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A facile analytical method for reliable selectivity examination in cofactor NADH regeneration

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I. Materials

β-Nicotinamide adenine dinucleotide hydrate (NAD^+, ≥ 96.5%), β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH, ≥ 94%), potassium phosphate monobasic (KH_2PO_4, ≥ 99%), potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$, ≥ 99%), VenPure SF sodium borohydride ($NaBH_4$, ≥ 98.5%), Triethanolamine (TEOA, ≥ 99.0%), acetaldehyde (CH_3CHO, ≥ 99.5%), alcohol dehydrogenase from *Saccharomyces cerevisiae* (≥ 300 units mg^{-1}), and platinum on activated carbon (1 wt. % loading) were obtained from Sigma-Aldrich. EnzyChrom™ NAD/NADH assay kit was purchased from Universal Biologicals. Invitrogen ultrapure tris buffer (≥ 99.99%) and hydrochloric acid solution (1 M) were purchased from Fischer Scientific. All the chemicals were used as received without further purification. The H_2 and N_2 gases of ultrahigh purity (≥ 99.99%) were supplied by BOC.

II. Apparatus

All the absorbance measurements were done using JENWAY 6850 double beam UV-Visible Spectrophotometer with wavelength range between 190 and 1100 nm. The Pt content of the catalyst was measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian Vista-MPX) from the diluted extract in HF. Nitrogen adsorption-desorption isotherms were obtained using a commercial automated Micromeritics Tristar II 3000 Analyser. Specific surface area was obtained from the adsorption isotherms using the standard BET method. Pore volume and pore size were determined by BJH analysis of desorption profiles. Transmission electron microscopy (TEM) images were taken on FEI Tecnai G2 F20 equipment. Particle size distribution was determined from TEM images by counting approximately 150 particles. X-ray diffraction (XRD) experiments were

performed on a Panalytical powder X-ray diffractometer. The diffraction patterns were recorded over an angular range of 10° < 2θ < 90° with a step-size of 0.02°.

III. Enzymatic kit procedure for the quantification of total NAD(H)

The supplied EnzyChrom™ kit consists of Assay Buffer, 1.5 mL of Lactate, 1.5 mL of MTT solution, 120 µL of Enzyme A (Lactate Dehydrogenase), 120 µL of Enzyme B (Diaphorase) and 500 µL of NAD (1 mM) stock solution (i.e. NAD_0). It is noteworthy that when not in use the enzymatic kit has to be stored at -20 °C. Prior to use, remove all components (except enzymes) and allow them to defrost at room temperature for at least 20 minutes. Prior to analysis, prepare diluted NAD solutions (i.e. two dilutions are required for a better accuracy) as follows:

NAD(H) concentration (µM)	First Dilution (NAD_1 solution)		Second Dilution (NAD_2 solution)	
	NAD stock solution (NAD_0) (µL)	Buffer (µL)	NAD_1 (µL)	Buffer (µL)
2.5	25	975	100	900
5.0	50	950	100	900
7.5	75	925	100	900
10.0	100	900	100	900

For each of the diluted standards, prepare working reagent (i.e. one at a time) by mixing 60 µL of assay buffer, 1 µL of Enzyme A, 1 µL of Enzyme B, 14 µL of Lactate and 14 µL of MTT solution. For each analysis, take 80 µL of the working reagent and mix it quickly with 240 µL of the prepared NAD_2 solution, then transfer the whole reaction mixture into a quartz cuvette (2 mm path length) and start recording the 565 nm change against buffer solution used as reference (i.e. time 0 is the time the reagents are mixed together).

IV. Enzymatic kit procedure for the quantification of 1,4-NADH

Prepare a stock solution of 0.5 mM 1,4-NADH (i.e. $NADH_0$) in phosphate buffer pH 7. Prepare the 1,4-NADH standards as follows:

1,4-NADH concentration (mM)	$NADH_1$ solution	
	NADH stock solution ($NADH_0$) (µL)	Buffer (µL)
0.01	20	980
0.05	100	900
0.1	200	800
0.2	400	600
0.3	600	400
0.4	800	200
0.5	1000	0

For each of the diluted standards, prepare working reagent (i.e. one at a time) by mixing 60 µL of assay buffer, 1 µL of Enzyme B and 14 µL of MTT solution. For each analysis, take 75 µL of the working reagent and mix it quickly with 240 µL of the prepared $NADH_1$ solution, then transfer the whole reaction mixture into a quartz cuvette (2 mm path length) and start recording the 565 nm change against buffer solution used as reference (i.e. time 0 is the time the reagents are mixed together).

V. Preparation of 1,2-NADH, 1,4-NADH and 1,6-NADH by sodium borohydride NAD^+ reduction

In a typical reduction reaction, 66.20 mg of $NaBH_4$ was added to 100 mL of 0.1 M potassium phosphate buffer, pH 7, containing 16.59 mg of NAD^+ (equivalent to 0.25 mM). The reaction was carried in a 100 mL Erlenmeyer flask in a fume hood under a mild stirring of 300 rpm to help the removal of hydrogen formed as a result of sodium borohydride decomposition in water. About 5 minutes were allowed for the reaction to complete. Samples

were taken and analysed in the UV to make sure there is no more increase in the absorbance at 340 nm. The same procedure was repeated but with higher initial concentration of NAD^+ (0.50 and 0.75 mM) while keeping the molar ratio of $NaBH_4$ to NAD^+ fixed at 70.

VI. Reoxidation of 1,4-NADH to NAD^+ over alcohol dehydrogenase (ADH)

The oxidation reaction was carried in a 3.5 mL cuvette containing 2 mL of reaction mixture (1,4- and 1,6-NADH), 1 mL of ADH (2 mg mL^{-1}) and 0.5 mL of acetaldehyde (0.5 M). It is very important that the acetaldehyde is added last and that high amounts of enzymes are in place to rapidly oxidise 1,4-NADH in such a way to avoid any loss of 1,4-NADH through addition to the 5,6-double bond.[1] The oxidation reaction was followed in the spectrophotometer at 340 nm until there was no more decrease in the absorbance.

VII. NADH regeneration by the hydrogenation of NAD^+ with hydrogen and Pt/C catalyst

The hydrogenation reaction was conducted in a Parr®5500 compact reactor (with a Parr® 4848 reactor controller) following the procedure in our previous works.[2] The hydrogenation reaction was conducted in a pressurized system at room temperature (22 °C) in a tris-buffer pH 8.5. In the experiment, 5 mg of the catalyst and 50 mL 0.1 M tris buffered solution (pH 8.5) containing NAD^+ (1 mM) were loaded into the reactor. The system was flushed (three times) with N_2 (3 atm). Hydrogen gas was then introduced, the system was pressurised to 8 atm and stirring (900 rpm) was engaged (time t = 0 for reaction). A non-invasive liquid sampling system via syringe/in-line filters allowed the controlled removal of aliquots from the reactor. Samples were taken periodically and the total absorbance at 340 nm was measured using JENWAY 6850 double beam UV-Visible Spectrophotometer.

VIII. Supporting Table and Figures

Table S1: A literature summary of analytical methods used for the quantification of NADH

Regeneration Method	Analytical Method					References
	UV-Vis Spectroscopy	Enzymatic Assay	HPLC	NMR	Fluorescence Spectroscopy	
Biocatalytic	☒	☒	☐	☐	☐	3
Biocatalytic	☒	☐	☐	☐	☐	4
Electrochemical	☒	☐	☐	☐	☐	5
Electrochemical	☒	☒	☐	☐	☐	6
Electrochemical	☒	☒	☐	☐	☐	7
Electrochemical	☒	☒	☐	☐	☐	8
Homogeneous Catalytic	☒	☒	☒	☐	☐	9
Homogeneous Catalytic	☒	☐	☐	☒	☐	10
Homogeneous Catalytic	☐	☐	☐	☒	☐	11
Homogeneous Catalytic	☒	☐	☐	☐	☐	12
Homogeneous Catalytic	☒	☐	☐	☐	☐	13
Photocatalytic	☒	☒	☐	☒	☐	14
Photocatalytic	☐	☐	☐	☐	☒	15
Photocatalytic	☐	☐	☐	☐	☒	16
Photocatalytic	☒	☐	☐	☐	☐	17
Photocatalytic	☒	☐	☐	☐	☐	18
Photocatalytic	☒	☐	☐	☐	☐	19
Photocatalytic	☒	☐	☐	☐	☐	20
Photocatalytic	☒	☐	☐	☐	☐	21
Photocatalytic	☒	☐	☐	☐	☐	22
Photocatalytic	☒	☐	☐	☐	☐	23

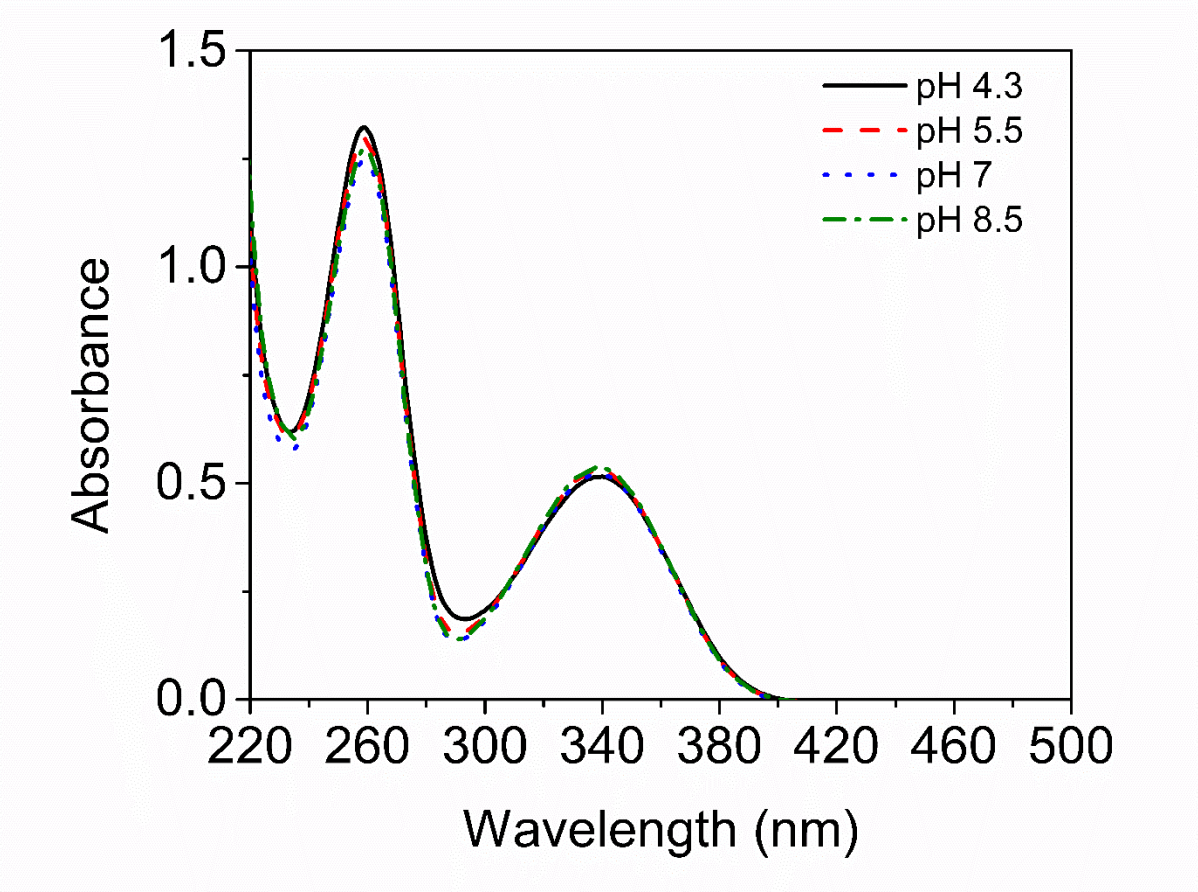

Fig. S1 Effect of initial pH on fresh 1,4-NADH solutions at 0.1 mM.

Fig. S1 confirms that the initial pH of a solution does not affect the UV spectra, and the epsilon obtained should be applicable at different values of pH.

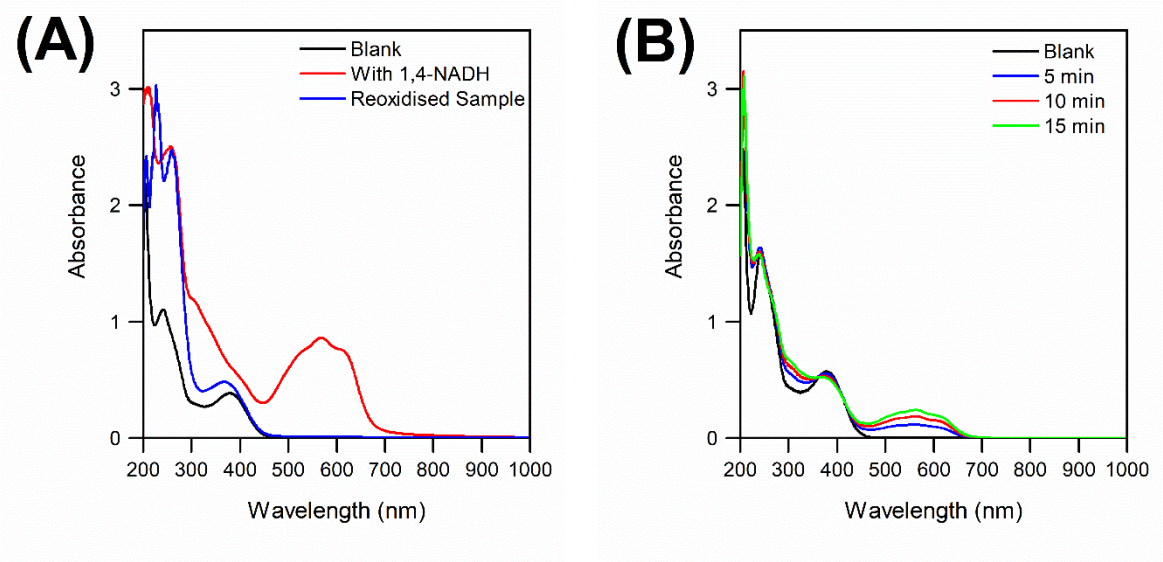

Fig. S2 (A) Half kit full UV-Vis scans (Blank: no 1,4-NADH in place; With 1,4-NADH: 1,4-NADH in place at C = 0.5 mM after 5 min assay time; Reoxidised Sample: From ADH catalysed reoxidation reaction containing NAD^+, 1,2-NADH and 1,6-NADH at a total concentration of 0.5 mM and after 5 min assay time. (B) Full kit UV-Vis scans for 10 µM NAD(H) throughout the kit measurement time (Blank: No NAD(H) in place)

The reoxidised 1,4-NADH sample demonstrated that 1,2-NADH and 1,6-NADH are enzymatically inactive isomers with their inability to transfer a hydride (i.e. the reducing equivalent) to an oxidised substrate (i.e. MTT in our case). As shown in the Fig. S2(A), the half kit (in blue) exhibits no absorbance at 565 nm as expected, whereas a 1,4-NADH sample at similar concentration (0.5 mM) presents a clear peak (in red). This confirms that the kit is only sensitive to 1,4-NADH but not to 1,2- and 1,6-NADH.

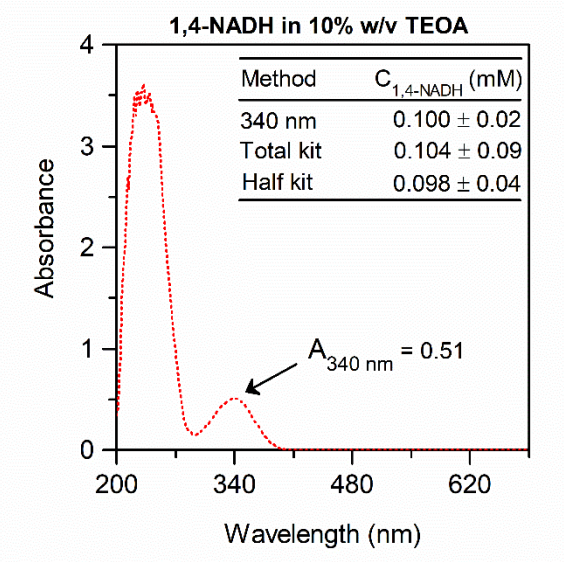

Fig. S3: Demonstration of the practical validity of the analytical method by mimicking a photocatalytic reaction sample containing 10% w/v TEOA and 1,4-NADH (scanned spectrum at 0.1 mM concentration); Inset: the concentration of 1,4-NADH determined by the three methods: 340 nm UV-Vis, total enzymatic kit and half enzymatic kit.

In Fig. S3, we have mimicked a "sample" from a photocatalytic reaction including 10 w/v% TEOA in the solution. The kit still works well and quantifies correctly the known concentration of 1,4-NADH. We suggest here if any potential interfering reagents exist (at high concentrations) or sample pH is too far off neutral, the sample volume should be kept as low as possible and balanced with pH = 7 buffer to minimise potential effects on the method.

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