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Affinity Capillary Electrophoresis Method to Assess Carboxylation of Multi-walled Carbon Nanotubes

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Abstract

Carboxylation of multi-walled carbon nanotubes is hypothesized to reduce the toxicity of these nanomaterials; however, literature reports are conflicting and the degree of carboxylic acid functionalization in different studies is not well quantified. The extent of carboxylation of multi-walled carbon nanotubes after acid treatment is quantified using affinity capillary electrophoresis. A polytryptophan peptide that contains a single arginine residue (WRWWWW) serves as a ligand in affinity capillary electrophoresis to assess the degree of carboxylation. The formation of peptide-nanotube ligand-receptor complex allows for the detection of the complex with a common UV absorbance detector based on light scatter rather than molecular absorbance. Dissociation constants (K_D) are obtained by observing the migration shift of the WRWWWW peptide in background electrolyte at increasing concentrations of multi-walled carbon nanotubes. The K_D values obtained from triplicate analyses of a single preparation of commercially available carboxylated multi-walled carbon nanotubes had a precision of 20% RSD (1.2 ± 0.2 mg/L). Preparations of multi-walled carbon nanotube remade from powdered samples (n = 3) generated K_D values with 20% RSD (1.1 ± 0.2 mg/L) as well. When applied to commercially available multi-walled carbon nanotubes with different degrees of carboxylation, the capillary electrophoresis method yielded K_D values that reflected higher levels of carboxylation. Zeta potential measurements of these preparations were not significantly different. The utility of the capillary electrophoresis method for evaluating acid treatment protocols was demonstrated by comparing K_D values obtained for multi-walled carbon nanotubes subject to six different acidification times. While K_D values were significantly different for acidification times ranging from 15 minutes to 3 hours, none of the zeta potential measurements of these samples were significantly different. This work is significant to research involving carbon nanotube toxicity because it provides a new metric to rapidly characterize carbon nanotubes obtained from different

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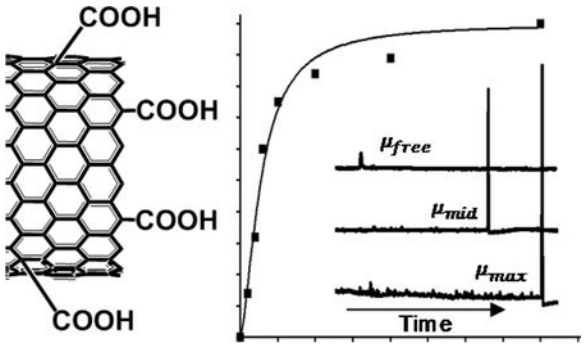
Supporting Information

Supporting information regarding ancillary data is available. This supporting information includes a stepwise description of the calculations of fractional binding, with the results of fractional binding calculations of a single affinity capillary electrophoresis binding analysis summarized in Table S-1. The results of reproducibility studies of sample stability and dried powder preparations are displayed as electropherograms and binding curves, with results summarized in Tables S-2 and S-3. Control runs demonstrating that complex formation is due to the combination of peptide and carbon nanotubes (Figure S-3) and electropherograms obtained with carbon nanotubes treated with acid at 0 °C (Figures S-4, 5, 6) are also found in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

vendors, synthesized in laboratories using different procedures, or subject to different acidification protocols. Affinity capillary electrophoresis is a cost-effective, rapid and simple alternative to current technologies used to distinguish the degree of carbon nanotube carboxylation.

Graphical Abstract



Keywords

multi-walled carbon nanotube; peptide affinity; tryptophan; Dissociation Constant; acid treatment; Carboxylation

Introduction

In 2011 carbon nanotube production ranged from 230,000–450,000 tons/year, with multi-walled carbon nanotubes more prevalent in manufacturing than single wall carbon nanotubes because the bulk cost for synthesis is lower [1]. The demand for multi-walled carbon nanotubes will accelerate dramatically as improvements in synthesis and processing bring the production cost down to that of carbon fiber [1] and carbon black [2]. With this increase in production imminent, there is a concerted effort to ensure that new materials meet appropriate levels of health and safety. A goal of these efforts is to minimize the potential for toxicity without sacrificing the material performance. A substantial number of studies aimed at exploring the health effects of carbon nanotube toxicity have been reported. Currently, the toxicity of carbon nanotubes is conflicting or poorly understood for a variety of reasons [3–5], although a specific type of multi-walled carbon nanotube has been demonstrated to be carcinogenic in mice and rats [3, 6]. Modifying carbon nanotubes by functionalizing them may reduce toxicity and improve the aqueous solubility. The most straightforward alteration is the generation of carboxylic acid functional groups on carbon nanotubes with mineral acid treatment. The effect of mineral acid treatment on toxicity is unclear, with some reports correlating carbon nanotubes functionalized with carboxylic acids with reduced toxicity [7, 8], while others indicate carboxylated carbon nanotubes show toxicity similar to untreated nanotubes [9–11]. Differences in these results may arise from the multi-walled carbon nanotubes used in these studies. The carbon nanotubes originate from different sources, are generated with different synthetic protocols, and use diverse acid treatment procedures that may lead to different degrees of carboxylation.

Differences in the carboxylation of carbon nanotubes lead to a change in the surface charge. This is assessed by measuring the zeta potential (ξ), which is, in part, a function of the surface charge of the nanoparticle. Zeta potential measurements are made by subjecting a nanoparticle suspension to an electric field and monitoring the resultant motion with laser scattering. Applications to nanoparticle analyses are performed under conditions that maintain a stable suspension. Zeta potential measurements have distinguished significant changes in surface charge due to protein adsorption [7], but have not successfully been used to distinguish subtle differences in changes of the degree of carboxylation that would be observed with different acid washing protocols. The primary factor that limits the use of this technique is the complexity of carbon nanotube samples. In spite of the potential of this method, the discrimination of surface charge by zeta potential can only differentiate the effects of rigorous acid treatment protocols [12]. These concerns have led to a call for new instrumentation [4].

Capillary electrophoresis is a microscale separation technique based on the mobility of charged analytes in an electric field that operates on the same fundamental principles observed for classical zeta potential measurements. Capillary electrophoresis has been used to measure zeta potential of charged nanoparticles including quantum dots [13], gold nanoparticles [14], latex particles [15], and single walled carbon nanotubes complexed with DNA [16]. While these two instrumental techniques are similar, detection with commercially available capillary electrophoresis instruments is typically limited to fluorescence or UV absorbance. Detection of carbon nanotubes with capillary electrophoresis requires some additional considerations, but has been accomplished using Raman spectroscopy [17] or absorbance with a source at 575 nm [16].

The separation selectivity of capillary electrophoresis can be increased by including a secondary selector such as a ligand or receptor in the background electrolyte. The method of affinity capillary electrophoresis is used to rapidly quantify dissociation constants of receptor-ligand complexes and although successfully applied to some nanoparticles [18, 19] it has not been used to assess carbon nanotubes. It is well established that carbon nanotubes bind to tryptophan residues in peptides [20] as a result of a strong π-π interaction between the indole ring of tryptophan and the carbon nanotube surface [21]. The complex formed between a polytryptophan carbon nanotube complex will result in only a slight change in the charge-to-size ratio; however, a polytryptophan peptide amidated on the carboxyl terminus and modified to contain a cationic arginine residue will create a measureable change in the charge-to-size ratio of carbon nanotubes-peptide complex. Furthermore the arginine residue will also interact electrostatically with carboxylated functional groups on the carbon nanotubes. The complex formed between the peptide tryptophylarginyltryptophyltryptophyltryptophyltryptophan (WRWWWW) and carbon nanotube scatters UV light and are detectable with common capillary electrophoresis instrumentation. By introducing a single arginine residue in a polytryptophan peptide the degree of carboxylation can be distinguished because carbon nanotubes with a higher degree of carboxylation will have stronger electrostatic interaction and the peptide-nanotube complexes will have lower dissociation constants. Affinity capillary electrophoresis is used to quantify the binding affinity of ligand-receptor systems by monitoring the change in the charge-to-size ratio upon receptor-ligand binding. The method of affinity capillary

electrophoresis is used to rapidly quantify dissociation constants and although successfully applied to other nanoparticles [18, 19] it has not been used to assess carbon nanotubes.

This is the first report of the adaptation of capillary electrophoresis to quantify the degree of carbon nanotube carboxylation. The approach is evaluated and the precision of the method is established using commercially available carboxylated multi-walled carbon nanotubes. The effect of acid treatment is quantified using affinity capillary electrophoresis to relate the dissociation constants with the time of acid exposure. Whereas, the affinity capillary electrophoresis method produces statistically different dissociation constants, no significant difference is detected using classical zeta potential measurements. The approach is used to compare commercially available carbon nanotubes from different manufacturers. Finally, the method is applied to an archived sample of acid treated carbon nanotubes that, when previously studied, displayed genotoxicity in immortalized human airway epithelial cells [9].

Experimental Section

Chemicals and Reagents

Carbon nanotubes from NanoLab Inc (Waltham, MA) included precarboxylated carbon nanotubes (PD15L1–5-COOH) with outer diameter 15 ± 5 nm and length 1– 5 μm, and carbon nanotubes (PD15L5–20) with outer diameter 15 ± 5 nm and length 5– 20 μm. Precarboxylated carbon nanotubes (US4358) with outer diameter 10 – 20 nm and length 0.5– 2 μm were from US Research Nanomaterial, Inc (US-Nano, Houston, TX). Morpholinopropane-1-sulfonic acid (MOPS), methanol, sodium hydroxide and mesityl oxide were from Sigma-Aldrich Corp. (St. Louis, MO). MOPS (25 mM) was prepared in deionized water obtained from an Elga Purelab ultra water system (Lowell, MA), and the pH adjusted to 7 using sodium hydroxide. Acid treatment was accomplished with 95% sulfuric acid purchased from J.T. Baker (Center Valley, PA) and 69% Nitric Acid purchased from GFS Chemicals (Columbus, OH). The amidated peptide WRWWWW-NH_2 was purchased from Bachem (Torrance, CA).

Capillary Electrophoresis

Analyses were completed using a P/ACE MDQ (Sciex, Redwood City, CA) with a photodiode array. A 25 μm inner diameter and 360 μm outer diameter bare fused silica capillary (Polymicro Technologies, Phoenix, AZ) with an effective length of 20.0 cm and a total length of 30.2 cm was used for all analyses. Before analysis the capillary was flushed daily with 1 M NaOH for 30 min at 138 kPa (20 psi), deionized water for 15 min at 138 kPa (20 psi), methanol for 15 min at 138 kPa (20 psi), and deionized water for 15 min at 138 kPa (20 psi). Prior to each electrophoretic separation the capillary was flushed with 1 M NaOH for 2 min at 138 kPa (20 psi), deionized water for 1 min at 138 kPa (20 psi), and 25 mM MOPS for 4 min at 138 kPa (20 psi). Before each run, the capillary was filled with background electrolyte, which contained carbon nanotubes at different concentrations ranging from 0 to 50 mg/L. The anodic and cathodic reservoirs contained the same background electrolyte that was loaded in the capillary. The WRWWWW peptide and mesityl oxide were diluted in 25 mM MOPS buffer to a final concentration 25 μM and 130 μM, respectively. The peptide and mesityl oxide sample was injected at 10 kV for 5 secs. All

separations were at an applied voltage of 10 kV (E = 333 V/cm) using normal polarity. The 32 Karat Software version 5.0 (Beckman Coulter) was used for data collection and analyses. Binding curves were obtained using Graphpad Prism Version 4.0 (Graphpad Software, San Diego, CA) curve-fitting software for nonlinear regression.

Sample Preparation

All carbon nanotube stock suspensions were prepared from dried powder that was weighed and suspended in deionized water to a concentration of either 0.5 mg/L (acid treated in-house) or 1 mg/L (acid treated by the manufacturer) and sonicated in an ice bath for 5 min then stored at 4 °C. For capillary electrophoresis analyses, the carbon nanotubes were diluted daily in 25 mM MOPS to make stocks ranging from 20 to 50 mg/L and sonicated in an ice bath for 5 min. This stock was then diluted in 25 mM MOPS to the concentrations required for experiments. Each sample was individually sonicated for 1 min in an ice bath. Although carboxylation of the carbon nanotubes improved the dispersion in deionized water, poorly dispersed carbon nanotubes produced random spikes during separation. Samples that produced these spikes were sonicated an additional minute and the separation was repeated.

Acid Treatment

Multi-walled carbon nanotubes from NanoLab Inc (PD15L5–20, research grade) were exposed to a mixture of sulfuric and nitric acid at 3:1 (v/v) ratio. The carbon nanotube powder was combined with the acid mixture to a concentration of 1 mg carbon nanotube powder per 5 mL acid, and sonicated with a Branson (Danbury, CT), model 2800, 40 KHz sonicator for the specified time. Ice was added as needed to maintain the temperature of the water in the sonicator bath to a range of 20 to 25 °C. Following sonication, the mixture of the carbon nanotubes and acid was diluted with water and filtered using a 0.2 μm polycarbonate filter (Whatman TrackEtch membrane filter part # 111106, GE Healthcare Bio-Sciences, Pittsburgh, PA). The carbon nanotubes were rinsed, covered, and allowed to dry on the filter under vacuum. The resulting pellet was rinsed from the filter into a secondary container with methanol in a fume hood. The carbon nanotubes were covered and allowed to dry in a fume hood before being weighed for analysis.

Zeta Potential Measurements

Measurements were done with a Zetasizer Nano ZS90 and analyzed using Zetasizer software version 7.11 (Malvern Instruments, Worcestershire, UK). Determination of zeta potential is based on first principles so the instrument cannot be calibrated; however, correct operation of this instrument as verified using NIST Standard Reference Material 1980 prior to use. The cells (DTS1070) were prepared by rinsing with 1 mL of methanol, 2 mL of deionized water and 2 mL of 25 mM MOPS. The carbon nanotube samples were prepared by diluting stocks to a final concentration of 5 mg/L in 25 mM MOPS and sonicated in an ice bath for 1 min. The cells were filled with carbon nanotube samples. A single value was obtained from 10 replicate measurements, which were in turn repeated 10 times for a total of 100 readings on a single sample loaded into the cell. Within sample reproducibility of 2.1% RSD was determined by measuring three aliquots of a single preparation of precarboxylated carbon nanotube. Across sample reproducibility of 1.6% RSD was determined with three

independent preparations of pre-carboxylated carbon nanotube. All other samples were measured with a single aliquot for a single analysis.

Safety Considerations

Due to the potential toxicity of respirable carbon nanotubes safe handling was required when working with dried powder. Appropriate personal protective equipment included gloves, a lab coat, and a respirator mask certified to handle a particulate size of 100 nm (NIOSH P100). The dry carbon nanotubes were only handled in a fume hood. The weight of an empty sample vial and cap was obtained outside of the hood, transported to the hood, filled with dry carbon nanotube, closed, the exterior surfaces cleaned, and weighed outside of the hood. Once a mass of approximately 2 mg was weighed, the sample vial was then placed back in the fume hood, opened, and diluted in water. The fume hood and any items inside of it were wiped with a damp cloth after use. Once the dry powder was suspended in water it could be safely handled outside of the hood. The error for this weighing technique was ± 0.2 mg determined by weighing, taring and reweighing a vial three times. As carbon nanotubes have problems with static electricity that can affect weighing accuracy, this method of weighing was also performed with the vial wrapped in aluminum foil. When the foil was used, the error for the weighing technique was ± 0.3 mg determined by weighing, taring and reweighing a vial three times. Therefore, the measurements were conducted without the use of aluminum foil.

Results and Discussion

Adaptation of Affinity Capillary Electrophoresis to Multi-walled Carbon Nanotubes

Classical affinity capillary electrophoresis was used to rapidly quantify the binding of ligand to receptor in-capillary. The peptide ligand was bound to the carbon nanotubes in background electrolyte and the migration of the peptide-carbon nanotube ligand-receptor complex measured. The measurement of migration was repeated using different carbon nanotube concentrations. Changes in the ligand migration were directly correlated to the concentration of the receptor dissolved in the capillary electrophoresis background electrolyte. Figure 1 depicts a schematic of affinity capillary electrophoresis used to assess peptide-carbon nanotube binding. For analysis, the peptide was injected at a fixed concentration (25 μM) and migrated in background electrolyte containing carbon nanotubes (Fig. 1A). The WRWWWW peptide interacted with the carbon nanotube suspension as it migrated through the capillary (Fig. 1B). The carbon nanotubes-peptide complex, which appeared when peptide and nanotubes were combined, scattered the incident UV light used for absorbance detection. This allowed for detection of the complex so that the migration time could be measured at various concentrations (Fig. 1C). The method of classical affinity capillary electrophoresis required accurate measurement of migration time, but not peak area. As the concentration of carbon nanotubes was increased, the complex size and migration was increased until migration shift reached the maximum binding because the peptide was saturated with nanotubes.

Calculating the K_D from Shifts in Migration Time

The dissociation constant, K_D, of the peptide-nanotube complex was calculated using the Hill equation, assuming that the binding interaction between ligand and receptor is homogeneous and that complex formation occurs without an intermediate state or with a short-lived intermediate. With the Hill equation, the K_D, as determined by evaluating the fraction, θ, of peptide that is bound to carbon nanotubes, is measured at a specified carbon nanotube concentration, [CNT] (eq 1),

$$\theta = \frac{[CNT]^n}{{K_D}^n + [CNT]^n} \quad \text{(eq 1)}$$

where n is the cooperativity of the binding interaction. In some systems, the value of n is an indicator of cooperativity where the binding of other ligands is enhanced (n > 1), decreased (n < 1), or unaffected by the presence of other ligands (n = 1). For the peptide-carbon nanotube studies, the results of non-linear regression revealed enhanced cooperativity, although conclusions were not made based on these values [22]. The fractional binding is plotted as shown in Figure 2 with the carbon nanotubes concentration as the x-axis and the fraction bound on the y-axis. The data were then evaluated using commercial software to find the best fit to the Hill equation (i.e. equation 1) using nonlinear regression to solve for K_D and n.

The migration shift of the bound peptide reflected the fractional time the peptide migrated in the free and bound forms. The mobility of the peptide partially bound to carbon nanotube, μ_{mid}, was measured with affinity capillary electrophoresis at a particular carbon nanotube concentration as described by equation 2 [18, 23, 24],

$$\mu_{mid} = f_1 \cdot \mu_{max} + f_2 \cdot \mu_{free} \quad \text{(eq 2)}$$

where f_1 is the fraction of bound peptide and μ_{max} is the mobility of the peptide at binding saturation (Fig. 2A, 20 mg/L trace). The fraction of the peptide that is free or unbound is f_2 and μ_{free} is the mobility of the peptide in the absence of carbon nanotube (Fig. 2A, 0 mg/L). The sum of these two fractions equals one ($1 = f_1 + f_2$), allowing the equation to be rearranged and simplified to equation 3.

$$f_1 = \theta = (\mu_{mid} - \mu_{free})/(\mu_{max} - \mu_{free}) \quad \text{(eq 3)}$$

The fastest migration of the cationic peptide, achieved at a mobility of μ_{free}, was obtained in the absence of carbon nanotubes in the background electrolyte (Fig. 2A, 0 mg/L trace) and the fraction bound was zero. The slowest migration time of the cationic peptide was observed at $\mu_{max,}$ because the injected peptide was fully complexed and the fraction bound was 1. At all other carbon nanotube concentrations the peptide existed in a partially bound state in between 0 and 1. For those concentrations, the migration time increased as the

carbon nanotube concentration increased in the background electrolyte. A stepwise calculation of fractional binding from migration time and the subsequent error propagation is described in the supporting information.

Curve Fitting Criteria

The K_D determinations were best achieved with a range of fractional binding to ensure that the error in the mobility shift measurements was reasonable and that the nonlinear fit was accurate. The affinity curves were derived with some guidelines because literature reports of measurements of carbon nanotube dissociation constants were limited to single wall carbon nanotubes and surfactant [25] or to multi-walled carbon nanotubes and wheat agglutinin protein [26]. No data have been published on the binding of WRWWWW and multi-walled carbon nanotubes. The K_D values were obtained at carbon nanotube concentrations that resulted in fractional binding that evenly spanned the full range of the curve. Each curve had a minimum of six carbon nanotube concentrations ranging from two points representing binding saturation, a single point before and after the curve inflection, a single point at or around the center of the linear region of the curve, and a single point at the lower end of the curve. The single point at the lower end of the curve was rejected if the relative standard deviation of the fraction bound was greater than 30%, as calculated from the systematic error in the measurement. This occurred if the migration shift was too small to maintain 2 significant figures when deriving $\mu_{mid} - \mu_{free}$. An example of an acceptable peak shift is found in Figure 2 in the 0.5 mg/L carbon nanotube trace obtained at 214 nm. A stepwise calculation for these data can be found in the supporting information. Fitted curves with a correlation coefficient below 0.96 were also rejected.

Reproducibility of Migration Shift Assays of the Same Carbon Nanotube Solutions

In order to obtain accurate K_D values the migration shift analyses must be reproducible within a sample preparation. To establish reproducibility of the affinity capillary electrophoresis binding method with the curve fitting criteria, replicate measurements of a commercial carbon nanotube were performed. Carbon nanotubes had the potential to settle out of solution. If this occurred, then the true concentration of serial dilutions would be unknown. To ensure that this had not occurred, reproducibility was tested using a single set of carbon nanotube concentrations made by dilution from a common 20 mg/L master stock into 25 mM MOPS buffer. For each K_D determination, all dilutions were made from the master stock at the same time and the peptide migration was analyzed sequentially from the lowest to highest carbon nanotube preparations. This analysis of the diluted carbon nanotube samples was repeated twice to generate three binding curves and three dissociation constants: $K_{D1} = 1.4 \pm 0.3$, $K_{D2} = 1.2 \pm 0.2$, and $K_{D3} = 1.1 \pm 0.2$ (see Fig. S-1 and Table S-1 in the supporting information). A comparison of these three dissociation constants, which was done using a one-way ANOVA followed by a Tukey test post analysis with Graphpad software, showed no significant difference. The average of the three dissociation constants is 1.2 ± 0.2 (20% RSD). These results indicated that the single samples were stable for a minimum of three runs and the K_D values were reproducible within a single set of carbon nanotube concentrations.

Reproducibility of K_D Determination of the Same Carbon Nanotube Powder Stock

To determine the reproducibility of preparing the carbon nanotubes from dry powder stock, the sample preparations were weighed, suspended, diluted and the peptide migration analyzed sequentially from the lowest to highest carbon nanotube concentration. The analyses of the diluted carbon nanotube samples were repeated twice to generate three binding curves and three dissociation constants for each sample made fresh from dry powder. Determinations were repeated for two additional dry powder stocks. A total of three carbon nanotube powders were analyzed in triplicate for a total of 9 K_D determinations. For each carbon nanotube stock three dissociation constants were determined from three independent binding curves and averaged into a single dissociation constant. A Tukey test of each values shows no significant difference from stock to stock. The K_D from each stock was averaged to yield an across sample K_D of 1.1 ± 0.2 (20% RSD). The data were summarized Figure S2 and Table S-2 in the supporting information. The sample preparations of carbon nanotubes were consistent across powder and the affinity capillary electrophoresis method showed a high level of reproducibility for measuring dissociation constants.

Effect of Acid Treatment on K_D

Acid treatment has been used to carboxylate surface defects and end caps on the carbon nanotubes and the methods, times, and acids used for treatments vary in the literature [9, 12, 27–29]. Affinity capillary electrophoresis was used to quantify the effects of acid treatment on pristine carbon nanotubes. Verification of carboxylated carbon nanotube product was an important aspect to evaluate the quality of the starting material. Dissociation constant data, summarized in Table 1, were collected for acid treated carbon nanotubes at various time points. As the acid treatment time increased the dissociation constant decreased, indicating higher binding affinity with WRWWWW model peptide. Previous studies revealed that longer acid treatment times led to a higher weight percent of carboxylation on the carbon nanotube surface [12, 30, 31]. Therefore, the increase in binding of the WRWWWW model peptide was due to the increase in the carboxylic acids on the carbon nanotube. The zeta potential of the samples was also measured to evaluate changes in the carbon nanotube surface composition as a function of charge. However, the zeta potential measurements for all time points were not significantly different and ranged from −35 to −39 mV. Both capillary electrophoresis separations and zeta potential were a measure of the migration of analytes in an electric field as a function of the analyte charge and size. However, with affinity capillary electrophoresis the measurement was enhanced through the additional aromatic and electrostatic binding of the peptide to differentiate the degree of carboxylation of treated carbon nanotubes. Small changes in the surface composition were exploited by the binding of the peptide. This provided a new and simple method to compare materials.

Comparison of K_D for Multi-walled Carbon Nanotubes from Different Commercial Sources

The affinity capillary electrophoresis method was applied to compare carboxylated carbon nanotube preparations across manufacturers. Information about manufactured carboxylated carbon nanotubes was limited to the average length and diameter of the carbon nanotubes and either a value for the percent carboxylation or a range of percent carboxylation. Therefore, authenticating the amount of carboxylation of carbon nanotubes was important

prior to exposure. Affinity capillary electrophoresis was used to assess carboxylation of carbon nanotubes of similar length and diameter from two different manufacturers (see Table 2). The NanoLab multi-walled carbon nanotubes with 2–7% carboxylation had a K_D of 1.2 ± 0.2 mg/L (n = 3), and a ξ of −39 ± 2 mV. The US-Nano multi-walled carbon nanotubes with 2% carboxylation had a K_D of 3.9 ± 0.9 mg/L (n = 3), and a ξ of −38 ± 1 mV. While the zeta potential measurements showed no significant difference as a function of charge, the affinity capillary electrophoresis method indicated a difference in the carboxylation consistent with the manufacturer description. The NanoLab carbon nanotubes with a higher level of carboxylation had a lower K_D, which reflected a stronger electrostatic interaction with the cationic WRWWWW peptide.

Measurement of K_D of Similar Acid Treated Protocol

Affinity capillary electrophoresis can be used as an effective tool to compare acid treatment methods to ensure that equivalent carboxylated products can be obtained from different laboratories. To demonstrate that the affinity capillary electrophoresis method was effective for differentiating changes in the acid treatment techniques, the method was applied to carbon nanotubes that were used in an exposure study [9]. The carbon nanotubes used for the exposure were prepared with an acid treatment protocol similar to what was used in this paper except that the temperature was maintained at 0 °C during acidification. The previously reported acid treated carbon nanotubes were prepared at NIOSH with an ice sonication bath and acid treatment at 1, 3, or 6 hours, and the 1-h sonication used in an exposure study [9]. These carbon nanotubes, which were functionalized for 1, 3, and 6 hours, produced a K_D of 11 ± 3 mg/L, 3.7 ± 0.6 mg/L, and 2.9 ± 0.8 mg/L, respectively. These data, depicted in Figure 3, indicated that for the 1 and 3 hour sonication, the rate of carboxylation was a function of temperature and that the material sonicated in ice produced a lower degree of carboxylation than the carbon nanotubes sonicated at room temperature for the same time. Based on K_D values, the 6 hour sonication in ice was comparable to the in-house acid treated carbon nanotubes sonicated at room temperature (20 to 25°C) for 2 h, K_D = 2.6 ± 0.5 mg/L (see Table 1). Sonication at room temperature for 6 hours was not performed as the maximum degree of carboxylation appeared to be achieved after 3 hours of sonication at room temperature. These observations were in agreement with a report that acid treatments based on reflux produced a higher carboxylation at higher temperature [30]. The larger error in the replicate measurements of K_D obtained for carbon nanotube samples treated with acid at 0 °C as compared to room temperature, may reflect incomplete functionalization at the lower temperature. The carbon nanotubes functionalized using the low temperature sonication generated electropherograms that had additional peaks (see Figures S-4, S-5, S-6 in the supporting information), which may be attributed to the production of a wider distribution of carboxylation at lower temperature. The potential to reduce the carbon nanotube toxicity through surface carboxylation was limited by conflicting results from exposure studies [7–11]. Although nanoparticle exposure studies are complex, the reduction in toxicity had been linked to the amount of carboxylation of the carbon nanotubes [12]. Thus, differences in the degree of carboxylation should be characterized with enabling technology to control this variable in future research on multi-walled carbon nanotubes. The affinity capillary electrophoresis method may hold potential to characterize the effects of temperature, acid composition, and sonication energy on carbon

nanotube functionalization. Studies are currently underway to further demonstrate the applicability of the affinity capillary electrophoresis method to monitor the impact of temperature on carboxylation.

Conclusions and Future Directions

Classical affinity capillary electrophoresis was adapted to rapidly characterize the degree of carboxylation of multi-walled carbon nanotubes. A polytryptophan peptide that contained a single arginine residue bound to carbon nanotubes through aromatic as well as electrostatic interactions. The dissociation constant was derived from the shift in electrophoretic mobility. The migration shift increased as the concentration of carbon nanotube in the background electrolyte increased until a maximum shift was observed. A range of carbon nanotubes was used to span the full range of fractional binding. The method was reproducible when applied to stable dispersions made from dry powders. The method was used to distinguish the preparations of functionalized carbon nanotubes that were subject to different acid treatment times. These changes in the degree of carboxylation could not be distinguished using classical zeta potential measurements. An important application of this capillary electrophoresis method was the comparison of carbon nanotubes obtained from different sources as well as carbon nanotubes subject to different acid treatment protocol. This provided a means to normalize acid treatment protocols described in the literature. The method can also be used to estimate the degree of carboxylation of preparations used in exposure studies reported in the literature. With additional experiments, the capillary electrophoresis method will shed light on different acid treatment protocols and may serve to standardize strategies for carbon nanotube functionalization.

For routine characterization of nanomaterials, an automated capillary electrophoresis instrument is easy to operate and can be considered a more accessible instrument than, for example, electron microscopy methods that provide elemental information. However, as implemented in this application, capillary electrophoresis can differentiate carboxylation substantially better than zeta potential. While this report focusses on multi-walled carbon nanotubes, future efforts will expand the application of method to single walled carbon nanotubes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

1. De Volder MFL, et al., Carbon Nanotubes: Present and Future Commercial Applications. Science, 2013 339(6119): p. 535–539. [PubMed: 23372006]

2. Zhang Q, et al., The Road for Nanomaterials Industry: A Review of Carbon Nanotube Production, Post-Treatment, and Bulk Applications for Composites and Energy Storage. Small, 2013 9(8): p. 1237–1265. [PubMed: 23580370]

3. Kuempel ED, et al., Evaluating the mechanistic evidence and key data gaps in assessing the potential carcinogenicity of carbon nanotubes and nanofibers in humans. Critical Reviews in Toxicology, 2016: p. 1–58.

4. Grassian VH, et al., NanoEHS - defining fundamental science needs: no easy feat when the simple itself is complex. Environmental Science: Nano, 2016 3(1): p. 15–27.

5. Aschberger K, et al., Review of carbon nanotubes toxicity and exposure--appraisal of human health risk assessment based on open literature. Crit Rev Toxicol, 2010 40(9): p. 759–90. [PubMed: 20860524]

6. Grosse Y, et al., Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. The Lancet Oncology. 15(13): p. 1427–1428.

7. Li R, et al., Surface Charge and Cellular Processing of Covalently Functionalized Multiwall Carbon Nanotubes Determine Pulmonary Toxicity. ACS Nano, 2013 7(3): p. 2352–2368. [PubMed: 23414138]

8. Visalli G, et al., Toxicological assessment of multi-walled carbon nanotubes on A549 human lung epithelial cells. Toxicology in Vitro, 2015 29(2): p. 352–362. [PubMed: 25499066]

9. Siegrist KJ, et al., Genotoxicity of multi-walled carbon nanotubes at occupationally relevant doses. Particle and Fibre Toxicology, 2014 11: p. 6. [PubMed: 24479647]

10. Magrez A, et al., Cellular Toxicity of Carbon-Based Nanomaterials. Nano Letters, 2006 6(6): p. 1121–1125. [PubMed: 16771565]

11. Hamilton RF Jr., et al., NLRP3 inflammasome activation in murine alveolar macrophages and related lung pathology is associated with MWCNT nickel contamination. Inhal Toxicol, 2012 24(14): p. 995–1008. [PubMed: 23216160]

12. Jain S, et al., Toxicity of Multiwalled Carbon Nanotubes with End Defects Critically Depends on Their Functionalization Density. Chemical Research in Toxicology, 2011 24(11): p. 2028–2039. [PubMed: 21978239]

13. Tekrony A and Cramb D, Determination of the mobility of amine- and carboxy-terminated fluospheres and quantum dots by capillary electrophoresis. Canadian Journal of Chemistry, 2016 94(4): p. 430–435.

14. Pyell U, et al., Characterization of gold nanoparticles with different hydrophilic coatings via capillary electrophoresis and Taylor dispersion analysis. Part I: Determination of the zeta potential employing a modified analytic approximation. Journal of Colloid and Interface Science, 2015 450: p. 288–300. [PubMed: 25828436]

15. Connah M, Kaszuba M, and Morfesis A, High Resolution Zeta Potential Measurements: Analysis of Multi-component Mixtures. Journal of Dispersion Science & Technology, 2002 23(5): p. 663.

16. Khripin CY, et al., Measurement of Electrostatic Properties of DNA-Carbon Nanotube Hybrids by Capillary Electrophoresis. The Journal of Physical Chemistry C, 2009 113(31): p. 13616–13621.

17. Doorn SK, et al., High Resolution Capillary Electrophoresis of Carbon Nanotubes. Journal of the American Chemical Society, 2002 124(12): p. 3169–3174. [PubMed: 11902906]

18. Li N, et al., Probing Nanoparticle−Protein Interaction by Capillary Electrophoresis. Analytical Chemistry, 2010 82(17): p. 7460–7466. [PubMed: 20672831]

19. Boulanos S, et al., Nanoparticle-protein interaction: thermodynamic and kinetic study of adsorption of bovine serum albumin to gold nanoparticle surface. Langmuir, 2013 29 (48): p. 14984–14996. [PubMed: 24215427]

20. Wang S, Peptides with selective affinity for carbon nanotubes. Nature Materials, 2003 2(3): p. 196. [PubMed: 12612679]

21. Tomásio SM and Walsh TR, Modeling the Binding Affinity of Peptides for Graphitic Surfaces. Influences of Aromatic Content and Interfacial Shape. The Journal of Physical Chemistry C, 2009 113(20): p. 8778–8785.

22. Weiss JN, The Hill equation revisited: uses and misuses. Faseb J, 1997 11(11): p. 835–41. [PubMed: 9285481]

23. Avila LZ, et al., Use of affinity capillary electrophoresis to determine kinetic and equilibrium constants for binding of arylsulfonamides to bovine carbonic anhydrase. Journal of Medicinal Chemistry, 1993 36(1): p. 126–133. [PubMed: 8421278]
24. Shimura K and Kasai K.-i., Affinity Capillary Electrophoresis: A Sensitive Tool for the Study of Molecular Interactions and Its Use in Microscale Analyses. Analytical Biochemistry, 1997 251(1): p. 1–16. [PubMed: 9300076]
25. Oh H, Sim J, and Ju SY, Binding affinities and thermodynamics of noncovalent functionalization of carbon nanotubes with surfactants. Langmuir, 2013 29(35): p. 11154–62. [PubMed: 23909509]
26. Chen X, et al., Biomolecular interaction analysis for carbon nanotubes and for biocompatibility prediction. Anal Biochem, 2016 505: p. 1–7. [PubMed: 27108187]
27. Gómez S, et al., Surface modification of multiwall carbon nanotubes by sulfonitric treatment. Applied Surface Science, 2016 379: p. 264–269.
28. Wu Z and Mitra S, Length reduction of multi-walled carbon nanotubes via high energy ultrasonication and its effect on their dispersibility. Journal of Nanoparticle Research, 2014 16(8): p. 2563.
29. Datsyuk V, et al., Chemical oxidation of multiwalled carbon nanotubes. Carbon, 2008 46(6): p. 833–840.
30. Jain AK, et al., Carbohydrate-conjugated multiwalled carbon nanotubes: development and characterization. Nanomedicine, 2009 5(4): p. 432–42. [PubMed: 19341818]
31. Zhou W, Sasaki S, and Kawasaki A, Effective control of nanodefects in multiwalled carbon nanotubes by acid treatment. Carbon, 2014 78: p. 121–129.

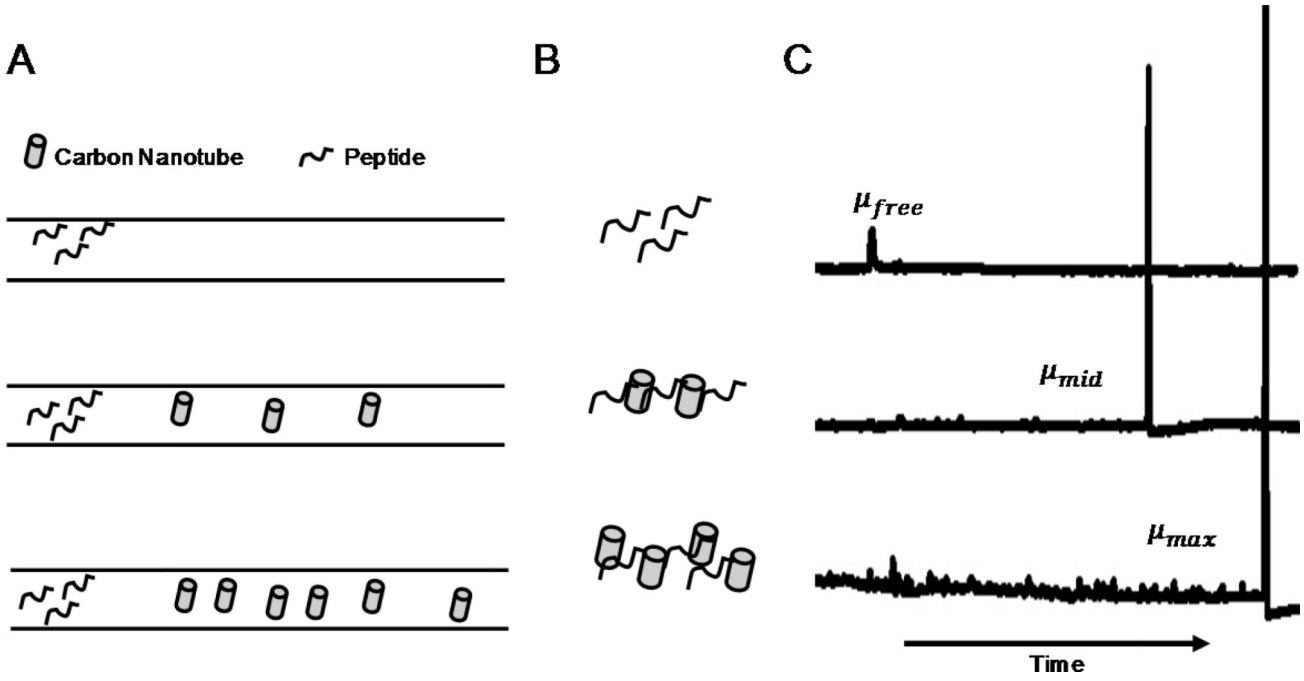

Figure 1.

Schematic of affinity capillary electrophoresis. 1A depicts the injection of WRWWWW model peptide into background electrolyte with increasing concentration of carbon nanotubes. 1B demonstrates the formation of peptide-carbon nanotube complex as the carbon nanotube concentration is increased in the background electrolyte. The electropherograms in 1C determine the mobility of the peptide in 0 mg/L (μ_{Free}), 5 mg/L (μ_{Mid}) and 20 mg/L (μ_{Max}) carbon nanotubes.

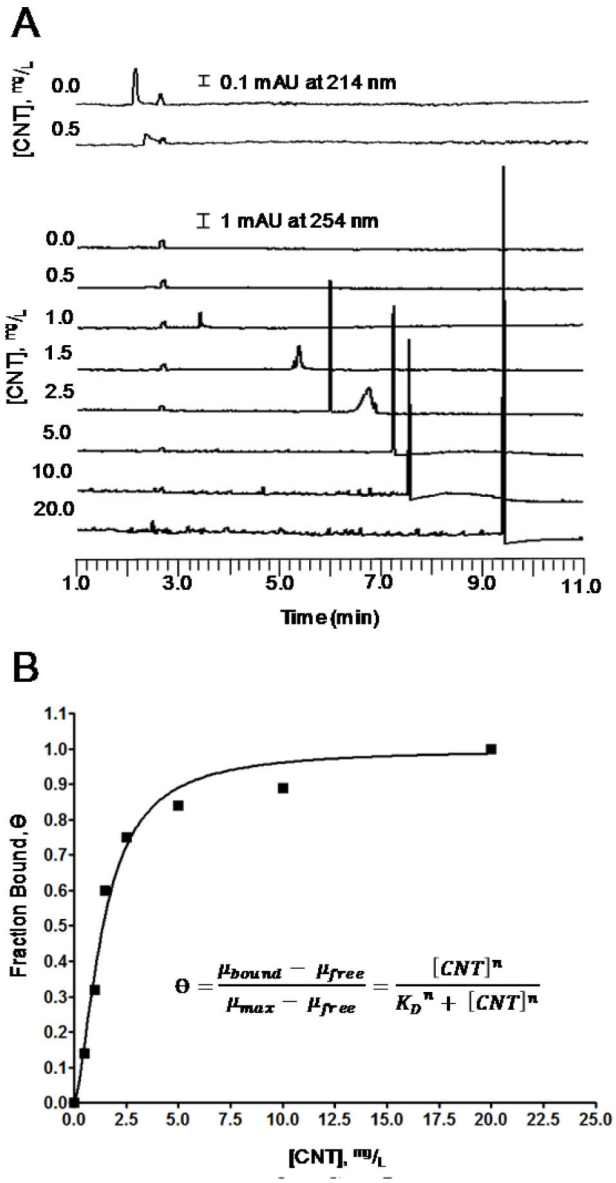

Figure 2A.

contains electropherograms from a single binding obtained with WRWWWW (25 μM) and carboxylated carbon nanotubes purchased from NanoLab. The upper traces labeled 0 mg/L and 0.5 mg/L are detected at 214 nm to better display the peptide migration. All other traces displayed in the figure are obtained at 254 nm. Mesityl oxide (130 μM) is the marker for electroosmotic flow. Separation conditions are specified in the Materials and Methods. These electropherograms are used to calculate the fractional binding, which is plotted as shown in **Figure 2B** to obtain the dissociation constant for peptide and carboxylated multi-walled carbon nanotubes purchased from NanoLab.

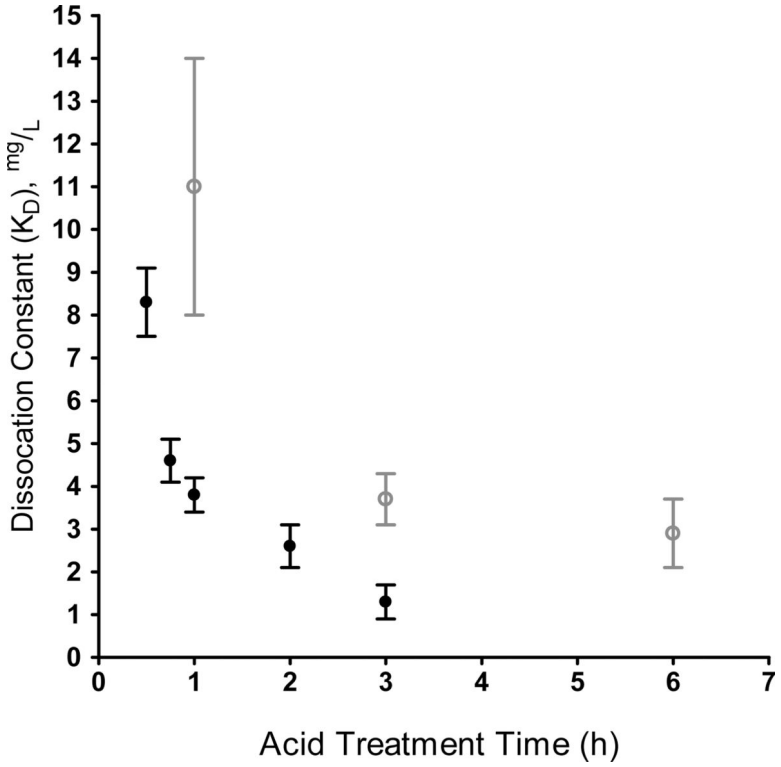

Figure 3.

A plot relating the effect acid treatment time on dissociation constant. The data were obtained at room temperature (●) or in an ice bath (○). Each point is an average of $n = 3$ binding curves per sonication time. Increased time of acid treatment increased the carboxylation, which is reflected by the decreased dissociation constant. The rate of carboxylation decreased with temperature. Separation conditions are specified in the Materials and Methods.

Table 1.

Effect of Acid Treatment on Dissociation Constant and Zeta Potential

Time in Acid (h)	K_D ± SD (mg/L)[1]	ξ ± SD (mV)[2]
3.0	1.3 ± 0.4	−38 ± 2
2.0	2.6 ± 0.5	−40 ± 1
1.0	3.8 ± 0.4	−37 ± 1
0.75	4.6 ± 0.5	−38 ± 2
0.5	8.3 ± 0.8	−35 ± 1

[1] Data are the average and propagated error from three curve fittings using 25 μM WRWWWW peptide carboxylated NanoLab carbon nanotube, 15 ± 5 nm OD, 1 –5 μm length

[2] Data collected from single 20 mg/L carbon nanotube sample

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Table 2.

Comparison of Commercial Carbon Nanotubes

% COOH[1]	K_D ± SD (mg/L)[2]	ξ ± SD (mV)[3]
2	3.9 ± 0.9	−38 ± 1
2 –7	1.2 ± 0.2	−39 ± 2

[1] Data provided by the manufacturer as follows: 2 wt% COOH- NanoLab (15 ± 5 nm o.d., 1 –5 μm long) and 2–7 wt% COOH US-Nano (10 – 20 nm o.d., 0.5–2 μm long)

[2] Average (n = 3 curves) using 25 μM WRWWWW

[3] Data collected with 5 mg/L multi-walled carbon nanotube