and the other as the mobile phase. One can create a combination of solvents to form a two-phase solvent system that has suitable solubility and provides partitioning to separate the components of the sample. There are many published methods for the different types of molecules. SWCNTs being extremely hydrophobic long molecules form tight bundles that are difficult to disperse in organic solvents. These bundles are most easily dispersed using strong detergents such as sodium dodecyl sulfate (SDS). CCC has been used to separate proteins with organic–aqueous solvent systems using detergents forming reverse micelles, and two-phase solvent systems with DEX have been developed with poly(ethylene glycol) (PEG) of various molecular weights (MWs). Thus, the published CCC literature contains many methods that can be applied.

The mixer–settler rotor that CC Biotech has been developing for large molecules was used in the first published CCC experiment. A recently modified mixer–settler spiral CCC rotor is shown in Figure 1.

![Figure 1](image)

**Figure 1.** Mixer–settler spiral CCC separation rotor described in the Methods section. Side view showing six disks sandwiched between black Viton gaskets. On the bottom edge, the screws are held with double nuts instead of nylon bolts to give a more even pressure.

**Figure 2A** shows the data of early fractions of a typical CCC separation of a 6,5i SWCNT preparation in the early stages of the SDS gradient. Low concentrations of SDS (0.1–0.3%) remove carbonaceous materials from the applied lower phase. Images of these collected fractions are shown in the insets. These data show that the early fractions (10–16) form a dark layer of precipitate between the upper PEG phase (UP) and the lower DEX layer (LP), indicating that they are not soluble in either phase with cholate and deoxycholate.

In the later part of the SDS gradient, distinctly colored nanotubes were separated. **Figure 2B** shows the continuation of the chromatogram in later stages of the SDS gradient and images of isolated fractions. These data show that the distinctly colored CNTs are separated into the DEX-rich lower phase.
The majority of the chiral species in the 6,5 preparation have been identified by the manufacturer as (6,5) (41%) and (7,3) (16%). The species (8,4), (7,5), and (9,2) compose around 5% each, with the rest (28%) consisting of nine other chiralities. As the spectral properties of these chiralities have not been thoroughly examined, we attempted to distinguish them by function. Different SWCNT colors can denote different chiralities and distinct properties. Therefore, we first examined the utility of these fractions in a model CNT-FET biosensor assay.

Previous studies have shown that lysine and arginine residues in proteins are particularly effective in affecting impedance in SWCNT-FETs. As all arginines and most lysine residues are protonated at physiological pH, we developed a high-throughput model assay using hexadimethrine bromide (polybrene) to simulate the effects that proteins have on SWCNT-FETs. Polybrene is a water-soluble quaternary amine polymer connected by three and six methylene groups that resemble polypeptide bonds. In addition, this polymer is inexpensive and readily available from many chemical vendors.

A key factor in most model and real-world assay development protocols for biotechnology is that the assays have to be dose dependent. Demonstration of assay saturation is vital for biotechnological applications because it shows that there are concentration-dependent limitations of ligand binding to a target. A saturable assay also provides limits to maximally and minimally observable signals that are possible and statistically relevant.

The impedance of the SWCNT-FETs in the presence of polybrene is shown in Figure 3. The assay involves coating SWCNTs onto a printed circuit as previously described. After baseline accrual, 2 μL of polybrene (50 μg/mL in D2O, final concentration) was added to the circuits as indicated by the arrow in Figure 3A, which shows a representative trace of the measured increase in SWCNT-FET impedance with time upon addition of polybrene. In general, there is a rapid increase in impedance for 15–30 s, followed by tapering of the response. The maximum response is generally achieved within 1 min of polybrene addition and also for most antibody–antigen interactions on FETs. The increase in impedance is reported as increase in signal over the baseline (normalized impedance).

To determine whether this polybrene response was saturable, we performed a titration of polybrene onto the SWCNT circuits and monitored the maximum impedance after 1.5 min of adding the polybrene solution. The results shown in Figure 3B indicate that the polybrene response exhibits a saturation binding curve. Each point represents at least five independent experiments with standard error of the mean.
measurements. As can be seen in this figure, the SWCNT-FET response saturates at around 50 μg/mL of polybrene. Saturation of the signal indicates that all responsive semiconducting SWCNTs that are solvent exposed are influenced by polybrene. Besides polybrene, we also observed that other amine-containing materials like polylsine and polymyxin also gave strong responses on the SWCNT-FET. In contrast, anionic detergents like SDS and deoxycholate do not elicit a response like polybrene (data not shown).

To compare a real-world sample to the model assay, we coated antibodies against *E. coli* O103:H8 onto SWCNT-FET circuits made with SWCNTs from Nano-C (Westwood, MA). We have previously used antibodies against *E. coli* O157:H7 coated onto a different preparation of SWCNTs to establish an assay for bacterial detection using site-specific modification of polyclonal antibodies for optimal adherence and directionality. In this case, shown in Figure 3, we were using a preparation of semiconducting SWCNT from Nano-C.

To analyze the properties of the spiral CCC-isolated CNTs using our model assay, we coated CNT fractions 52–60 directly onto circuits and measured their impedance. These results are shown in Figure 4.

Figure 4. Assay of semiconductor activity of fractions 52–60 using polybrene as described. Dotted line across the graph indicates the polybrene activity of the starting 6,5i material. Inset shows images of the CNT fractions tested.

These results demonstrate that there is a clear difference in the response of the isolated CNT fractions in the model assay. To determine whether the model assay reflects real-world biosensor assays, we examined their response to bacteria binding to CNT-coated circuits. We have previously shown that CNT-FET circuits can detect *E. coli*, and in Figure 5, we demonstrate that circuits coated with CNTs from Nano-C using antibodies to *E. coli* O103:H8 are able to detect 10^4 cfu/mL of *E. coli*.

To compare the results of the polybrene model assay to those of the bacterial assay, we coated fractions #53 and #55 from the CCC isolation onto circuits. These fractions represent the highest and lowest responsiveness, respectively, in the model polybrene assay. These results are shown in Figure 6 and demonstrate that the polybrene assay corresponds to the bacterial detection assay.

To further analyze the difference in these isolated CNT fractions, we compare their spectra. Figure 7 shows the spectra of fractions 53 and 55 and demonstrates that the light-blue fractions (#52 and #53) are more uniform, consisting of less than three absorption maxima, compared to that of fraction #55, which has at least five absorption maxima.

Whether these CNT species isolated by CCC have uniform chiralities will need to be determined in future studies, but it is evident that CNT species are not equal in activity on FET biosensors. Although the spectra shown in Figure 7 do not demonstrate SWCNT purity and lack of MWCNT, they do demonstrate consistency with Figures 4 and 6 in terms of the responsiveness of the CNT circuits.

**METHODS**

**Materials.** DEX MW 75 000 was obtained from Fisher Scientific (Boston, MA) (Tokyo Chemical Co. manufacturer) or Spectrum Chemical (Gardena, CA), PEG MW 8000 was from Fisher Scientific or Spectrum Chemical, sodium cholate (SC), sodium deoxycholate (SDC), and SDS were from Fisher Scientific or Spectrum Chemical, and CNT 6,5 enriched powder was obtained from South West Nanotechnologies Inc. (now Chasm Advanced Materials, Norman, OK). Polybrene (hexadimethrine Br) was purchased from Sigma Chemical Co (St Louis, MO) and dissolved in H2O. BacTrace *E. coli* O103:H8 and a polyclonal antibody against *E. coli* O103 were obtained from KPL (Gaithersburg, MD). Printed circuits on 4 in. silica wafers were produced and measured as previously described. Water was purified in a Neu-Ion system.
Instrument. The CNTs were separated in a new mixer−settler spiral disk CCC rotor (17.5 cm OD, cat. no. 205-20001, CC Biotech, Rockville, MD) operated in a planetary centrifuge (CentriChrom, Inc. Buffalo, NY). The rotor shown in Figure 1 is built with six plastic spiral disks made of SOMOS NeXT by stereolithography. The 4 mm thick disks are sandwiched between Viton gaskets or sheets. The disks have four interwoven 2.6 mm wide × 2.0 mm deep channels with segments divided by 1.6 mm wide “pins” that allow flow on either side. In every fourth segment is a glass bead for “mixing”, and the remaining spaces are for “settling”, which allows the phases to flow on either side of the pins helping to retain the stationary phase. The gaskets hold the liquid flow in the spiral channels. The flow passes into the rotor and through to the next disk via a hole in the gasket near the center. The liquid volume held in the rotor is 84 mL.

Solvent System. The ATPS system consists of PEG MW 8000 and DEX MW 75 000 containing SDC. The gradient of SDS from 0 to 0.7% in the upper PEG-rich phase as the mobile phase served to elute the CNTs during chromatography. The solvent system was made by combining 300 mL of 10% PEG/water by weight and 200 mL of 16% DEX and 1 mL of 10% SDC, all of which formed two phases, SS#1. To the same solvent system, 3.5 g SDS was added to make SS#2, which was used for the gradient. The solvent composition and gradient method described previously were used.

Sample Preparation. The method for sample preparation of powdered CNTs was to sonicate with a Branson Sonifier 450 UltraSonic Processor Homogenizer Disruptor with 3−5 2 s pulses, followed by incubation in a sonicating water bath for 5 min. A suspension of 10 mg in 0.5 mL 10% SC and 0.5 mL 10% SDS is thus solubilized, and 0.25 mL is removed and added to 3 mL UP SS#1 and 3 mL LP SS#1, vortexed, and allowed to settle into two phases. The upper phase is removed, and 1 mL of fresh UP SS#1 is added, which is dispersed by water bath sonication and allowed to settle; the total volume is then loaded into the CCC. This was the procedure for the 2.5 mg sample load.

Elution Conditions. The rotor is filled with lower-phase SS#1; the sample is then pumped into the rotor at 0.5 mL/min. When it is in the rotor, the centrifugation is started at 990−1000 rpm, and the mobile-phase SS#1 is eluted at a rate of 0.5 mL/min. At this point, the gradient is started, that is, elution from UP SS#1 (A) to 100% UP SS#2 (B) over a 9 h period.
Fractions of 8 mL are collected using an automated fraction collector. The elution mode is the upper-phase flow entering the bottom of the rotor and eluting out of the top with rotation being CCW in the head-to-tail direction (U-o-H).27

**Analysis of Fractions.** Generally, a 0.5 mL aliquot is taken out of the vortexed fraction to include both phases, and 0.5 mL 2% sq. SDS is added to dissolve all of the phases and is then sonicated to make a clear solution. Absorbance is read with water as the blank in a Cary 3E VC-Vis Spectrophotometer (Varian, division of Agilent, Santa Clara, CA). The absorbance of the fractions were measured from 200 to 900 nm to analyze the peaks, which help identify chiral SWCNT species.

**Semiconductor Activity.** The fractions containing color were assessed for semiconductor activity in the model polybrene impedance assay. Briefly, for measuring the sample FET impedance, the SWCNT-coated circuits were incubated with 2 μL of DI H2O for 20–30 s to obtain baseline values for each circuit. After baseline accrual, 2 μL of polybrene (50 μg/mL in DI H2O, final concentration) was added. Further description of this assay procedure is in the Results and Discussion section.

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**Notes**

The authors declare no competing financial interest.

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