Quantitative determination of oxidized carbon nanotube probes in yeast by capillary electrophoresis with laser-induced fluorescence detection

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Received 24 May 2006; received in revised form 25 July 2006; accepted 26 July 2006
Available online 5 August 2006

Abstract

Short oxidized multi-walled carbon nanotubes were functionalized with fluorescein isothiocyanate to form carbon nanotube probes (CNTP). The distribution of CNTP in yeast was quantitatively determined by capillary electrophoresis coupled with laser-induced fluorescence detection. The detection sensitivity for CNTP was greatly improved comparing with UV absorbance and Raman detection. The time- and temperature-dependent influx patterns of CNTP into yeast were obtained. The apparent permeability coefficient for influx of CNTP into yeast was calculated, which suggested that CNTP might permeate into yeast through endocytosis. This study implies that CNTP could be a fine drug transporter and might be wildly used in multidrug resistance research and microorganism detection.

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Keywords: Capillary electrophoresis; Laser-induced fluorescence detection; Carbon nanotube probes; Yeast; Apparent permeability coefficient

1. Introduction

Carbon nanotubes (CNT) are cylinder-shaped macro-molecules with a radius as small as a few nanometers, which comprise one or multiple layers of graphene sheets. CNT are characterized by their high surface areas and outstanding electrical, chemical, mechanical and conducting properties. These characteristics have made CNT the subject of intensive investigation since their discovery \cite{1}.

The exceptional interest in CNT resides in their possible applications as molecular transporters \cite{2,3}. They possess hollow cores, which can store guest molecules. CNT have been shown to shuttle various cargoes across cellular membrane without cytotoxicity. The exploration of the intrinsic physical properties of CNT can help impart unique features to nanotube biocarriers \cite{4}.

CNT are versatile carriers and can also be well functionalized \cite{5}. The transporting capabilities of CNT combined with suitable chemical functionalization and their intrinsic optical properties can lead to new classes of novel materials for drug delivery and cancer therapy \cite{4,6–8}. As a potential application, the drug delivery efficiency could be greatly improved by the magnetic-field-assisted biointeraction when using the multi-walled CNT to carry the drug inside \cite{9}. Single-walled CNT to penetrate mammalian cells and further transport substances inside cells have also been demonstrated recently \cite{4,6,10,11}.

CNT can be separated by capillary electrophoresis (CE) and detected by UV absorbance and Raman detection \cite{12,13}. Due to very weak self-fluorescence, CNT themselves are quite difficultly determined by fluorescence detector, thus the detection limit for CNT is hard to satisfy the real application demands in biological and medical research. Fluorophores were generally functionalized onto the CNT to improve the sensitivity of detection. There have been some reports on determination of the fluorescence of those carbon nanotube probes (CNTP) by flow cytometry and laser scanning confocal microscopy \cite{14,15}. However, there are seldom reports on the quantitative determination of CNTP by CE coupled with laser-induced fluorescence detection (CE-LIF). In this study, short oxidized multi-walled CNT were functionalized with fluorescein isothiocyanate (FITC) to form CNTP, and the distribution of them in yeast was quantitatively determined by CE-LIF. The time- and
temperature-dependent influx of CNTP into yeast were investigated. The uptake pathway of CNTP in yeast was also concluded from the experimental results.

2. Experimental

2.1. Apparatus

A lab-built CE-LIF system as described previously [16] was used in this study. An Ar-ion laser (488 nm) was used for excitation with operating at 6 mW. The emission fluorescence of FITC was detected at 515 nm. A HV3-2 valve (Hamilton, Reno, Nevada, USA) connected to a hydraulic differential at the capillary outlet was used to generate a pulse of siphoning force for the injection. Unless otherwise stated, samples were injected into capillary by siphoning at the anode with a 10.8 kPa × 5 s pulse.

UV spectra were obtained on Jasco V-550 UV/vis spectrophotometer (Tokyo, Japan). Fluorescence spectra were obtained on Hitachi F-2500 fluorescence spectrophotometer (Tokyo, Japan) with Xe lamp as light source.

The oxidized and the original multi-walled CNT were characterized by JEM-2000EX transmission electron microscopy (TEM, Tokyo, Japan) operated at 120 kV.

2.2. Capillary for CE

A fused-silica capillary obtained from polymicro with 365 μm o.d., 25 or 75 μm i.d. and 65 cm length (Phoenix, Arizona, USA) was used for CE separation. The capillary was first treated with 500 μL 0.2 M sodium hydroxide for 1 h, and washed with 500 μL methanol then 500 μL water for 1 h, respectively. The activated capillary was then filled and reacted with γ-methacryloxypropyltrimethoxysilane at 45 °C for 12 h, washed with 500 μL methanol, and filled with a 4% acrylamide solution containing 0.1% ammonium persulphate and 0.1% N,N,N′,N′-tetramethylethylenediamine, according to the procedures described by Hjerten [17]. After polymerization at room temperature for 12 h, the capillary was rinsed with 500 μL water for 30 min and the excessive polyacrylamide was pushed out of the capillary.

2.3. Materials

Unless otherwise stated, all chemicals were analytical grade. Multi-walled CNT were kindly offered by Professor F. Wei (Tsinghua University, Beijing, China). FITC was purchased from Fluka (Buchs, Switzerland). Phosphate-buffered saline (PBS) buffer was used in all yeast rinse procedures. EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] was purchased from Pierce (Rockford, IL, USA). MES buffer (pH 6.0) was used as reaction buffer for CNT and hexamethylene diamine (pH 6.0). The mixed solution was stirred at room temperature for 2 h. The oxidized CNT bonded with hexamethylene diamine (CNT–CONH(CH2)6NH2) was obtained by centrifuging at 25000 rpm. Fluorescently labeled CNT were obtained by reacting the CNT–CONH(CH2)6NH2 with FITC to afford CNTP. The whole procedure for preparation of CNTP reported elsewhere [19]. The excess FITC was removed by rinsing and centrifuging at 25000 rpm for three times. The obtained CNTP were dissolved in PBS at a concentration of 0.5 mg mL−1.

2.4. Preparation of CNTP

The oxidation procedures of CNT were described previously [18]. It involved the reaction of CNT with concentrated HNO3 and H2SO4 (v/v, 1:3) by stirring and refluxing at 120 °C for 30 min. The oxidized CNT in water solution were centrifuged at 8000 rpm to remove any large un-reacted CNT from the solution. The oxidized CNT were then reacted with hexamethylene diamine in the presence of EDC to afford a linker between the CNT and the subsequent fluorescent probe. Briefly, 2 mg of oxidized CNT were mixed with 5 mg hexamethylene diamine and 1 mg EDC in 0.1 M MES buffer (pH 6.0). The mixed solution was stirred at room temperature for 2 h. The oxidized CNT bonded with hexamethylene diamine (CNT–CONH(CH2)6NH2) was obtained by centrifuging at 25000 rpm. Fluorescently labeled CNT were obtained by reacting the CNT–CONH(CH2)6NH2 with FITC to afford CNTP. The whole procedure for preparation of CNTP reported elsewhere [19]. The excess FITC was removed by rinsing and centrifuging at 25000 rpm for three times. The obtained CNTP were dissolved in PBS at a concentration of 0.5 mg mL−1.

2.5. Yeast and sample preparation

Yeast S. cerevisiae ATCC26786 was grown in YPD overnight at 30 °C with shaking. The harvested yeast cells were rinsed three times with PBS before experiments. Prior to incubation, the cells were collected by centrifugation and resuspended in PBS at a cell density of 3.75 × 10⁸ cells mL⁻¹. Unless otherwise stated, incubation was done by mixing 3.75 × 10⁷ yeast cells with corresponding volume of CNTP solution and keeping each tube total volume as 100 μL. The incubation was always with shaking. After incubation, the suspension of yeast cells was obtained by centrifugation at 5000 rpm for 5 min. The yeast cells were resuspended in PBS for further analysis.

3. Results and discussion

3.1. Characterization of oxidized CNT and CNTP

Original CNT and the oxidized CNT and the FITC-labeled CNT (CNTP) have been characterized by TEM images [19], and the oxidized CNT became thinner and shorter because the exterior walls of CNT were oxidized. The FT-IR spectra of oxidized CNT [18] also showed that the carboxylic groups were introduced onto CNT through the oxidation process.

The prepared CNTP were characterized by UV/vis and fluorescence spectra, and the obtained spectra are shown in Fig. 1A–D. It can be seen that there is a peak of CNTP different from oxidized CNT but similar with FITC in Fig. 1A, indicating that FITC was covalently bonded with oxidized CNT. The...
Fig. 1. Samples were characterized by: (A) UV/vis spectra; (B) fluorescence emission spectra excited at 258 nm; (C) fluorescence excitation spectra emitted at 520 nm; (D) fluorescence emission spectra excited at 488 nm. Samples of (A)–(D): (a) oxidized CNT (0.5 µg mL⁻¹); (b) CNTP (0.5 µg mL⁻¹); (c) FITC (1.5 nM).

maximal fluorescence excitation and emission wavelength of oxidized CNT are 258 and 520 nm, respectively. In the fluorescence emission spectra excited at 258 nm, as shown in Fig. 1B, there is little difference between the peak area of oxidized CNT and CNTP. But as shown in Fig. 1C, there are some new peaks in the fluorescence excitation spectra emitted at 520 nm of CNTP, which indicated new chemicals were formed between FITC and oxidized CNT. The emission spectra with the maximal emission wavelength at 520 nm after excitation at 488 nm are shown in Fig. 1D, which proves the presence of fluorescence absorbance group in the structure of CNTP, while oxidized CNT themselves have very weak fluorescence absorbance under our experimental conditions. According to the above results, it is clearly indicated that FITC has covalently bonded with oxidized CNT.

3.2. CE of yeast

The CE separation of yeast was carried out following the method of Armstrong et al. [20]. The electropherograms of yeast that incubated with and without 10 µg mL⁻¹ CNTP are shown in Fig. 2. There is a little displacement of migration time between the two electropherograms, which may be caused by the changed charge/mass ratio after the influx of CNTP into yeast. But there is obvious difference in peak area between the two electropherograms which proved that CNTP have been absorbed by yeast. The relative standard deviations (R.S.D.) for the total peak area and migration time are 5.27% (n = 4) and 2.27% (n = 4), respectively. Albeit the reproducibility of the separation is fine, the quantitative determination of CNTP in yeast cannot be realized by electrophoresis analyses of yeast cells because of the difficulty in peak area integral. Due to the heterogeneity of yeast cells, those spiny peaks have not accurately reflected the corresponding fluorescence signal. Meanwhile, even the lysate of yeast cells can be analyzed by CE, the interaction between CNTP and cellular components will result in broader peaks than stan-

Fig. 2. Electropherograms of yeast incubated with (A) and without (B) 10 µg mL⁻¹ CNTP for 3 h under 37°C. Experimental conditions—capillary: 65 cm × 75 µm (an effective length of 15 cm); separation buffer: 3.94 mM Tris-0.56 mM boric acid-0.013 mM EDTA, 0.05% PEO at pH 9.02; injection: −1 5 3Vcm⁻¹ for 20 s; applied voltage: −153 V cm⁻¹.
standard CNTP. As a result, it will ruin the direct relation with the standard curve and induce incorrect quantitation. The quantitative determination was simply realized by indirect detection of the decrease of CNTP in the yeast incubation suspension in this study.

### 3.3. CE of CNTP

Since CNTP were negatively charged, the micellar electrokinetic chromatography analysis was conducted in negative polarity. Under this electrophoresis separation mode, the electroosmotic flow is deleterious which should be controlled. Polyacrylamide-coated capillary was used in all the experiments in order to diminish the electroosmotic flow and wall adsorption. And the polyacrylamide coating provided reproducible separation for CNTP. TBS buffer was chosen as the running buffer in the CNTP analysis experiments [12,13]. The electropherograms of standard CNTP with different concentrations are shown in Fig. 3A. Fig. 3C shows the electropherograms of CNTP before and after incubation with yeast. There is unconspicuous decrease of the peak areas with migration time before 20 min. The offset of those peaks is quite serious which may be caused by their heterogeneity. There is conspicuous decrease of the peak areas with migration time after 20 min. The calibration curve was then established between the peak area of peaks with migration time after 20 min and the concentration of CNTP. The calibration curve of integral peak area of CNTP (0.25, 0.5, 1, 2 and 2.5 μg mL\(^{-1}\)) are shown as follows:

\[
A = 16.93C - 4.13, \quad R = 0.9972,
\]

where \(A\) is the peak area (\(\times 10^{-5}\)) and \(C\) is the concentration of CNTP (μg mL\(^{-1}\)). It can be seen that there is a good linearity which can be used for CNTP quantitation. The sodium dodecyl sulfate (SDS) both in the separation buffer and in the sample solution improved the analysis reproducibility. The R.S.D. values for migration time and peak areas of peaks with migration time after 20 min are 2.42 and 0.97%, respectively, with five consecutive injections. The inter-day RSD for quantitation is 8.52% (\(n = 3\), which may be caused by photobleaching. The limit of detection for CNTP is about 2.5 fg (S/N > 3) which is far below than that of UV absorbance and Raman detection (about pg) [12,13].

The quantitation of CNTP in yeast was carried out based on the decrease of peak area of CNTP in the suspension before and after incubation with yeast. The corresponding electropherograms of CNTP in suspension solutions after incubation with yeast are shown in Fig. 3B.

### 3.4. Effects of CNTP on viability of yeast

The effects of CNTP on yeast viability were investigated by cell counter. Yeast cells were incubated for 12 h with 25 μg mL\(^{-1}\) of CNTP, which is far above the highest CNTP incubation concentration used in the experiments. No appreciable yeast death was observed. This result suggested that CNTP themselves exhibit no apparent cytotoxicity to yeast and it is also consistent with the previous report [21].

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Fig. 3. Electropherograms of: (A) the CNTP standard solutions; (B) the suspension solutions of CNTP after incubated with yeast; (C) 1 μg mL\(^{-1}\) CNTP before (up) and after (down) incubated with yeast. Samples: CNTP concentration: (a) 0.25; (b) 0.50; (c) 1.00; (d) 2.00; (e) 2.50 μg mL\(^{-1}\). Experimental conditions—capillary: 65 cm × 25 μm (an effective length of 50 cm); separation buffer: 10 mM Tris–10 mM boric acid–10 mM SDS at pH 7.4; injection: –10.8 kPa × 5 s; applied voltage: –153 V cm\(^{-1}\).
3.5. Effects of incubation time on influx of CNTP into yeast

The influence of incubation time on CNTP influx into yeast was investigated. CNTP with the concentration of 1 μg mL⁻¹ was added to equal number of yeast (3.75 × 10⁷ cells) and incubated for 0, 15, 30, 60, 90 and 120 min, respectively. It can be found from the experimental results that the influx of CNTP into yeast increased with prolonging of the incubation time. It shows a fast increase curve before 15 min, but flatness after 30 min, which means that the balance of the CNTP influx into yeast is achieved very quickly. No obvious increase of CNTP in yeast was found at the given concentration after incubation for longer than 60 min. Therefore, 60 min was taken as the balance time for CNTP incubation with yeast in all the experiments.

3.6. Effects of CNTP concentration on its influx into yeast

The amount of CNTP in yeast increased with the increasing concentrations (0, 0.25, 0.5, 1, 2 and 2.5 μg mL⁻¹) of CNTP, which was characterized by CE-LIF. Quantitation of CNTP in yeast was accomplished based on the calibration curve. The amount of CNTP that permeated into a single yeast cell was also calculated as shown in Table 1. Only several feta grams of CNTP permeated into a single yeast cell statistically. The ratio of inner concentration of CNTP in yeast to that in incubation solution was about 38, with R.S.D. of 6.28% calculated from five incubation concentrations of CNTP. This implied the uniformity of yeast’s membrane. It also proved the possibility of CNTP as a molecular transporter of cell membrane.

3.7. Effects of temperature on CNTP influx into yeast

The uptake of 1 μg mL⁻¹ CNTP incubated at 4 °C for 15 min showed 23.42% of that incubated at 37 °C for 15 min. And the uptake of CNTP incubated at 4 °C for 1 h showed 28.18% of CNTP uptake incubated at 37 °C for 1 h. The influx of CNTP into yeast was dramatically affected by temperature, which implied that the uptake pathway was energy dependent. This is also consistent with the previous report [14].

3.8. Apparent permeability coefficient ($P_{app}$)

The $P_{app}$ was calculated through the CNTP amount in single yeast cell. The $P_{app}$ (in cm s⁻¹) was calculated as follows [22]:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}$$

where $\Delta Q/\Delta t$ is the amount of fluorophore appearing in the receiver compartment in function of time (nmol s⁻¹), $C_0$ the initial concentration in the donor compartment (μM) and $A$ is the surface area (cm²) across which transport occurred. This is a little similar with that of apical to basolateral and basolateral to apical [22,23]. The approximate yeast cell diameter is 5 μm, and the corresponding yeast cell surface area is 7.85 × 10⁻⁷ cm². The apparent permeability coefficients of 1 μg mL⁻¹ CNTP at 4 and 37 °C for 15 min were 3.49 × 10⁻⁷ and 7.22 × 10⁻⁷ cm s⁻¹, respectively. They were 3.94 × 10⁻⁷ and 7.98 × 10⁻⁷ cm s⁻¹ at 4 and 37 °C for 1 h, respectively. It suggested that the transport of CNTP into yeast membrane through endocytosis [21].

4. Potential applications

The rapid and sensitive determination of pathogenic microorganisms plays an important role in biotechnology and medical diagnosis [24]. Since CNTP permeate into yeast through endocytosis, the inner amount of CNTP in yeast increased with the increasing CNTP concentration. Thus the distribution of CNTP in yeast will improve the detection sensitivity. And the sensitivity for yeast detection was improved about two orders of magnitude when the incubation concentration of CNTP is 10 μg mL⁻¹. This can also be found obviously from Fig. 2.

In multidrug resistance research, drugs can be pumped out by energy-dependent transporters [25]. Since CNTP can permeate into cells through endocytosis, it suggests that CNTP might increase the absorption of drugs by target cells in cancer therapy [26]. It may also provide essential breakthroughs in the fight against cancer as a drug transporter [27].

5. Conclusion remark

The influx of CNTP into yeast was quantitatively determined by CE-LIF. The detection sensitivity for CNTP is greatly improved in comparison with UV absorbance and Raman detection. In this study, it shows that CNTP permeate into yeast through endocytosis. Based on the rapid and sensitive properties by influx of CNTP into yeast cells, it suggests that CNTP can be used as molecular transporter and slow release device for drug delivery. It might find that CNTP would be widely used in further multidrug resistance research, cancer therapy and microorganism sensitive detection.

Table 1

<table>
<thead>
<tr>
<th>CNTP incubation concentration (μg mL⁻¹)</th>
<th>CNTP mass in single yeast cell (μg)</th>
<th>CNTP concentration in single yeast cell (μg mL⁻¹)</th>
<th>Concentration enrichment multiple^a</th>
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</thead>
<tbody>
<tr>
<td>0.25</td>
<td>$6.86 \times 10^{-10}$</td>
<td>10.38</td>
<td>41.95</td>
</tr>
<tr>
<td>0.5</td>
<td>$1.20 \times 10^{-9}$</td>
<td>18.34</td>
<td>36.69</td>
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<td>38.07</td>
<td>33.93</td>
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<td>2</td>
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<td>71.86</td>
<td>35.93</td>
</tr>
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<td>2.5</td>
<td>$6.49 \times 10^{-9}$</td>
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<td>39.69</td>
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</tbody>
</table>

^a The ratio of inner concentration of CNTP in yeast to that in incubation solution.
Acknowledgements

The financial supports from the National Natural Sciences Foundation of China (No. 20520120220), the China State Key Basic Research Program Grants (2003CB716002), and the Knowledge Innovation Program of DICP to H.Z. are gratefully acknowledged.

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