

Autonomous Propulsion of Carbon Nanotubes Powered by a Multi-Enzyme Ensemble.

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Supplementary Information

All the reagents and solvents were obtained from commercial suppliers and used without further purification. Multiwalled carbon nanotubes (CNTs) were purchased from Nanostructured and Amorphous Materials, Inc. (Houston, USA). Catalase from bovine liver and Glucose oxidase from *Aspergillus Niger* were purchased from Sigma Aldrich and used as received.

Characterization of CNTs

The acid treatment of commercial CNTs was carried out to dissolve metal species, breakdown amorphous carbon, and introduce reactive groups along the sidewalls of the nanotubes.¹ After treatment, the CNTs were characterized by electron microscopy (TEM, SEM) and atomic force microscopy (AFM).

For AFM, a drop of the diluted nanotube suspension in pure water or distilled dichloromethane was deposited onto a clean flat silicon surface. The solvent was allowed

to evaporate over 10 min. The substrate was analyzed by tapping mode AFM (Digital Instruments Nanoscope III).

TEM characterization was carried out on a JEOL 1200EX operating at 100 KV. A drop of diluted nanotube suspension was deposited on a carbon film copper grid. The drop was dry after 5 min. Negative staining was not required.

The SEM measurements were performed on a JEOL JSM 7000F field emission SEM operating at 2.0 kV. A nanotube suspension (150 μ l) was deposited on a glass slit covered with a 120 nm Al top-layer on which a 1 nm Cr layer was sputtered. These samples were allowed to dry at ambient temperatures and used without additional staining.

Quantitative analysis of carboxylic sites was performed using the quantitative Kaiser test² after further functionalisation of the oxidized nanotubes with 1,8-diamino-3,6-dioxaoctane (*vide infra*).

Preparation of water soluble CNTs

After gently grinding, commercial CNTs (160.0 mg) were suspended in 150 ml of a mixture of concentrated H₂SO₄/HNO₃ (3/1 v/v) and sonicated for 5 h. The suspension was then cooled to RT and diluted to 500 ml with 2 M aqueous NaOH in an ice bath. The solution was filtered through a PTFE filter (pore size 0.22 μ m) under vacuum. The black residue collected was washed thoroughly with deionized water until the filtrate was pH 6 and subsequently dried under vacuum at 60 °C.

The product, 92.0 mg, was collected and suspended in 30 ml of Piranha solution (H₂SO₄ 98% / H₂O₂ 30 vol 4/1 v/v) and stirred r.t. for 1 h. The solution was filtered over a 0.22 μ m pore size polycarbonate membrane and washed extensively with deionised water. The

black material on the collected was dried under vacuum at 60 °C to yield 88.0 mg of product.

The material was stored as a solid or as a stable solution (concentration of 1 mg/ml).

The IR spectroscopy shows absorptions at 1710 cm⁻¹ (carbonyl stretching) and a broad absorption at 1100 cm⁻¹ (C-O stretching).

The loading was calculated using a quantitative Kaiser test² after functionalization of oxidized carbon nanotubes (typically 20 mg) first with an excess of oxalyl chloride and, after removal of the excess, by treatment with 1,8-diamino-3,6-dioxaoctane in tetrahydrofurane (THF) at 60°C for 2 h. The resulting suspension was filtered and the black solid washed with methanol and dried. To perform the Kaiser test three solutions were prepared in parallel. (I) 10 g of phenol dissolved in 20 mL of ethanol, (II) 2 mL of KCN 1 mM (aqueous solution) dissolved in 98 mL of pyridine, (III) 1.0 g of ninhydrine dissolved in 20 mL of ethanol. Approximately 20 mg of free amino CNTs was weighed accurately. Non-amino CNT was tested also as a control solution and treated in the same way. 75 µl of solution I, 100 µl of solution II and 75 µl of solution III were added to a haemolysis test tube. The test tube is incubated in a heating block at 100°C for 7 min and then removed and added immediately to 4.8 mL of 60% ethanol to make a final volume of 5 mL. The tube was then mixed to render the violet colour homogenous. The absorbance of each sample was measured relative to the blank CNT solution (pale yellow) at 570 nm. The amine loading was calculated from the equation:

$$\frac{\mu\text{mol}}{\text{g}} = \frac{[\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}] \times \text{dilution}(\text{ml}) \times 10^6}{\text{Extinctioncoefficient} \times \text{sampleweight}(\text{mg})}$$

(Dilution=5mL and Extinction coefficient = 15000 m⁻¹cm⁻¹).

The values are expressed as micromole of amino groups per gram of material and are an average of at least ten measurements at different dilutions.

Covalent enzyme conjugate 1

Oxidized CNTs (2.0 mg, loading 357 ± 14 $\mu\text{mol/g}$) were dispersed in 500 μl of deionised water and activated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) solution (0.2 M in PBS pH 7) for 2 h at room temperature. 500 μl of Catalase (62000 U or 1.5 mg) in phosphate buffer solution (PBS pH 7.6) were added. The reaction was carried out within 5 h at r.t. The final suspension was filtered on a 0.22 μm pore size nitrocellulose filter and washed with 30 ml of pure water. The black solid obtained (3.4 mg) was suspended in 2.0 ml of pure water and centrifuged twice at 4000 rpm with discharge of the supernatant. The precipitate collected was dissolved in 2.0 ml of water and stored at 4 °C. A small amount was used to determine the enzymatic activity to calculate the amount of enzyme present (*vide infra*). The enzymatic activity was conserved for at least 4 months.

Covalent enzyme conjugate 2

Oxidized CNTs (2.0 mg, loading 357 ± 14 $\mu\text{mol/g}$) were dispersed in 500 μl of deionised water and activated with EDC solution (0.2 M in water) for 2 h at room temperature. 500 μl of a solution of β -D-Glucose oxygen 1-oxidoreductase (1060 U or 3.6 mg) were added. The reaction was carried out within 5 h at r.t. The final suspension was filtered using a 0.22 μm pore size nitrocellulose filter and washed with 120 ml of pure water. The black solid obtained (3.3 mg) was suspended in 4 ml of pure water and centrifuged twice at 2000 rpm separated from the supernatant. The precipitate collected was redissolved in 1.0

ml of water and stored at 4 °C. A small amount was used to determine the enzymatic activity and allowed to calculate the amount of enzyme present (*vide infra*). The enzymatic activity was conserved for at least 4 months.

Covalent enzyme conjugate 3

Oxidized CNTs (5.0 mg, loading 357 ± 14 $\mu\text{mol/g}$) were dispersed in 1 ml of deionised water and activated with EDC solution (0.2 M in water) for 2 h at room temperature. 1 ml of a solution of Catalase (62000 U) in PBS (pH=7.6) were mixed with 500 μl of a solution of β -D-Glucose oxygen 1-oxidoreductase (GOx) (1060 U) and added slowly to the activated nanotubes. The reaction was continued for 5 h at r.t. The final suspension was filtered through a 0.22 μm pore size nitrocellulose filter and washed with 30 ml of pure water. The black solid obtained (8.2 mg) was suspended in 2 ml of pure water and centrifuged twice at 4000 rpm and separated from the supernatant. The precipitate collected was dissolved in 10 ml of water and stored at 4 °C. A small amount was used to determine the enzymatic activity to calculate the amount of each enzyme present (*vide infra*).

Catalase enzymatic activity assay

Catalase activity was measured using H₂O₂ as substrate.³ Catalase disproportionates H₂O₂ to form oxygen and water in a pH 7.0 PBS buffer at 25 °C. For a typical assay the disappearance of H₂O₂ was monitored by UV spectroscopy at 240 nm.

Table 4. Enzymatic assay activity of conjugate 1. Results from three independent experiments.

Conjugate 1 (mg)	Measured time ^a (min)	Enzymatic units ^b	Amount of enzyme ^c (μg)	Amount of catalase (μg /mg)
1.20	2.18	15797	355	295.83
1.30	2.35	17088	384	295.38
0.80	3.08	10502	251	200.80

^aTime expressed in min to decrease absorbance at 240nm from 0.45 to 0.40.

^bUnits/ml = (3.45*dilution factor)/(time*volume of enzyme in ml)

^c From supplier 44500 Units/mg

A solution of 2.9 ml of 0.0036% (w/w) H₂O₂ in PBS was prepared with a stable absorbance at 240 nm between 0.55 and 0.52. 100μl of conjugate 1 solution (weight amount dissolved in 2 ml) was added by inversion (at 25°C) and the kinetics in the loss of absorbance at 240 nm from 0.45 to 0.40 units was recorded and the number of enzymatic unit was calculated by:

$$\text{Enzymatic Units/ml} = (\Delta A_{500}/\text{min Gox} - \Delta A_{500}/\text{min Blank}) * 3.1 * (dF) / (7.5 * 0.1)$$

Glucose oxidase enzymatic assay

GOx activity was detected measuring the production of H₂O₂ in a 0.1 M acetate buffer at 35 °C and pH 5.0 upon addition of D(+)-glucose.⁴ The concentration was monitored with time at 240 nm using a spectrophotometer.

Table 5. Enzymatic assay activity of conjugate **2**. Results from three independent experiments.

Conjugate 2 (mg)	($\Delta A_{500}/\text{min}$ Gox- $\Delta A_{500}/\text{min}$ Blank) dF=100	Enzymatic units ^a	Amount of enzyme ^b (mg)	Amount of GOx ($\mu\text{g}/\text{mg}$)
2.21	0.721	300.4	1.50	678.73
2.32	0.763	315.4	1.57	676.72
4.10	1.349	557.6	2.78	678.04

^a Enzymatic Units/ml = ($\Delta A_{500}/\text{min}$ Gox- $\Delta A_{500}/\text{min}$ Blank)*3.1*(dF)/(7.5*0.1)

^b From supplier 200 Units/mg

A solution of 2.9 ml combining 24 ml of 0.21 mM o-dianisidine in 50mM sodium acetate buffer (pH 5.1) and 5 ml of 10% w/v β -D-Glucose in deionized water were mixed with 100 μl of prepared solution of 60 purpurogallin units/ml of peroxidase type II in cold water, equilibrated at 35°C and monitored at 500 nm absorbance until stable. Then 100 μl of glucose oxidase solution in 50 mM sodium acetate buffer was added and the increasing in absorbance at 500 nm was recorded for approximately 5 min. For the blank test 100 μl

of simple acetate buffer solution was added instead of glucose oxidase solution. The concentration of enzymatic units was calculated by⁴:

$$\text{Enzymatic Units/ml} = (\Delta A_{500}/\text{min Gox} - \Delta A_{500}/\text{min Blank}) * 3.1 * (\text{dilution factor}) / (7.5 * 0.1)$$

where 3.1 is the total volume, 7.5 is the millimolar extinction coefficient of oxidized o-dianisidine at 500 nm and 0.1 is the volume of enzyme used in the test.

FT-IR Spectroscopy

The secondary structure of native and adsorbed enzymes was determined using FT-IR spectroscopy. All spectra were collected using a Nicolet Magna 550 series II. The spectrometer was equipped with a mercury cadmium telluride detector cooled by liquid nitrogen. The data collection and second-derivative calculations were carried out using the software OMNIC 6.1a provided by Thermo Nicolet Corporation. An average of 170 scans were obtained at a resolution of 2 cm⁻¹ with a gain of 2 and an aperture of 50. Typically, lyophilized compound **1**, **2** and **3** (1 mg) were ground gently with IR grade KBr to form a homogeneous grey powder. The grinding was performed under nitrogen atmosphere to prevent absorption of atmospheric water. The spectra of the enzymes, both native and attached to the nanotubes were baseline corrected over the entire spectral window. Secondary structural content of the enzymes was obtained by analysis of their FT-IR spectra in the region from 1600 to 1700 cm⁻¹. To obtain the second-derivative spectrum, a baseline correction was applied to the corrected spectra in the region from 1750 to 2000 cm⁻¹, which is critical to obtain quantitative structural information using the second-derivative analysis method⁵. The second-derivative spectrum was then corrected

in the region $1600\text{-}1700\text{ cm}^{-1}$ to obtain a flat baseline. This spectrum was used to obtain the β -helix and α -sheet contents of the enzymes^{5,6}.

Table 2. Secondary structure of enzymes at 20 °C from FT-IR amide I band analysis compared with the reference native enzyme.

Assignment of components	Native Catalase	Conjugate 1	Native GOx	Conjugate 2
β -sheets (%) (1621 cm^{-1})	12.3	13.2	18.4	22.5
α -helix (%) (1647 cm^{-1})	26.0	35.3	28.8	27.6

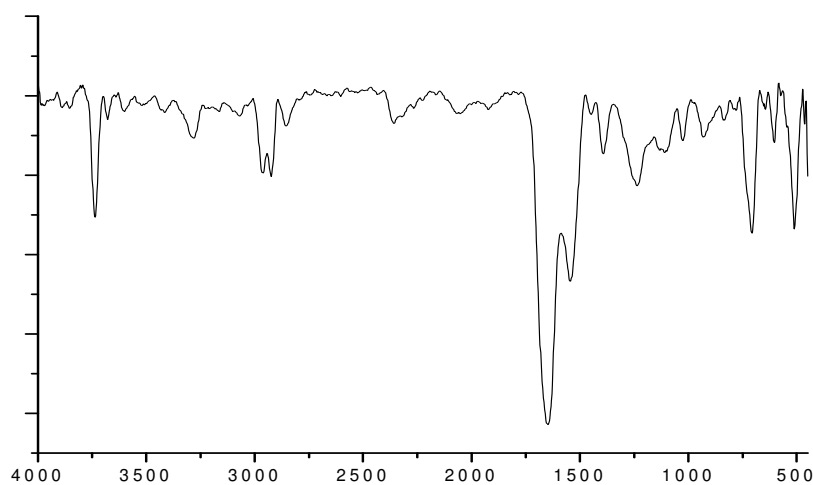


Figure 8. FT-IR reflectance spectrum of lyophilized catalase

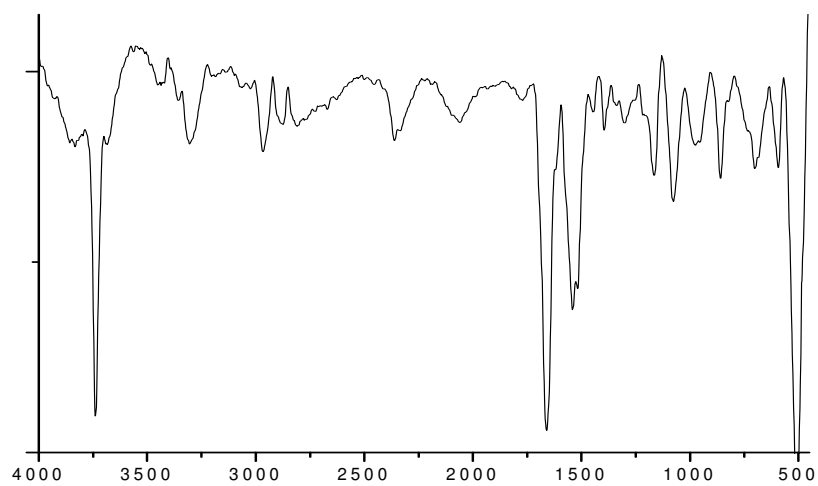


Figure 9. FT-IR reflectance spectrum of conjugate **1**.

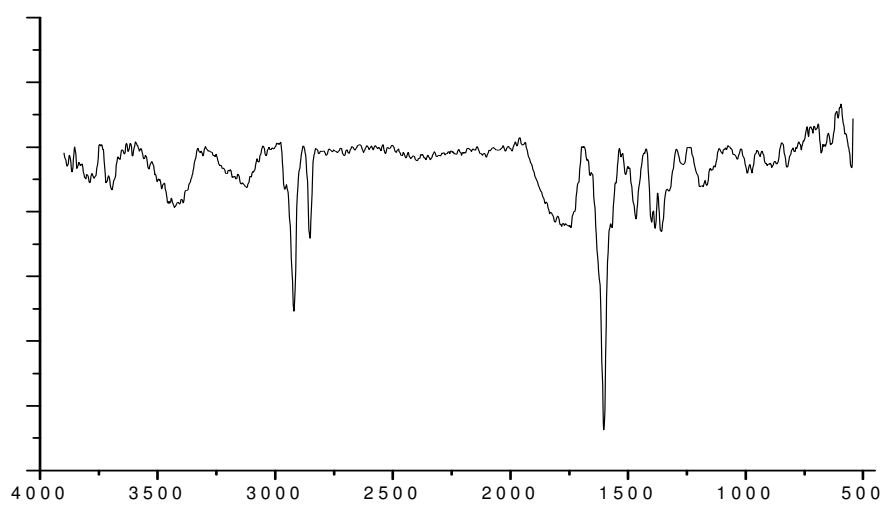


Figure 10. FT-IR reflectance spectrum of lyophilized GOx enzyme.

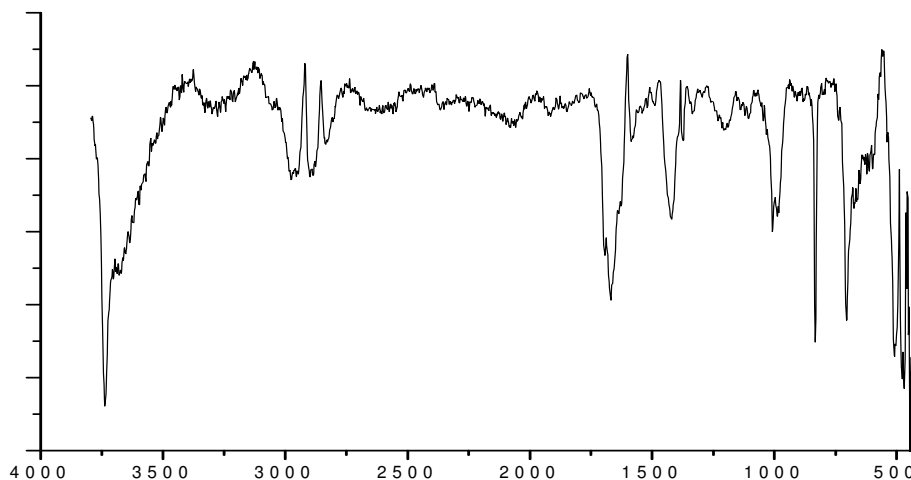


Figure 11. FT-IR reflectance spectrum of conjugate **2**.

Microscopy characterization

AFM images were taken by spreading a drop of the diluted conjugate suspension in pure water onto a clean silicon surface. The solvent was removed by evaporation by a gentle air stream for 20 min. The substrate was then analyzed by a tapping mode AFM (Digital Instruments Nanoscope III). Pictures were analyzed using WSxM 4.0.

TEM characterization was carried out on a JEOL 1200EX operating at 100 KV. A drop of diluted enzyme-nanotube water suspension was deposited on a carbon film copper grid. After 5 min the drop was blow dried and stained for 10 min with a saturated solution of uranyl acetate.

The SEM measurements were performed on a JEOL JSM 7000F field emission SEM operating at 2.0 kV. 150 μ l of enzyme-nanotube water suspension was deposited on a glass slit covered with a 120 nm Al top-layer on which a 1 nm Cr layer was sputtered. These samples were allowed to dry at ambient temperature and used with an additional sputtered staining.

Optical microscopy analysis: Images and movies of the moving nanotubes were recorded using a Olympus BX 60 microscope, equipped with a Sony 3CCD DXC 950P digital camera, attached to a personal computer with Matrox Inspector 2.1 imaging software.

Typically, a 50 μ l of aqueous enzyme-nanotube conjugate solution was deposited onto a glass coverslip and examined for the presence of aggregates under by optical microscopy. After careful addition of hydrogen peroxide (0.3% v/v) or D-glucose (100 mM), the movement of the carbon nanotube bundles was recorded and analyzed.

Video 1. Observation by optical microscopy of conjugate **3** after addition of a glucose solution (100 mM) under constant air flow. The CNT aggregates are visible as black irregular shape. Round shape oxygen bubbles generated from the aggregate are visible. An amount of non-enzyme-modified CNTs was added also.

Video 2. Observation by video-microscopy of conjugate **3** after addition of a glucose solution 100 mM under constant air flow. The CNT aggregates are visible as irregular black shapes. The round shapes are oxygen bubbles generated by/at the aggregates. An amount of non enzyme modified CNTs was added also.

Video 3 Observation by video-microscopy of a static Brownian diluted solution of conjugate **3** at 25°C. The very slow movement of fiber-like aggregate is attributed to Brownian motion.

Video 4. Observation by video-microscopy of conjugate **3** after addition of a glucose solution (100mM) under constant nitrogen flow.

References

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