Supplementary information

H_2-driven Biocatalysis in Continuous Flow using Enzyme-Modified Carbon Nanotube Columns

Ceren Zor,[a,b] Holly A. Reeve,[a] Jonathan Quinson,[a,b] Lisa A. Thompson,[a] Thomas H. Lonsdale,[a] Frank Dillon,[b] Nicole Grobert[b] and Kylie A. Vincent[a]*

Methods for generation of carbon nanotube-lined quartz columns

Carbon nanotube-lined quartz columns (CNCs) were generated using 20 mm long quartz tubes quartz tubes (2 mm inner diameter, 4 mm outer diameter) as substrates in a conventional aerosol assisted chemical vapour deposition (AACVD) experiment typically used for the production of vertically aligned multi-wall carbon nanotubes (VA-MWCNTs).[S1-4] These quartz tubes were placed inside a larger quartz reaction tube located inside a tube furnace and connected to an aerosol unit at one end and to an acetone bubbler at the other end. A standard 5 wt % ferrocene in toluene solution was used as a carbon precursor to generate the CNCs at 850 °C in an Ar atmosphere. CNC AACVD experiments were conducted for 90 minutes. Once the furnace cooled to room temperature the VA-MWCNTs-coated quartz tubes were removed from the furnace (Figure S1(a)) and any MWCNTs that formed on the outside of the 20 mm quartz tube were mechanically removed resulting in a VA-MWCNT-filled quartz column – CNC (Figure S1(b)).

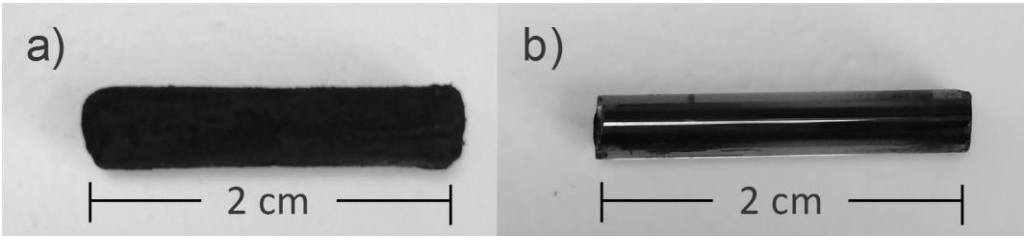

Figure S1: Photographs of a a) CNC directly after the synthesis, b) CNC after cleaning the outer deposit consisting of MWCNTs, ready to use as a flow device

S1. S. S. Meysami, F. Dillon, A. A. Koós, Z. Aslam and N. Grobert, *Carbon N. Y.*, 2013, **58**, 151–158.
S2. S. S. Meysami, A. A. Koós, F. Dillon and N. Grobert, *Carbon N. Y.*, 2013, **58**, 159–169.
S3. S. S. Meysami, A. A. Koós, F. Dillon, M. Dutta and N. Grobert, *Carbon N. Y.*, 2015, **88**, 148–156.
S4. Grobert et al, manuscript in preparation 2017

Flow materials and methods

The carbon nanotube-lined quartz column (CNC) comprised quartz tubing (2.4 cm x 2 mm inner diameter) lined with carbon nanotubes (image shown in Figure S2).

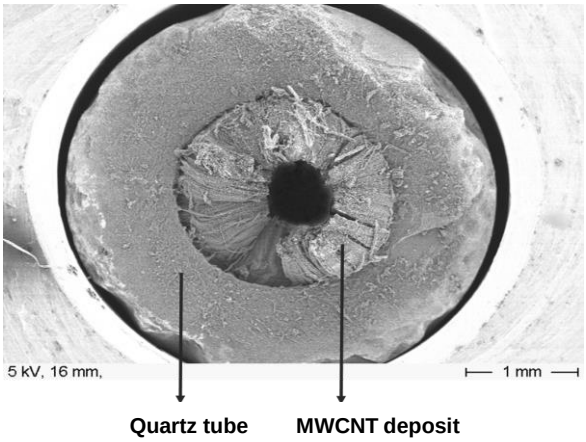

Figure S2: SEM micrograph of a CNC cross-section revealing the radially grown multi-walled carbon nanotubes (MWCNT) deposited on the inside of the quartz tube, i.e. flow reactor.

For all flow reactions the reaction solution was infused into the CNC reactor using a Watson Marlow 120U peristaltic pump or an Asia syringe pump (Syrris). The pump was connected to the CNC using 1/32" inner diameter PTFE tubing, 1/32" inner diameter Marprene tubing and standard HPLC fittings.

Confirmation of the extent of conversion of NAD^+ to NADH using an enzyme-modified carbon nanotube column

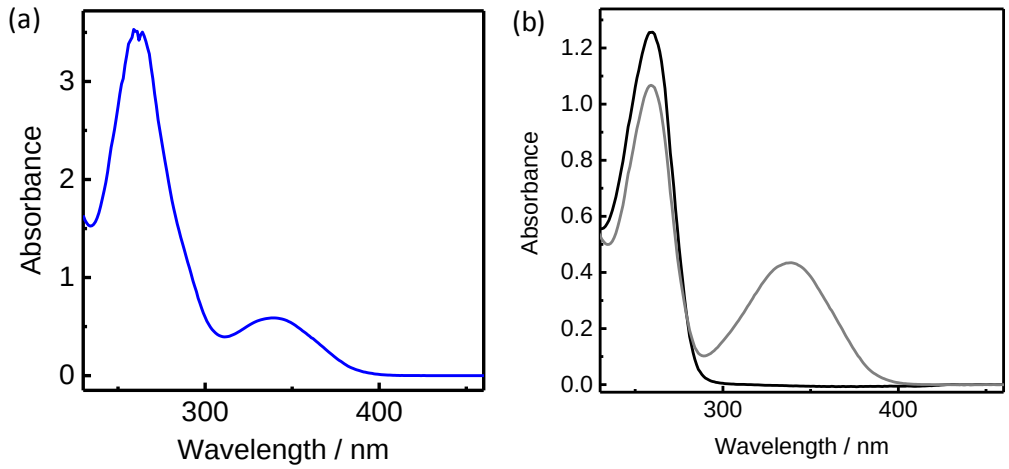

Figure S3: NADH generation by hydrogenase and NAD^+ reductase immobilised in a CNC, with H_2-saturated NAD^+ solution cycling through. This UV-vis spectrum represents the end-point for the reaction sequence shown in Figure 1 of the main text, and was recorded after dilution of the final reaction solution to enable UV-visible quantification of the 260 nm peak. The ratio of the absorbance (A) at 260 nm and 340 nm indicates *ca* 67 % conversion of NAD^+ to NADH by comparison with a calibration curve for $A_{260\ nm}/A_{340\ nm}$ vs $[NAD^+]/[NADH]$ described previously.[S5] (b) UV-vis spectra of NAD^+ (black) and NADH (grey) at 77 µM.

Additional experimental conditions: A 50 µL solution containing a mixture of hydrogenase (*E. coli* hydrogenase 2, 70 µg) and NAD^+ reductase (I64A, 40 µg) was flowed into the CNC and allowed to adsorb over *ca* 1 hour at 4 °C. The CNC was washed with buffer (5 mL, 50 mM Bis-Tris, pH 6) to remove any unadsorbed enzyme. A H_2-saturated solution (at ambient pressure) containing NAD^+ (0.5 mM) in 50 mM Bis-Tris buffer (1.2 mL) was then cycled through the CNC (flow rate = 25 µL min^{-1}) with H_2 continuously bubbled through the buffer reservoir. The solution was monitored continuously, in-line, at 340 nm using a UV-visible spectrophotometer to determine the concentration of NADH.

[S5] H. A. Reeve, L. Lauterbach, O. Lenz, K. A. Vincent, *ChemCatChem* **2015**, *7*, 3480–3487 (Electronic Supplementary information).

Chiral GC for determination of 1-phenylethanol enantiomer

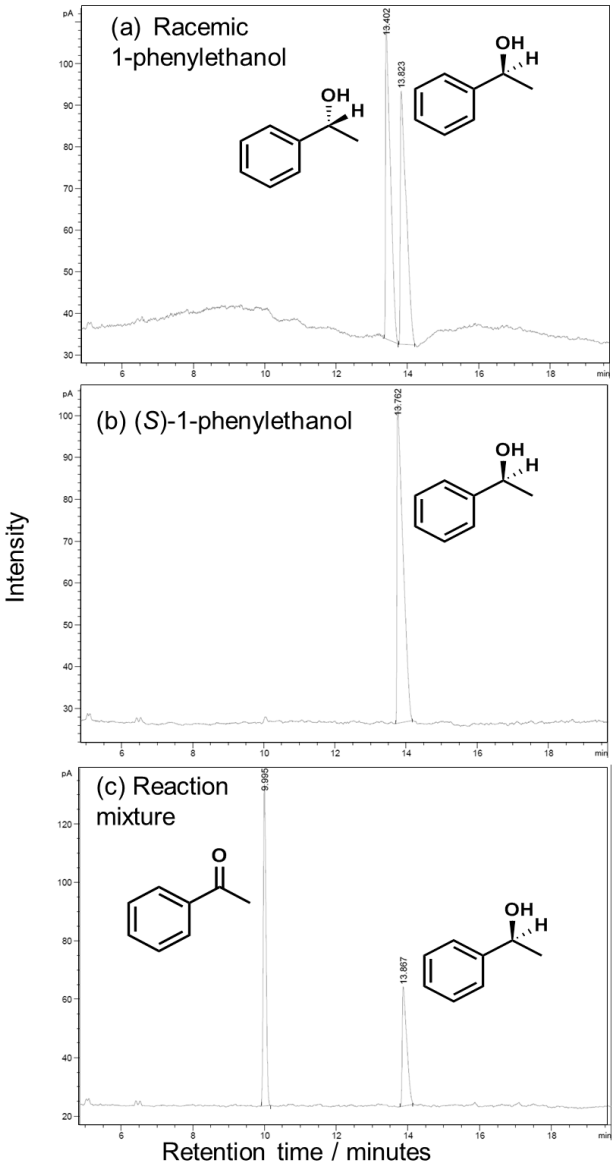

Figure S4: Chiral gas chromatograms of (a) racemic 1-phenylethanol standard (Sigma), (b) (*S*)-1-phenylethanol standard (Sigma) and (c) the final reaction solution after H_2-driven acetophenone reduction using a CNC modified with hydrogenase, NAD^+ reductase and alcohol dehydrogenase. Only the (*S*)-enantiomer of 1-phenylethanol was observed in the reaction solution, demonstrating that the ADH retains its (*S*)-selectivity when immobilised in the CNC.

Additional experimental conditions: A 75 µL solution containing a mixture of hydrogenase (*E. coli* hydrogenase 2, 90 µg), NAD^+ reductase (I64A, 64 µg) and ADH (1 mg) was flowed into the CNC and allowed to adsorb over *ca* 90 minutes at 4 °C. The CNC was washed with buffer (1 mL of 50 mM Bis-Tris, pH 6) to remove any unadsorbed enzyme. The H_2-saturated solution containing NAD^+ (1 mM) and acetophenone (8.7 mM) in Bis-Tris buffer (3.25 mL) was then cycled through the CNC (flow rate = 25 µL min^{-1}). The mixture was analysed by chiral GC after 26 hours. GC conditions: Supelco β-dexTM 325 column (30 m, 0.25 mm, 0.25 µm film thickness), 120 ^oC isocratic, He carrier gas (flow rate 21.5 cm s^{-1}).

Control experiment to test for background reaction of acetophenone with the carbon nanotube column

A carbon nanotube column without enzyme modification was used to confirm that there was no H_2-driven acetophenone reduction in the absence of enzymes.

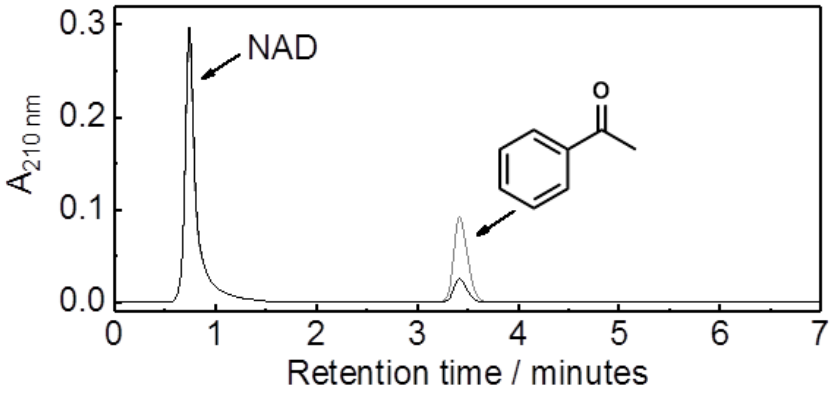

Figure S5: HPLC traces of the reaction mixture before (grey) and after (black) cycling through a pristine carbon nanotube column. The final reaction mixture shows a smaller acetophenone peak, likely due to substantial adsorption of acetophenone at the unmodified carbon, however, the absence of additional peaks demonstrates that reduction of acetophenone does not occur at the bare CNC. Conditions: H_2-saturated solution containing NAD^+ (1 mM) and acetophenone (8.7 mM) in Bis-Tris buffer.

When using enzyme-modified CNCs, adsorption of substrate occurs to a much lower extent, but nevertheless, substrate adsorption has been taken into consideration when calculating the conversions recorded in Table 1 in the manuscript.

H_2-driven biocatalysis in flow using whole soluble hydrogenase (HoxHYFU) for cofactor recycling

A carbon nanotube column modified with soluble hydrogenase and alcohol dehydrogenase was used to demonstrate that H_2-driven cofactor recycling can be achieved using the native soluble hydrogenase (HoxHYFU)[S6] in place of the hydrogenase and NAD^+ reductase constructs used in the previous experiments. In this experiment, the carbon nanotube column is only acting as a convenient support for enzyme immobilisation; the enzyme system does not rely on transfer of electrons *via* the carbon network.

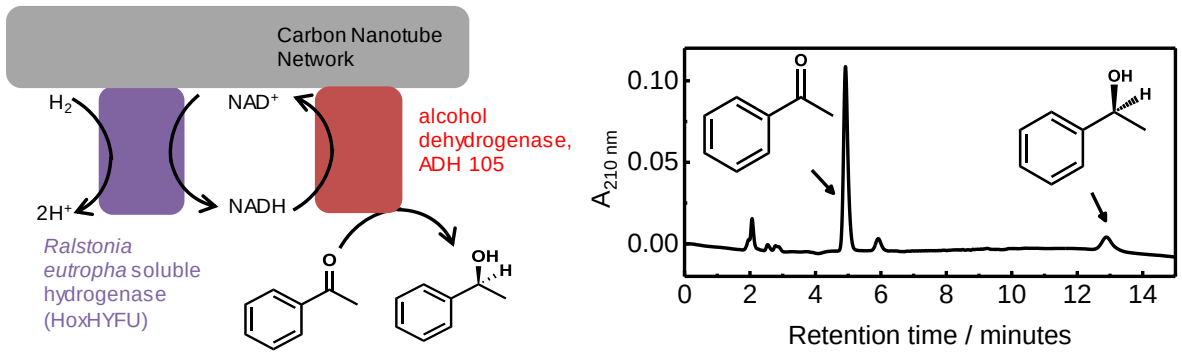

Figure S6: Chiral HPLC traces of the reaction mixture after H_2-driven acetophenone reduction using a CNC modified with *Ralstonia eutropha* soluble hydrogenase (HoxHYFU) and ADH. The final reaction mixture shows generation of (*S*)-1-phenylethanol. Conditions: H_2-saturated solution containing NAD^+ (1 mM) and acetophenone (10 mM) in Tris-HCl buffer, pH 8.0.

Additional experimental conditions: A 40 µL solution containing a mixture of soluble hydrogenase (*Ralstonia eutropha* HoxHYFU, 0.4 U, activity determined under the same reaction conditions in a solution assay), and ADH (0.6 mg in 20 µL buffer) was flowed into the CNC (75 µL reactor volume) and allowed to adsorb over *ca* 60 min at 4 °C. Buffer (0.8 mL of 100 mM Tris-HCl, pH 8.0) was pumped through the CNC to remove any unadsorbed enzyme. A H_2-saturated solution containing NAD^+ (1 mM) and acetophenone (10 mM) in Tris-HCl buffer (3.5 mL total volume) was then cycled through the CNC (flow rate = 67 µL min^{-1}). The mixture was analysed by chiral HPLC after 19 hours (approx. 22 full cycles), after extraction into heptane/*i*PrOH (99:1). HPLC method: Chiralpak IA column (15 cm x 4.6 mm, 5 µm particle size), heptane/*i*PrOH (99:1) mobile phase, isocratic over 15 min, 1 mL min^{-1}, 40 °C, 210 nm. The product was determined to be a single enantiomer, and by comparison to standards this enantiomer was shown to be (*S*)-1-phenylethanol.

[S6]L. Lauterbach, O. Lenz and K. A. Vincent, *FEBS J.*, 2013, **280**, 3058–3068; L. Lauterbach and O. Lenz, *J. Am. Chem. Soc.*, 2013, **135**, 17897–905.

Detection of pyruvate and alanine

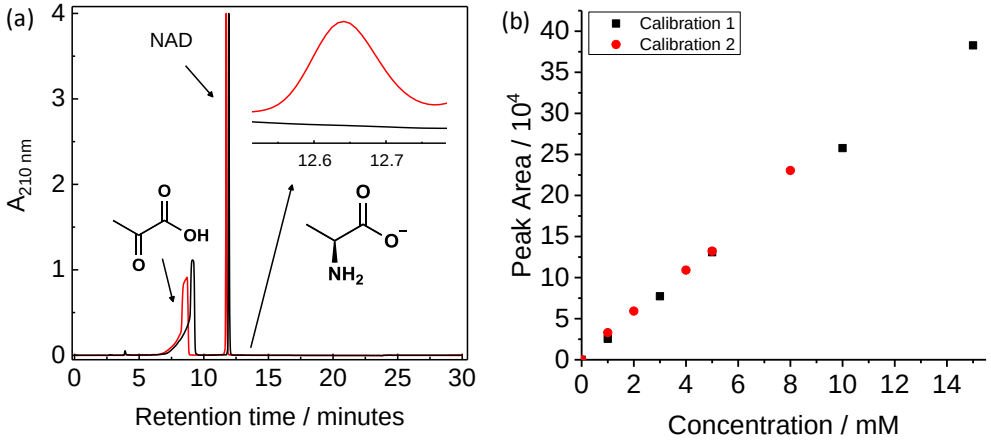

Figure S7: (a) Chromatogram of the starting reaction mixture (black) and final reaction solution (red) for a typical H_2-driven pyruvate reduction experiment using a CNC modified with hydrogenase, NAD^+ reductase and L-alanine dehydrogenase. (b) Two calibrations recorded on two separate days were compared to show that HPLC could be used for quantitative determination of the alanine concentration.

Additional experimental conditions: A 75 µL solution containing a mixture of hydrogenase (*E. coli* hydrogenase 2, 120 µg) and NAD^+ reductase (I64A, 48 µg) and L-alanine dehydrogenase (300 µg) was flowed into the CNC and the enzymes were allowed to adsorb over *ca* 90 minutes at 4 °C. The CNC was washed with buffer (1 mL of 50 mM Bis-Tris, pH 6) to remove any unadsorbed enzyme. A H_2-saturated solution containing NAD^+ (1 mM), pyruvate (1.99 mM) and ammonium chloride (100 mM) in Bis-Tris buffer was then cycled through the CNC (flow rate = 25 µL min^{-1}). The mixture was analysed by HPLC after 26 hours. Pyruvate and alanine were separated using a Chromolith® Performance 100-3 mm column (10 cm x 3 mm, 2 µm particle size), H_2O/MeCN (5:95) to H_2O/MeCN (80:20) over 20 min, 0.75 mL min^{-1}, 40 °C, 210 nm. Note that the product, L-alanine, absorbs very weakly in the UV-visible and therefore gives a very small peak in the chromatogram. For this reason, two separate calibration curves were obtained to confirm that the concentration of L-alanine could be determined using HPLC. The L-alanine concentration was determined by comparison to the HPLC response for known-concentration standards.

Single pass continuous flow

A CNC modified with hydrogenase, NAD^+ reductase and ADH 105 was used to demonstrate that H_2-driven ketone reduction can be achieved in a single pass. In this experiment, lower concentrations of NAD^+ and acetophenone were used such that the concentration of acetophenone was approximately equivalent to the solubility of H_2 in water (1 mM).

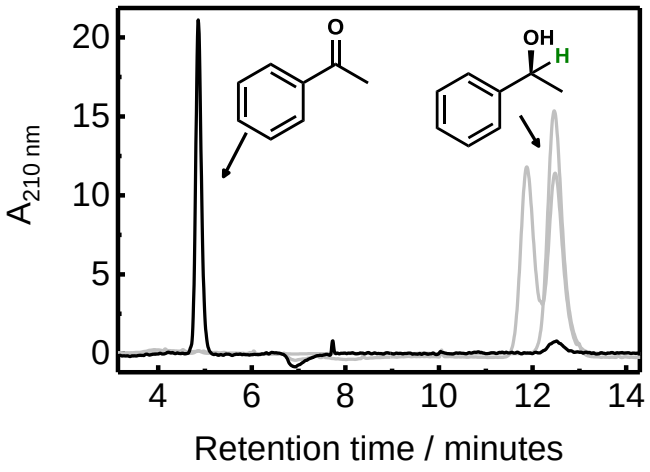

Figure S8: Chiral HPLC trace of the reaction mixture after H_2-driven acetophenone reduction using a CNC modified with hydrogenase, NAD^+ reductase and ADH 105. The H_2-saturated reaction mixture was pumped through the reactor in a single pass with a residence time (tRes) of 58 min. The final reaction mixture shows generation of (*S*)-1-phenylethanol. Conditions: H_2-saturated solution containing NAD^+ (0.1 mM) and acetophenone (1 mM) in Tris-HCl buffer, pH 8.0.

Additional experimental conditions: A 66 µL solution containing a mixture of hydrogenase (*E. coli* hydrogenase 2, 70 µg), NAD^+ reductase (I64A, 22 µg) and ADH 105 (0.6 mg) was flowed into the CNC (75 µL reactor volume) and allowed to adsorb over *ca* 90 min at 4 °C. Buffer (1 mL of 100 mM Tris-HCl, pH 8.0) was pumped through the CNC to remove any unadsorbed enzyme. A H_2-saturated solution containing NAD^+ (0.1 mM) and acetophenone (1 mM) in Tris-HCl buffer (2 mL total volume) was then pumped through the CNC (flow rate = 1.3 µL min^{-1}, tRes = 58 min) as a single pass. For this experiment an Asia syringe pump (Syrris) was used to achieve lower flow rates. The eluent was analysed by chiral HPLC after extraction into heptane/*i*PrOH (99:1). HPLC method: Chiralpak IA column (15 cm x 4.6 mm, 5 µm particle size), heptane/*i*PrOH (99:1) mobile phase, isocratic over 15 min, 1 mL min^{-1}, 40 °C, 210 nm. HPLC analysis showed 8% conversion to 1-phenylethanol after a single pass, with only the (*S*)-enantiomer detected.