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

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This study demonstrates a novel method to quantify selective (1,4-NADH) and unselective products (1,2- and 1,6-NADH) in NADH regeneration using combined UV-Vis spectroscopy and biological assays. The validity of the proposed method was tested in the Pt/C promoted NAD+ hydrogenation using hydrogen as reducing agent.

1,4-NADH is a valuable cofactor that is used in tandem with enzymes (i.e. oxidoreductases) in the chemical and pharmaceutical industries for the manufacture of intermediates and drugs. Notable examples of these products include the Lipitor (atorvastatin), the antilipemic agent ZETIA (ezetimibe), the HIV protease inhibitor REYATAZ (atazanavir) and L-tert-leucine building block, among others.1 Cofactor 1,4-NADH is very expensive ($2600 per mol) making its stoichiometric supply in a bioreaction not economically viable and necessitating its in situ regeneration.2 1,4-NADH regeneration has been the area of extensive research over the past ~40 years with various methods having been investigated, including biocatalytic, electrochemical, chemical, photocatalytic, homogeneous catalytic, and heterogeneous catalytic.3

Besides the commercialised enzymatic method, other regeneration strategies, currently under development, may suffer from non-selective reduction of NAD+ to a mixture of the enzymatically active 1,4-NADH, and its inactive 1,2-NADH and 1,6-NADH isomers4 (see Scheme 1). These molecules absorb light at 340, 395 and 345 nm, respectively.5 It is furthermore noteworthy that 1,2-NADH exhibits high instability in phosphate buffer solutions (PBS) at pH ≤ 7 with a life time of only ~30 min.6 Considering these facts, it is surprising to note that a significant number of literature has been relying solely on UV-Vis at 340 nm to quantify 1,4-NADH concentration.7 Moreover, Morrison et al.8 stated that unusual situations (e.g. discrepancies in HPLC analyses of NADH isomers) were observed in isomers preparation. This may lead to inaccurate or misleading conclusions, as other isomers were potentially not considered or distinguished.

In the course of a reaction, the reaction medium may consist of all four molecules as shown in Scheme 1. The most important indicator for catalytic performance and kinetic analysis is the conversion of NAD+ and the selectivity to 1,4-NADH. We have accordingly customised an enzymatic assay kit to determine both 1,4-NADH and NAD+. The kit works as an enzymatic

Scheme 1 Representative scheme for a non-selective NADH regeneration process in which NAD+ reacts into 1,2-, 1,4- and 1,6-NADH.
The difference in absorbance at 565 nm between the sample and blank (i.e. with cofactors) was recorded with time using eqn (1) below:

\[ \Delta A_{\text{Sample}} = \Delta A_{\text{Sample}} - \Delta A_{\text{Blank}} \]  

where \( \Delta A_{\text{Sample}} = A_{\text{Sample}} \) at time \( t \) min \(-\) \( A_{\text{Sample}} \) at 0 min; \( \Delta A_{\text{Blank}} = A_{\text{Blank}} \) at time \( t \) min \(-\) \( A_{\text{Blank}} \) at 0 min. The difference in absorbance at 565 nm is directly proportional to the amount of NAD(H) in the system provided it stays linear with time.\(^9\) Fig. 1C shows the results of the kinetic measurements using our customised procedure which shows an excellent linear fit for the range of NAD(H) concentrations considered. Fig. 1D shows the calibration curve at \( t = 15 \) min for the total cycle and the resulting slope can be used for the quantification of the total amount of NAD(H) as follows:

\[ C_{\text{NAD(H)}}(\mu M) = \frac{\Delta A_{\text{Sample}}}{\text{Slope} (\mu M^{-1})} = \frac{\Delta A_{\text{Sample}} - \Delta A_{\text{Blank}}}{\text{Slope} (\mu M^{-1})} \]  

With the concentration of NAD(H) known, either of the two components can be quantified, the other can be directly determined. To determine the concentration of 1,4-NADH, we have innovatively used the reduction side (right half) of the kit as depicted in Fig. 1B, where 1,4-NADH reacts with MTT to form both NAD\(^+\) and formazan. The working reagent was prepared by mixing 60 \( \mu L \) of the assay buffer, 1 \( \mu L \) of diaphorase and 14 \( \mu L \) of the MTT. The whole working reagent (75 \( \mu L \)) was then added to 240 \( \mu L \) of 1,4-NADH sample (in the range of 0.01 to 0.5 mM) and the absorbance was recorded with respect to time (see ESI,\(^\dagger\) Part IV for the detailed procedure). The kinetic results are shown in Fig. 1E. The absorbance increases rapidly and then stabilises. The absorbance at the plateau is an indication of the concentration of 1,4-NADH in solution and it was plotted against the actual concentration as seen in the calibration curve (Fig. 1F). Eqn (3) is used for the quantification of 1,4-NADH:

\[ C_{1,4-\text{NADH}}(\text{mM}) = \frac{\Delta A_{\text{Sample}}}{\text{Slope} (\text{mM}^{-1})} = \frac{\Delta A_{\text{Sample}} - \Delta A_{\text{Blank}}}{\text{Slope} (\text{mM}^{-1})} \]  

The validity of the half cycle’s novel use was tested by preparing a mixture of both NAD\(^+\) (0.3 mM) and 1,4-NADH (0.2 mM). The absorbance measurement fit exceptionally well the calibration curve (see Fig. 1F), confirming that the existence of NAD\(^+\) in solution has no practical effect on the 1,4-NADH analysis. The amount of NAD\(^+\) is then deduced by subtracting the concentration of 1,4-NADH from the total NAD(H) using eqn (4):

\[ C_{\text{NAD\(^+\)}}(\text{mM}) = C_{\text{NAD(H)}}(\text{mM}) - C_{1,4-\text{NADH}}(\text{mM}) \]  

To elucidate the reaction pathway and mechanism, knowing only the concentrations of NAD\(^+\) and 1,4-NADH is not sufficient, unless the amount of reacted NAD\(^+\) is equal to that of 1,4-NADH produced, i.e. the reaction is 100% selective. When this is not the case, the key information to obtain is the concentrations of the NADH isomers produced, which is hampered by the fact that 1,4- and 1,6-NADH exhibit similar characteristic absorbance peak (~340 nm) and 1,2-NADH decays quickly in PBS with pH ≤ 7 (which is commonly used in NADH regeneration). We have therefore developed a systematic approach to distinguish and quantify the three isomers. In order to quantify
1,2- and 1,6-NADH in a reaction mixture, their molar absorptivities must be determined. Unfortunately, there is no supplier providing commercial isomer products (apart from 1,4-NADH). For this reason, we have synthesised a mixture of 1,2-, 1,4- and 1,6-NADH with known concentrations according to an established experimental procedure, i.e. sodium borohydride reduction of NAD+ (see ESI,† Part V). We conducted first the synthesis in PBS at pH = 7 as the high instability of 1,2-NADH at this condition (exhibits rapid decay) leaves only 1,4- and 1,6-NADH in the reaction medium.

In order to distinguish between the two isomers at 340 nm, active 1,4-NADH was reoxidised to NAD+ by alcohol dehydrogenase (ADH) catalysed acetaldehyde reduction11 (see the ESI,† Part VI). A reduction of almost half of the initial 340 absorbance was observed (Fig. 2A), in line with previous studies.10c,12 Upon 1,4-NADH oxidation, the absorbance at 260 nm increased dramatically indicating the formation of NAD+ that has a high molar absorptivity at 260 nm compared to all isomers.5 The residual absorbance at 340 nm (due to 1,6-NADH) at five different initial concentrations of NAD+ was then translated into a calibration curve of 1,6-NADH, which is depicted in Fig. 2B. A standard calibration curve for commercial 1,4-NADH at 340 nm was generated in the range of 0.05 to 0.3 mM. The slope of the linear curve corresponds to the molar absorptivity of the 1,4-NADH in mM⁻¹ cm⁻¹ (Fig. 2B).

In a course of a reaction, the absorbance of a sample at time t at 340 nm, A340(t, total), is a combination of both 1,6-NADH and 1,4-NADH absorptions. The latter can be determined by converting 1,4-NADH concentration (determined from the enzymatic half cycle) using ε(1,4-NADH) according to eqn (5):

$$A_{340(1,4-\text{NADH})} = \varepsilon_{1,4-\text{NADH}} \left(\frac{mM}{cm}\right) \times L (cm)$$

The absorbance and concentration of 1,6-NADH are then calculated using its molar absorptivity at 340 nm and the difference between the A340(total) and A340(1,4-NADH) (see eqn (6) and (7)):

$$A_{340(1,6-\text{NADH})} = A_{340(\text{total})} - A_{340(1,4-\text{NADH})}$$

$$C_{1,6-\text{NADH}} (mM) = \frac{A_{340(1,6-\text{NADH})}}{\varepsilon_{1,6-\text{NADH}} \left(\frac{mM}{cm} \cdot cm^{-1}\right) \times L (cm)}$$

$$A_{395} = \frac{C_{1,2-\text{NADH}} \left(\frac{mM}{cm} \cdot cm^{-1}\right) \times L (cm)}{\varepsilon_{1,2-\text{NADH}} \left(\frac{mM}{cm} \cdot cm^{-1}\right) \times L (cm)}$$

Fig. 3A presents the UV-Vis spectra of the products formed in the borohydride reduction of NAD+ in PBS at pH 7. The reaction produced in the first few seconds a bright yellow mixture, which disappeared 1 minute later forming a very pale-yellow colour product (Fig. 3A inset). There is also no clear peak at 395 nm, characteristic of 1,2-NADH, suggesting 1,2-NADH has decayed.10b In contrast, when NAD+ was reduced in Tris buffer at pH 8.5, it generated a persistent bright yellow mixture with a wide spectrum between 300 and 480 nm (Fig. 3B). This vivid colour is associated with an absorbance band at 395 nm, i.e. presence of 1,2-NADH. The difference spectrum between the two sets of reduction data (Fig. 3C) clearly shows the presence of the 395 nm absorbing species in Tris buffer. These results prove the impossibility to track the formation of 1,2-NADH in a PBS system at pH 7. The difference in absorbance at 395 nm was then used for a calibration curve of 1,2-NADH (Fig. 3D). It is important to mention that the A395 values in Fig. 3C are not identical to those in Fig. 3D because different dilution factors were used. The concentration of 1,2-NADH can be calculated using the absorbance of the sample at 395 nm following eqn (8):

$$C_{1,2-\text{NADH}} (mM) = \frac{A_{395}}{\varepsilon_{1,2-\text{NADH}} \left(\frac{mM}{cm} \cdot cm^{-1}\right) \times L (cm)}$$

The validity of the proposed method was tested in the regeneration of NADH via the hydrogenation of NAD+ (see ESI,† Part VII) using a commercial Pt/C catalyst, as previously reported.11 The concentration of each species was determined using the
The method described before, and the selectivity of each component was calculated using eqn (9):

\[
S_{\text{Compound}_i} (%) = \left( \frac{C_{\text{Compound}_i} \text{ (mM)}}{C_{\text{Reacted NAD}^+} \text{ (mM)}} \right) \times 100 \tag{9}
\]

The reaction profile in the hydrogenation of NAD\(^+\) was first tracked by measuring 340 nm absorbance with time (Fig. 4A). The scanned reaction mixtures (Fig. 4A inset) clearly show wide absorbance spectra, indicating the formation of all isomers. Based on Fig. 4B, the catalyst exhibits its maximum conversion at 5.5 h. The analysis of the reaction mixture using the reductive cycle of the kit after 1 h showed that only 60% of the total 340 nm absorbance amounted to the enzymatically active 1,4-NADH, with the remaining 40% being a contribution from 1,6-NADH. The colour of the sample taken at 1 h was slightly yellowish, but persistent, suggesting the formation of 1,2-NADH as well. This was confirmed by UV-Vis analysis at 395 nm. The amount of reacted NAD\(^+\) was determined using the total cycle of the kit by subtracting the concentration of NAD\(^+\) left from the initial NAD\(^+\). Subtracting the concentration of all isomers (1,2-, 1,4-, and 1,6-NADH) from the reacted NAD\(^+\) resulted in a mole balance closure of 89% with the rest being attributed to NAD\(^+\) left from the initial NAD\(^+\). Notwithstanding, it is noted that the reported analytical method can also be used in other more complicated regeneration systems relying on sacrificial organic electron donors or hole scavengers (e.g. TEOA, see Fig. S3, ESI\(^{1}\)).

In conclusion, we reported a method that uses UV-Vis spectrophotometry alongside biological assays for the determination of the concentrations of NAD\(^+\) and both selective and non-selective compounds in NADH regeneration. The developed method, validated in a Pt/C catalysed regeneration reaction, can be applied to other NADH regeneration systems, providing critical information for mechanistic/pathway analysis and further process optimisation.

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Conflicts of interest
There are no conflicts to declare.

Notes and references