Biotechnological production of reduced and oxidized NAD^+ precursors

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ABSTRACT

Dysregulation of nicotinamide adenine dinucleotide (NAD^+) homeostasis by increased activity of NAD^+ consumers or reduced NAD^+ biosynthesis plays an important role in the onset of prevalent, often age-related, diseases, such as diabetes, neuropathies or nephropathies.

To counteract such dysregulation, NAD^+ replenishment strategies can be used. Among these, administration of vitamin B_3 derivatives (NAD^+ precursors) has garnered attention in recent years. However, the high market price of these compounds and their limited availability, pose important limitations to their use in nutritional or biomedical applications. To overcome these limitations, we have designed an enzymatic method for the synthesis and purification of (1) the oxidized NAD^+ precursors nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR), (2) their reduced forms NMNH and NRH, and (3) their deaminated forms nicotinic acid mononucleotide (NaMN) and nicotinic acid riboside (NaR). Starting from NAD^+ or NADH as substrates, we use a combination of three highly overexpressed soluble recombinant enzymes; (a) a NAD+ pyrophosphatase, (b) an NMN deamidase, and (c) a 5'-nucleotidase, to produce these six precursors. Finally, we validate the activity of the enzymatically produced molecules as NAD^+ enhancers in cell culture.

Keywords: NAD precursor, enzymatic synthesis, NaMN

1. INTRODUCTION

Nicotinamide adenine dinucleotide (NAD^+) and its reduced form (NADH) are essential metabolites for a wide variety of electron exchange-dependent biochemical reactions, in which they act as metabolic cofactors. Apart from this known function, NAD^+ also acts as a substrate for a number of key protein families, including the sirtuins (SIRTs), poly(ADP-ribose)polymerases and ADP-ribose cyclases. Together, these enzymes are involved in most major biological processes in cells; from metabolism, aging or cell death, to DNA repair and gene expression [1].

The cellular pool of NAD^+ is maintained through the dynamic balance between its breakdown by NAD^+-consuming enzymes and its synthesis, which can occur via *de novo* biosynthesis from tryptophan or through its salvage from precursors. The alteration of this fine regulation by increased activity of NAD^+-consuming enzymes or reduced biosynthesis has been observed in several pathological conditions, such as diabetes, liver disease, mitochondrial myopathy and nephropathies, and are a common feature of the natural process of aging [2].

This relationship between decreased NAD^+ levels and disease has attracted the attention to NAD^+ replenishment strategies as potential therapeutic avenues in recent years. In this regard, supplementation with NAD^+ precursors in order to activate NAD^+ salvage pathways and increase NAD^+ levels, has proven particularly effective in ameliorating or preventing disease in a number of animal models of metabolic disorders, neurodegeneration, and aging, and also in humans [2].

The classical NAD^+ precursors nicotinic acid (NA) and nicotinamide (NAM), generically known as niacin (a form of vitamin B_3), have long been used as the curative agents for pellagra, the first human disease associated with a deficiency in NAD^+ precursors due to general malnutrition [3], and which can be effectively prevented or treated through a diet rich in fresh products such as fish, meat, milk and enriched flour [4] . In the last two decades, other vitamin B3 derivatives, such as nicotinamide mononucleotide (NMN), nicotinamide riboside (NR) and, more recently, nicotinic acid mononucleotide (NaMN) and nicotinic acid riboside (NaR), have been identified as upstream metabolites in the NAD^+ salvage pathway [5-9]. Some of these derivatives are also present in

natural food sources. In fact, NR is present in cow milk [10] in concentrations of approximately 0.4 mg per liter, while NMN has been identified in vegetables, fruit, meat and seafood in quantities that range from 0.6 to 18.8 mg per kilo [11].

Although efficient as NAD^+ enhancers, NA and NAM have downsides, since the former induces flushing triggered by NA binding to the GPR109A receptor and NAM can act as an inhibitor of sirtuins, which could limit the desired activation of these enzymes. In addition, elevated NAM levels can lead to increased NAM methylation, which has been associated with the pathogenesis of type 2 diabetes and other diseases [12]. In contrast, NMN and NR lack these side effects and, since they are considered naturally-occurring compounds, they have become the preferred alternatives to NA and NAM in recent years. This has fueled a multimillion-dollar market for NMN and NR, which are sold as dietary supplements, and it is expected to increase exponentially in the coming years as NR, for example, has recently been approved as a novel food by the European Food Safety Authority (EFSA) and, as an ingredient in foods for special medical purposes and total diet replacement for weight control [13]. Finally, NaR, the deaminated form of NR, has been described as a potential neuroprotective molecule [7].

The promising results obtained with these compounds in animal models have turned attention to the potential benefits that their intake may have in humans. Early clinical trials with NMN and NR demonstrated that these compounds are well tolerated and safe [14-24], even at doses of 2g per day for 12 weeks [18]. Moreover, NR administration increases blood NAD^+ levels [14] and enhances the NAD^+ metabolome in muscle [23]. These findings make NMN and NR commercially attractive, as evidenced by their rapid growth in the dietary supplement market. Yet, NMN and NR have some limitations of their own, including maximal NAD^+-enhancing effects of around two-fold, or the rapid degradation in plasma to NAM, at least in the case of NR [25].

With the aim to overcome some of these limitations, new molecules with a more pronounced effect on the NAD^+ intracellular pool are now being studied. We and others have recently reported that the reduced forms of nicotinamide riboside (NRH) and nicotinamide mononucleotide (NMNH)

act as very powerful NAD^+ enhancers in cell lines and in mice [26, 27], via their conversion to NADH, which is subsequently oxidized to NAD^+ by cellular dehydrogenases [28, 29], and that supplementation with these compounds has potential renoprotective effects [26, 27]. Interestingly, benefits derived from NAD^+ enhancement may have an upper limit. In fact, raising NAD^+ levels too high might be deleterious, as NMNH supplementation can suppress the glycolytic rate and the TCA cycle [30], while NRH has been recently shown to induce a pro-inflammatory state in macrophages [31].

To understand the role of reduced precursors in metabolism, further research is needed. However, these molecules are only available through custom chemical synthesis, making them extremely expensive, difficult to obtain, and of variable quality, not free of toxic side products.

The discovery of scalable and efficient synthesis methods for the synthesis of NAD^+ precursors is of great interest from the nutritional and biomedical perspective, and can achieve significant market volumes once a suitable and cost-effective process of manufacturing has been established. This has stimulated us to design a method that relies on the activity of three enzymes; an NAD^+ diphosphatase/pyrophosphatase, an NMN deamidase and a 5'-nucleotidase, to produce a plethora of NAD^+ precursors, both in their oxidized and reduced forms (Figure 1). This method enables the production of six different NAD^+ precursors, namely NMN, NR, NaMN, NaR, NMNH and NRH, using the inexpensive starting substrates NAD^+ and NADH. The major advance of our method is the use of a low-price scalable aqueous C18 resin in a low-pressure FPLC system, capable of handling large volumes. This, together with the high expression and easy purification of the required enzymes, makes this method ideal for large-scale production of these NAD^+ precursors, which could lead to reduced production costs, increased commercial availability and, above all, increased accessibility to these compounds for the treatment of disease.

2. MATERIALS & METHODS

2.1. Overexpression and purification of recombinant enzymes

Escherichia coli Rosetta 2(DE3)pLysS harboring pET24b plasmid containing the gene encoding the NAD^+ diphosphatase from *Rahnella aquatilis* (RaNADD, Uniprot code H2IVT9) or the 5'-nucleotidase from *Maridesulfovibrio hydrothermalis* (MhNuc, Uniprot code L0REK7) was grown at 37 °C in Terrific Broth (TB) supplemented with 50 μg/mL kanamycin and 34 μg/mL chloramphenicol, until the optical density of the culture at 600 nm (OD_{600}) reached ~4.0. Protein overexpression was induced by addition of 0.75 mM isopropyl-β-thiogalactoside (IPTG) in both cases. Cultures were kept at 25 °C during 16 hours on a shaker at 150 rpm. Pellets were then harvested by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl pH 8.0 containing 300 mM NaCl) and disrupted using a Bead Beater homogenizer (BioSpec). After clearance by ultracentrifugation, RaNADD or MhNuc were purified from the supernatant by Ni^{2+}-chelating affinity chromatography in a FPLC system (ÄKTA Prime Plus, GE Lifesciences) using a HiPrep IMAC 16/10 FF 20 mL column (GE Lifesciences). For both enzymes, elution was performed with a linear imidazole gradient from 40 to 250 mM. Fractions containing the protein of interest were desalted in a HiPrep 26/10 desalting column (GE Lifesciences) and stored at -20 °C in 10% glycerol. *Escherichia coli* NMN deamidase (EcCinA) overexpression and purification was performed as previously described [32].

2.2. NMN and NMNH enzymatic synthesis and purification

Oxidized (NMN) and reduced (NMNH) nicotinamide mononucleotide were produced through the cleavage activity of RaNADD using NAD^+ or NADH as substrates, respectively. 5 mM of the corresponding substrate (a total of ~165 mg) were added to 3.38 μM RaNADD in a total volume of 50 mL 50 mM Tris-HCl pH 7.5 containing 0.5 mM $MnCl_2$. Samples were incubated at 37 °C in a water bath for one or 16 hours for NADH or NAD^+ cleavage, respectively. After this time, full conversion to NMN(H) and AMP was analyzed by HPLC.

NMN and AMP were resolved through ion exchange chromatography in a FPLC system (ÄKTA Prime Plus, GE Lifesciences) using a Dowex 1X8 100-200 mesh resin (Alfa Aesar) packed in a XK 26/20 holder (GE Lifesciences) previously activated with three column volumes (CV) of 3M NaOH and equilibrated with 3CV of 3M formic acid and water, until a pH of 3.8 was reached. After sample injection, NMN was collected using isocratic chromatography with water until no absorbance at 260 nm was detected in the fractions. Residual AMP bound to the column was eluted by flushing with 3CV of 0.9M formic acid. Finally, the column was re-equilibrated with water to pH 3.8. All fractions containing pure NMN were concentrated in a rotary evaporator at 30 °C to a volume of approximately 20 mL, frozen in liquid nitrogen and freeze dried for long-term storage at -20 °C.

NMNH and AMP were separated through ion pairing chromatography in a Redisep Rf Gold® C18Aq preparative column (Teledyne Isco) packed with 100g of resin and coupled to a FPLC system (ÄKTA Prime Plus, GE Lifesciences). After pre-activation with 6CV of pure methanol, the column was equilibrated with 6CV of 10 mM triethylamine acetate (TEAA) pH 7.8 buffer. After sample injection, NMNH was eluted by isocratic chromatography with TEAA buffer until no absorbance at 340 nm was detected in the fractions. Residual AMP bound to the column was eluted by flushing 3CV of pure methanol, followed by re-equilibration with 3CV of 10 mM TEAA buffer pH 7.8. Fractions containing NMNH were brought to an alkaline pH of 8.0-9.0 by addition 45 µL of 0.5 M NaOH per mL, and concentrated in a rotary evaporator at 40 °C until complete dryness. The precipitate obtained was then resuspended in 5-10 mL water, filtered through a 0.45 µm pore size polytetrafluoroethylene (PTFE) membrane (Merck-Millipore) and desalted through a XK 26 (GE Lifesciences) packed with approximately 350 mL of Sephadex LH-20 (GE Lifesciences) previously equilibrated with 2CV of water. The desalted fractions containing salt-free NMNH were directly frozen in liquid nitrogen and freeze dried for long-term storage at -20 °C.

2.3. NaMN preparation

Nicotinic acid mononucleotide (NaMN) was directly synthesized from enzymatically produced NMN by deamination of the nicotinamide ring using *Escherichia coli* NMN deamidase (EcCinA), as we have previously described [32]. Briefly, 5 mM NMN (a total of 84 mg) was added to 14 µM EcCinA and incubated at 37 °C in a water bath during 16 hours in a total volume of 50 mL 50 mM phosphate buffer pH 7.4. After confirmation of reaction completion by HPLC, reactions were filtered through 10 kDa cut-off cellulose centrifugal filters (Amicon, Merck-Millipore). The permeate containing pure NaMN was then snap frozen in liquid nitrogen and freeze dried for long-term storage at -20 °C.

2.4. NR, NRH and NaR preparation

Oxidized (NR) and reduced (NRH) nicotinamide riboside, and nicotinic acid riboside (NaR) were prepared from their mononucleotide forms NMN, NMNH and NaMN, respectively, by enzymatic dephosphorylation using *Maridesulfovibrio hydrothermalis* 5'-nucleotidase (MhNuc). 5 mM NMN, NMNH or NaMN (a total of 84-86 mg) were added to 2.89 µM, 0.16 µM, or 25.36 µM MhNuc, respectively, in 50 mL 50 mM Tris-HCl pH 7.5 containing 5 mM $MgCl_2$. Samples were incubated for 16 hours at 37 °C in a water bath. After this time, full conversion to the corresponding products was analyzed by HPLC. Endpoint reactions were filtered through 10 kDa cut-off cellulose centrifugal filters (Amicon, Merck-Millipore) and the permeates containing the pure compounds frozen in liquid nitrogen and freeze dried for long-term storage at -20 °C.

2.5. High Performance Liquid Chromatography (HPLC) analyses

Preliminary identification of the different NAD^+ precursors was performed by HPLC (Thermo-Fisher UltiMate 3000) using a reverse-phase C18 250 x 4.6 mm column (Supelcosil LC-18-DB). The method consisted in a solvent A (20 mM ammonium acetate pH 6.9) running at 1 mL/min for 10 minutes, followed by a gradient from 0 to 10% solvent B (methanol) in 10 minutes. All

compounds were eluted between 3 and 15 minutes. Before each injection, the column was washed with 100% solvent B for 10 minutes and re-equilibrated with 100% solvent A for an additional period of 10 minutes. Under these conditions, NMN and NMNH eluted after 4.5 and 4.2 minutes, respectively. NR and NRH showed retention times of 9 and 13.2 minutes, while NaMN and NaR eluted after 3.2 and 4.7 minutes, respectively. NAD^+ and NADH had very similar retention times of 14.7 and 14.6 minutes. Finally, AMP eluted after 6 minutes.

2.6. Degradation of the produced NAD^+ precursors in culture medium

NMN(H), NR(H), NaMN or NaR were added to Dulbecco's modified Eagle's medium (DMEM) at a concentration of 500 µM in the presence or absence of 10% fetal bovine serum (FBS, BioWhittaker) and incubated at 37 °C during 16 hours. Aliquots were taken at times 0 (control) and 16 hours and analyzed by HPLC (Agilent 1200 series) using a reverse-phase C18 250 x 4.6 mm column (Phenomenex Gemini NX) running under the method described in section 2.5. Under these conditions, NMN and NMNH eluted at 2.6 and 3.6 minutes, respectively. NR and NRH showed retention times of 4.3 and 14.6 minutes, while NaMN and NaR eluted after 2.2 and 3.8 minutes, respectively. NAM and NA had retention times of 13.5 and 4 minutes.

2.7. Mass spectrometry identification of the NAD^+ precursors

MS/MS identification of the different NAD^+ metabolites was performed using a Waters Acquity Ultra-High Performance Liquid Chromatography (UHPLC) system coupled to a Bruker Impact II™ Ultra-High Resolution Qq-Time-Of-Flight mass spectrometer. Compounds were dissolved in methanol:water 3:2 (v/v) at a final concentration of 100 µM. Samples were kept at 12 °C during the whole analysis and 5 µL of each sample was injected onto a Merck Millipore SeQuant ZIC-cHILIC column (PEEK 100 x 2.1 mm, 3 µm particle size). Column temperature was held at 30 °C. Mobile phase consisted of (A) 1:9 (v/v) acetonitrile:water and (B) 9:1 (v/v) acetonitrile:water, both containing 5 mmol/L ammonium acetate. Using a flow rate of 0.25 mL/min, the LC gradient

consisted of: 100% B for 0-2 min, reach 54% B at 13.5 min, reach 0% B at 13.51 min, 0% B for 13.51-19 min, reach 100% B at 19.01 min, 100% B for 19.01-19.5 min. Column equilibration was achieved using a 0.4 mL/min flow at 100% B for 19.5-21 min. Fragmentation spectra were acquired using Multiple Reaction Monitoring (MRM) in positive ionization with 35 eV collision energy for all compounds except for NMNH, which was fragmented using 15 eV. Selected m/z values (±0.5) for MRM were: NMN (M+): 335.0639, NMNH (M+H): 337.0795, NaMN (M+): 336.0479, NR (M+): 255.0975, NRH (M+H): 257.1132, NaR (M+): 256.0816. MS/MS data were analyzed using Bruker Compass DataAnalysis Version 5.2 (Build 211.232.5075) (64 bit).

2.8. Enzyme kinetics determination

RaNADD pyrophosphatase activity towards NAD^+ and NADH was analyzed at 37 °C using different substrate concentrations in 50 mM Tris-HCl pH 7.5, 5 mM $MnCl_2$, containing 0.17 µM or 0.03 µM RaNADD, respectively. For MhNuc nucleotidase activity assays, different concentrations of NMN, NMNH or NaMN were incubated at 37 °C in 50 mM Tris-HCl pH 7.5, 0.5 mM $MgCl_2$, containing 2.89 µM, 0.16 µM or 25.36 µM MhNuc, respectively.

In all cases, aliquots were taken at different time points and reactions stopped by ultrafiltration at 4 °C through 10 kDa cut-off cellulose centrifugal filters (Amicon, Merck-Millipore). RaNADD activity was determined by HPLC following NMN or NMNH production. MhNuc activity was calculated following NR, NRH or NaR production.

2.9. Cell culture and supplementation with NAD^+ precursors

AML12, HepG2, HeLa cells and human skin fibroblasts were cultured at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS, BioWhittaker), 100 U/mL of penicillin, and 10 mg/mL of streptomycin (Life Sciences). Supplementation with the different enzymatically-obtained NAD^+ precursors was made at the concentrations and times indicated in fresh medium.

2.10. NAD^+ content determination

NAD^+ extraction was performed in 12-well plates by addition of 400 µL 2M $HClO_4$ to the PBS-washed cells. Each well was thoroughly scrapped and volume transferred to 1.5 mL tubes. After centrifugation at 16.000*g* for 5 minutes, 100 µL of the acidic supernatant was neutralized with 150 µL 2 M/0.6 M KOH/MOPS and centrifuged again to remove precipitated salts. NAD^+ content was determined using an enzymatic spectrophotometric cycling assay based on the coupled reaction of malate and alcohol dehydrogenases, as previously described [33].

3. RESULTS

3.1. Enzymatic production and chromatographic purification of NMN and NMNH

The first step of the process involves NAD^+ and NADH cleavage by an NAD^+ diphosphatase. We searched for known diphosphatase DNA sequences in commonly found biosafety 1-level bacteria, such as those found in natural sources. BLAST sequence analysis of the NAD^+ diphosphatase from *Escherichia coli* (Uniprot code: P32664) revealed Uniprot entry H2IVT9 as a putative diphosphatase from the mesophile microorganism *Rahnella aquatilis* CIP 78.65 (ATCC 33071), isolated from a drinking water source and with putative activity towards NAD^+ and NADH. The DNA sequence encoding for this enzyme was cloned into pET24b and overexpressed in *E. coli* Rosetta 2 cells. The recombinant N-terminal His_6-tagged protein (RaNADD) was purified in a single Ni^{2+}-chelating chromatographic step (Supplementary Figure 1), obtaining a yield of 40 mg of pure enzyme per liter of bacterial culture.

We next set out to determine the kinetic properties of RaNADD towards both NAD^+ and NADH under neutral pH (50 mM Tris-HCl pH 7.5 containing 0.5 mM $MnCl_2$) and mild temperature (37 °C) conditions. Under these conditions, RaNADD showed a Michaelis-Menten constant of 0.51 mM (Figure 2A) and a k_{cat}/K_M of 5.58 $mM^{-1} \cdot s^{-1}$ towards NAD^+, while it was 40-fold more active towards NADH, with a K_M of 0.14 mM (Figure 2B) and a k_{cat}/K_M of 224.38 $mM^{-1} \cdot s^{-1}$ (Table 1).

Once the activity of RaNADD towards NAD^+ and NADH was confirmed, we aimed to study its biotechnological application for NMN and NMNH synthesis. To do this, 5 mM NAD^+ or NADH were added to 3.38 µM RaNADD in the above described standard reaction medium. NADH-containing reactions were incubated during one hour, while NAD^+ incubation times were prolonged up to 16 hours due to the lower activity of the enzyme over this substrate. Reactions were analyzed by HPLC to confirm NAD(H) full conversion into NMN(H) and AMP, being the latter compound the byproduct of the reaction in both cases.

Separation of NMN and AMP from the RaNADD-NAD^+ reaction mix was achieved in a single ion-exchange chromatographic step using a Dowex 1X8 resin (Figure 2C). Under these conditions, NMN was eluted in water, while AMP retained in the column. Fractions containing pure NMN were concentrated by evaporation, freeze dried and stored at -20 °C. NMN purity was analyzed by HPLC at 260 and 340 nm (Figure 2C), and its exact mass and fragmentation pattern analyzed by mass spectrometry (Figure 2D, Supplementary Table 1), confirming the expected mass of m/z = 335.063, and the presence of the nicotinamide ring at m/z = 123.054. Using this small-scale setup, we were able to obtain 67 mg of pure NMN from 166 mg of NAD^+, which corresponds to a yield of ~80%.

Due to the net charge similarity between NMNH and AMP, the ion-exchange Dowex 1X8 resin used for NMN purification was unable to resolve these two molecules. Only HPLC-based reverse phase C18 chromatography has been able to resolve these compounds, opening the possibility to use expensive preparative HPLC systems to produce NMNH. However, after a considerable effort scouting different mobile phases and resins that could be used in a low-pressure chromatography system (FPLC), we found that the separation of NMNH from AMP could be carried out using a new volatile-phase ion pairing chromatography based on the use of 10 mM triethylamine acetate (TEAA) and a water-compatible low-pressure preparative C18Aq column. This volatile mobile phase avoids any salt contamination in the downstream processing of the samples. With this setup, NMNH was collected in the flow through, while AMP remained bound

to the column. To increase NMNH stability, fractions containing pure NMNH were brought to pH 8.0 with NaOH, prior to concentration under vacuum at 40 ºC. After complete evaporation, the precipitate was resuspended in water, filtered and desalted by molecular exclusion chromatography in a Sephadex LH-20 column. After this step, fractions containing pure and salt-free NMNH were freeze-dried, and the resulting yellow powder stored at -20°C. NMNH purity and identification analyses were performed by HPLC and UV spectrometry, showing its characteristic absorbance at 340 nm (Figure 2E), in contrast to NMN. Mass spectrometry analysis also confirmed its expected mass of m/z = 337.079 (Figure 2F) and the presence of the dihydronicotinamide ring at m/z = 125.069 (Figure 2F, Supplementary Table 1). Using this approach, the yield was ~65%, which corresponds to 22 mg pure NMNH from 71 mg of NADH used in the reactions.

Collectively, our results provide an efficient, simple, inexpensive and low-cost scalable method to produce the phosphorylated NAD^+ precursors NMN and NMNH.

3.2. Enzymatic conversion of NMN into NaMN

The use of the RaNADD diphosphatase towards NAD^+ allowed us to produce pure and cheap NMN from NAD^+. We have previously reported that the NMN deamidase from *E. coli* (EcCinA) is highly active towards NMN [32]. Since we had this enzyme readily available in our laboratory, we leveraged its deaminase activity to produce nicotinic acid mononucleotide (NaMN) from NMN. After reaction completion, the enzyme was easily removed by using a 10 kDa cut-off centrifugal filter and the pure product obtained was freeze-dried and stored at -20 ºC.

Complete conversion of NMN into NaMN was confirmed by HPLC (Supplementary Figure 2A) and its identity verified by mass spectrometry (Supplementary Figure 2B), confirming the expected mass of m/z = 336.047 and the presence of the nicotinic acid ring at m/z = 124.039. Since NMN is fully converted to NaMN through the reaction carried out by EcCinA, and considering the minimum volume that is retained in the membrane of the filter, the yield obtained

for this conversion was of approximately 95-98%. Surprisingly, there was no activity of EcCinA towards NMNH, even when a different NMN deamidase, such as that from *Agrobacterium tumefaciens* (AtCinA), also available in our laboratory [34], was used.

3.3. Enzymatic production of riboside NAD^+ precursors

Once we had established a reliable method for the production of the phosphorylated NAD^+ precursors NMN, NMNH and NaMN, we explored the possibility of using them as enzymatic substrates to produce their ribosides. To do this, we searched for bacterial sequences of enzymes able to dephosphorylate 5'-nucleotides, such as NMN(H) and NaMN, and which could be expressed as stable soluble proteins in a recombinant system. We found that UniProt entry L0REK7 belonged to a 5'-nucleotidase from the extremophile sulfate-reducing bacterium *Maridesulfovibrio hydrothermalis* AM13 (MhNuc), with putative activity towards ribonucleosides 5'-phosphate. The gene encoding this protein was cloned into pET24b, overexpressed in *E. coli* Rosetta 2, and the enzyme was purified in a single Ni^{2+}-chelating chromatographic step, in a similar fashion to the purification of RaNADD. The yield obtained for MhNuc was of 55 mg of pure soluble protein per liter of bacterial culture (Supplementary Figure 3).

Next, we analyzed the putative phosphatase activity of MhNuc towards NMN, NaMN and NMNH, in the same reaction conditions used for RaNADD (50 mM Tris-HCl containing 5 mM $MgCl_2$; 37 °C). NMNH was by far the preferred substrate for MhNuc (K_M = 0.26 mM), followed by NMN (K_M = 0.99 mM) and NaMN (K_M = 2.89 mM), with 4 and 11-fold lower activities, respectively (Figures 3A-C and Table 1). MhNuc catalytic efficiency was also higher for NMNH (k_{cat}/K_M = 2.71 $mM^{-1} \cdot s^{-1}$) than for either of the other two precursors. In fact, the catalytic efficiencies for NMN (k_{cat}/K_M = 6.88 x 10^{-2} $mM^{-1} \cdot s^{-1}$) and for NaMN (k_{cat}/K_M = 7.74 x 10^{-4} $mM^{-1} \cdot s^{-1}$) were 39 and 3509-fold lower than that for NMNH, respectively (Table 1).

Once we had proven MhNuC able to dephosphorylate NMN, NaMN and NMNH, we studied its potential biotechnological application for NR, NaR and NRH production. Following a similar

approach to that used for NMN(H) synthesis, 5 mM NMN, NaMN or NMNH were incubated at 37 ºC during 16 hours with 2.89 µM, 25.36 µM or 0.16 µM MhNuc, respectively, in 50 mM Tris-HCl pH 7.5 containing 5 mM $MgCl_2$. Full conversion of the phosphorylated precursors to their dephosphorylated forms was analyzed by HPLC and mass spectrometry for NR (Figure 3D-E), NaR (Figure 3F-G) and NRH (Figure 3H-I). Analysis of the fragmentation patterns and total mass confirmed the identity of the three dephosphorylated compounds (Supplementary table 2).

After conversion, all reactions were filtered through 10 kDa cut-off column filters, freeze dried and stored at -20°C. Since the starting material consisted of the pure substrates and MhNuc was able to fully dephosphorylate NMN(H) and NaMN, we achieved a yield of 95-98%, considering the minimum volume that is retained in the filter membranes.

The above results demonstrate that MhNuc has the potential to be used as an inexpensive biocatalyst for the production of the dephosphorylated NAD^+ precursors NR, NaR and NRH using their phosphorylated forms as starting substrates.

3.4. *In vitro* validation of the enzymatically-produced NAD^+-precursors

Once we had confirmed the identity of the different NAD^+ precursors by chromatography and mass spectrometry, we aimed to validate their activity as NAD^+ enhancers in cell cultures. To do so, we supplemented AML12 hepatocytes with vehicle (PBS) or 500 µM of each of the enzymatic NAD^+ precursors for 16 hours, and measured NAD^+ levels (Figure 4A). The reduced forms of NMN (NMNH) and NR (NRH) had the strongest effects on NAD^+ levels in these cells, showing a 6.6 and 5.8-fold increase, respectively, while their oxidized counterparts led to an increase of 2.8-fold in both cases. Interestingly, both deaminated NAD^+ precursors, NaMN and NaR, served as extracellular NAD^+ enhancers in this cell line, achieving NAD^+ increases above 2-fold in both cases (Figure 4A). These results are in agreement with previous reports describing that reduced precursors are incorporated into the NAD^+ pool via their conversion to NADH, which is subsequently oxidized to NAD^+ by cellular dehydrogenases [26-29].

Extracellular NAD^+ intermediates have been reported to be degraded when added to cell culture medium [8]. To determine whether degradation products may contribute to the observed increases in NAD^+ levels in cell culture, we incubated the enzymatically-produced intermediates in DMEM (without NAM) containing 10% water (control) or FBS during 16 hours at 37 °C and checked for degradation via HPLC (Supplementary Figure 4). Our results confirm that NAD^+ precursors are degraded to their corresponding nucleosides or bases, either by the enzymatic activities present in FBS or by spontaneous hydrolysis in aqueous solution (Supplementary Figure 4), in line with previous results [8].To the best of our knowledge, the effect of NaMN supplementation over cellular NAD^+ levels has never been studied, even though its activity as an extracellular NAD^+ precursor has already been demonstrated [8, 9] . To determine the effect of this precursor in cellular NAD^+ content, we performed a dose-response experiment in AML12 (Figure 4B). NaMN was able to significantly increase NAD^+ levels already at a concentration of 25 μM, steadily enhancing NAD^+ content as NaMN concentration was increased, up to 500 μM, when a plateau was reached. Finally, the effect of NaMN on cellular NAD^+ levels was also tested in other cell lines. At a concentration of 500 μM, NaMN increased NAD^+ levels 2.3-fold in HeLa cells, 1.55-fold in skin fibroblasts and 1.8-fold in HepG2 hepatocytes (Figure 4C), further confirming that NaMN can act as an extracellular NAD^+ enhancer.

Overall, our results in cells confirm the identity of the different enzymatic NAD^+ precursors and demonstrate their NAD^+-enhancing activity when administered to cell cultures.

4. DISCUSSION

Supplementation with NAD^+ precursors is an attractive intervention to target NAD^+ metabolism in the fight against metabolic disease, as demonstrated by the growing number of clinical trials carried out with NMN [19, 22] and, especially, with NR [14-18, 20, 21, 23, 24].

399 However, while the oxidized NAD^+ precursors have shown promising effects in many preclinical
400 models of disease [5], in humans these effects are only mild. In fact, oral administration with NR
401 has not shown strong metabolic benefits in humans [18, 20, 21], and while NMN administration
402 led to improved insulin sensitivity in the muscle of prediabetic women, it did not affect insulin
403 sensitivity in the liver, body composition, plasma glucose, or lipid content [22].
404 To overcome the limitations of the oxidized precursors, the classical repertoire of NAD^+-enhancing
405 molecules has been recently expanded with the incorporation of the reduced forms of NR and
406 NMN, termed NRH and NMNH [26, 27]. These reduced precursors have shown an outstanding
407 potential to increase NAD^+ levels *in vitro* and in mice, as well as potential therapeutic effects for
408 renal disease [26, 27]. Therefore, these reduced precursors could represent a good alternative to
409 the oxidized ones, and may allow stronger modulations of NAD^+ metabolism in humans.
410 Interestingly, the deaminated forms of NMN and NR, called NaMN and NaR, have also recently
411 garnered attention in the context of neuroprotection. In fact, NMN accumulation, a common
412 feature observed *in vitro* after supplementation with NMN or NR, triggers axonal death via
413 activation of the Sterile alpha and Toll/interleukin-1 receptor motif-containing 1 (SARM1) [35, 36].
414 NaR is incorporated into the NAD^+ pool through a pathway that involves its phosphorylation to
415 NaMN by NR kinase. NaMN is then adenylated to NaAD by the NMN adenylyltransferases and
416 NaAD is finally amidated to NAD^+ by the NAD^+ synthase[37]. Since this pathway bypasses NMN
417 synthesis and accumulation it is proposed that the deaminated forms of NAD^+ precursors might
418 be a safer approach to target NAD^+ metabolism in the brain [7].
419 In light of the high interest that these compounds attract, new methods for their obtention and
420 purification from cheaper substrates are highly relevant. Here we describe an enzyme-based
421 method for the synthesis of four oxidized (NMN, NR, NaMN, NaR) and two reduced (NMNH, NRH)
422 NAD^+ precursors from the starting substrates NAD^+ or NADH, respectively. These compounds
423 are easily purified via ion exchange or ion pairing chromatography in an affordable FPLC system

able to handle larger volumes and faster flows than a conventional HPLC system, and which is cheaper than a preparative HPLC.

Decades ago, NMN was synthesized via NAD^+ cleavage by the pyrophosphatase activity present in the extract obtained from potatoes [38-41], or from snake venom [40], and was further used for NR synthesis by the phosphomonoesterase activities of human seminal plasma or rat ventral prostate glands [40]. Alternatively, NMN was synthesized from nicotinamide (NAM) and the expensive phosphoribosyl pyrophosphate (PRPP); or from NAM, ribose-5-phosphate and ATP, through the NAM phosphoribosyl transferase (NAMPT) activity obtained from erythrocyte acetone powder or hemolysates [42]. Back then, NMNH and NRH were also prepared through pyrophosphatase-mediated cleavage of NADH, followed by dephosphorylation, although the purification of the compounds was not performed, with the consequent equimolecular contamination of NMNH and NRH with the reaction by-product, AMP [40]. In general, these protocols had important limitations, as they required laborious extraction protocols to obtain the enzymes [38-40], and were prone to contamination due to the use of crude extracts. Moreover, these methods had very poor yields and were quite variable, as they depended, for example, on the age and variety of the potatoes used [38]. Additionally, the identification, purity and cellular activity of the compounds obtained were not described in any of these studies.

Our method lacks these limitations, as it is based on the use of recombinant purified enzymes that can be produced in large scale, rendering a cleaner synthesis process. Moreover, the use of only NAD^+ or NADH as starting substrates avoids the need of costly substrates such as PRPP, ribose-5-phosphate or ATP to synthesize the compounds.

In the last few years, new methods have focused on the biotechnological synthesis of NMN. Qiang *et al.* [43] developed a biocatalytic process for the phosphorylation of chemically synthesized NR to NMN using the purified NR kinase from *Kluyveromyces marxianus.* In the presence of an external ATP regeneration system, they were able to convert up to 100 g per liter of NR into NMN.

Other methods rely on the use of engineered strains of *E. coli* able to use lactose or glucose as the carbon source for the synthesis of PRPP, via the pentose phosphate pathway. NAM is also added to the culture which, via recombinant NAM phosphoryl transferase (Nampt), is condensed with PRPP to form NMN [44-46]. Through this approach, Marinescu *et al*. [44] were able to obtain up to 15.42 mg of intracellular NMN per liter of bacterial culture, starting from 1 g NAM and 10 g lactose. Once NMN has been synthesized, bacteria are disrupted with an ultrasound generator and NMN is purified by size-exclusion chromatography [47].

A few years later, Shoji *et al.* [45] refined the method by overexpressing seven endogenous genes of the pentose phosphate pathway in *E. coli*, as well as the *Nampt* gene from *Chitinophaga pinensis*. This was coupled to the expression of a niacin transporter (NiaP), able to incorporate NAM into the bacterial intracellular space, as well as the expression of a nicotinamide mononucleotide transporter (PnuC), which allowed NMN to go to the extracellular medium once synthesized. In this protocol, bacteria are grown in LB, collected via centrifugation and resuspended in M9 minimum medium. This suspension is then used to produce NMN by addition of D-glucose and NAM. Through this system, Shoji and colleagues were able to produce 6.79 g of extracellular NMN per liter of culture.

Xylose has also been used as the carbon source for PRPP synthesis in NMN production. In fact, Ngivprom and collaborators [46] constructed a metabolically engineered *E. coli* strain with the knockout of two transketolase enzymes of the pentose phosphate pathway and the glucose-specific IICB component, allowing the conversion of xylose into D-ribose. To synthesize NMN, the supernatant containing D-ribose is directly used in a biocascade reaction using purified ribokinase, PRPP synthase and NAM phosphoribosyl transferase. ATP is constantly regenerated by the activity of a polyphosphate kinase, while pyrophosphate, an inhibitor of Nampt, is broken down by the activity of a pyrophosphatase.

These works have expanded the array of methods for NMN production at scale, making the process more efficient than classical protocols that used crude extracts. However, there are still

some limitations that need to be considered. For example, the use of NR as the starting substrate for NMN production [43] would make the process too expensive, as NR is a compound with a very similar market price. This can be partially solved by using engineered strains of *E. coli* that can convert cheaper substrates into PRPP and, ultimately, NMN, although there are pitfalls that will need to be overcome in future studies. In fact, in the protocol described by Marinescu *et al*. [44], the synthesized NMN remained inside the bacterial cells, requiring the disruption of the bacterial membrane and the purification of the compound from the crude extracts. This purification entailed an expensive poly(2-hydroxyethylaspartamide) derivatized silica preparative column coupled to an HPLC system [47], which does not allow large-scale purification of the compound. Alternatively, Ngivprom *et al.* [46] use the D-ribose produced from xylose, as a substrate for NMN production in a biocascade. This method does not require cell disruption, since D-ribose is converted into NMN with purified enzymes, but it does require the use of five different enzymes and the addition of ATP and polyphosphate to the reaction medium, making the process less cost-efficient and increasing the number of contaminants accompanying the synthesized NMN.

Shoji and collaborators [45] solved this issue by overexpressing an NMN transporter (PnuC), which allows NMN direct excretion to the extracellular medium, avoiding the need of cell disruption or the use of several enzymes and substrates. However, although they described a highly efficient bacteria-based method for NMN production, their engineered *E. coli* strain was not able to convert all the NAM fed to the culture into NMN, which remains in the extracellular medium in large amounts, even after several hours of biocatalysis, and thus hindering subsequent purification.

Although these novel methods can help reducing the price of NMN and make it more accessible, they do not allow for the production of the reduced precursors NMNH and NRH, or the deaminated forms NaMN and NaR. In fact, these compounds are only available through expensive custom synthesis processes, making new synthesis methods for their scalable production of outstanding relevance. This has stimulated us to design a new biotechnological process that would allow for the synthesis of not only NMN and NR, which are already available in the market, but also of other

NAD^+ precursors whose availability is very limited and at very high cost. We, therefore, propose the use of three different enzymes, an NAD^+ pyrophosphatase, an NMN deamidase and a 5'-nucleotidase which, together, allowed us to synthesize six different NAD^+ precursors from readily available and cost-effective substrates such as NAD^+ or NADH. We also designed a rapid and straightforward purification protocol for these enzymes, which are overexpressed as soluble proteins in large quantities. The use of highly pure enzymes avoids potential contaminations and the numerous purification steps required to obtain the final product when crude extracts, such as potato or bacterial homogenates, are used.

The cleavage of NAD^+ or NADH by RaNADD activity resulted in an equimolar solution of NMN or NMNH, and of the reaction by-product AMP. The removal of this by-product was accomplished through two low-pressure chromatographic separation methods (ionic or ion pairing), avoiding the use of expensive preparative HPLC systems and columns, being especially novel and economical the use of a C18Aq column with a volatile ion pairing mobile phase that simplifies the purification of NMNH. Subsequently, we used the purified phosphorylated compounds (NMN or NMNH) to synthesize their riboside forms (NR or NRH) via dephosphorylation with MhNuc.

Through the activity of an NMN deamidase (EcCinA), NaMN could also be obtained. Moreover, by taking advantage of the dephosphorylating activity of MhNuc, NaMN could be directly converted into NaR. Interestingly, MhNuc was particularly active towards NMNH, but its dephosphorylating activity was lower when NMN and, especially NaMN, were used as substrates. In fact, complete conversion of NaMN to NaR could only be achieved by adding a significant amount of enzyme to the reaction, indicating that, for this particular substrate, it might be desirable to search for new, more specific phosphatases.

It may be noted that this method does not necessarily require NAD^+ or NADH as starting substrates. Other advanced intermediates, such as NMN or NMNH, could be used to perform the deamination or dephosphorylation reactions with the corresponding enzymes, although the cost of NMN is currently higher than that of NAD^+, while NMNH is not commercially available.

Finally, to validate the *in vitro* activity of each of the enzymatically-synthesized compounds, and to further confirm their identity, we also tested their NAD^+-enhancing potential in AML12 hepatocytes. Our results showed that, as expected, the reduced forms NMNH and NRH were more potent than the oxidized forms, both amidated and deaminated, although supplementation with all of these precursors led to an increase in NAD^+ content in these cells. Moreover, our degradation tests confirmed that, according to previous results [8], NAD^+ precursors can be degraded to their corresponding nucleosides or bases in the extracellular medium either by the enzymatic activities present in FBS or, in some cases, also by spontaneous hydrolysis in aqueous solution.

Importantly, we have corroborated the ability of NaMN to act as an extracellular NAD^+ precursor, in line with previous reports [8, 9], demonstrating that it can be incorporated into the cellular NAD^+ pool in a dose-dependent manner, and in different cell types, even when added to the culture medium.

In conclusion, thanks to the efficient expression and purification of three different bacterial enzymes, we were able to design a cost-effective, straightforward and rapid method for the purification of six different NAD^+ precursors using either NAD^+ or NADH, which are much less expensive. The low price and high re-usability of the chromatographic resins, together with their easy scalability to larger volumes, make this method suitable for large-scale production of these NAD^+-enhancing molecules, which may contribute to (1) reduce the price of those currently available in the market, making them more accessible for their use as nutritional supplements, and especially (2) to increase the availability of the reduced and deaminated forms of NAD^+ precursors. This would make these molecules more accessible for their use as nutritional supplements or in biomedical applications.

AUTHOR CONTRIBUTIONS

RZP designed the experiments, analyzed data, and wrote the original draft of the manuscript. BVS and MvW performed the experiments regarding mass spectrometry and analyzed the data. AMLS, AGGS and RRR performed the cell-based experiments, analyzed the data and edited the manuscript. ASF and RHH designed experiments, analyzed data, edited the manuscript and provided funding. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Overview of the enzymatic production of oxidized and reduced NAD^+ precursors from NAD^+ and NADH. (A) NAD^+ is cleaved via NAD^+ pyrophosphatase from *Rahnella aquatilis* (RaNADD) to obtain NMN, which is chromatographically separated from AMP in a DOWEX 1X8 100-200 mesh column and used as the substrate for NR synthesis via dephosphorylation with the 5'-nucleotidase from *Maridesulfovibrio hydrothermalis* (MhNuc), or to produce NaMN through the activity of the NMN deamidase from *Escherichia coli* (EcCinA). NaMN is further used as a substrate for MhNuc to produce NaR. **(B)** NADH is cleaved via RaNADD pyrophosphatase activity to produce NMNH, which is separated from AMP through a preparative C18Aq column and used as a substrate for MhNuc to synthesize NRH.

Figure 2. Kinetic properties of *Rahnella aquatilis* NAD^+ diphosphatase (RaNADD) and enzymatic production of NMN and NMNH. (A-B) Steady-state saturation curves of RaNADD towards NAD^+ and NADH. Reactions were carried out at 37 °C in 50 mM Tris-HCl pH 7.5 containing 0.5 mM $MnCl_2$ and 1.68 µM RaNADD. NMN(H) production was analyzed by HPLC at different time points. Data were fit to the Michaelis-Menten equation using GraphPad Prism. Each data point corresponds to n=3 independent experiments. **(C)** HPLC chromatograms of RaNADD reaction towards NAD^+ before (I) and after (II) 16-hour incubation. NMN and AMP were resolved by ion-exchange chromatography in a Dowex 1X8 resin (III). **(D)** MS-MS analysis of the enzymatically produced NMN confirming the presence of the nicotinamide ring (c: 123.054, colored in green) and the expected mass of m/z= 335.063. **(E)** HPLC chromatograms of RaNADD reaction towards NADH before (I) and after (II) incubation, showing its characteristic absorbance at 340 nm. NMNH and AMP were chromatographically resolved in an aqueous-compatible C18Aq column (III). **(F)** MS-MS analysis of enzymatic NMNH confirming the presence of the dihydronicotinamide ring (f: 125.069, colored in red) and the expected mass of m/z = 337.079.

The letters above each peak correspond to the structures represented in Supplementary Table 1 (NMN and NMNH fragmentation analysis).

Figure 3. Kinetic properties of *Maridesulfovibrio hydrothermalis* 5'-nucleotidase (MhNuc) and enzymatic production of NR, NaMN and NRH. **(A-C)** Steady-state saturation curves of MhNuc towards NMN, NaMN and NMNH. Reactions were carried out at 37 °C in 50 mM Tris-HCl pH 7.5 containing 5 mM $MgCl_2$ and 2.89 µM, 25.36 µM or 0.16 µM MhNuc for NMN, NaMN or NMNH dephosphorylation, respectively. Data were fit to the Michaelis-Menten equation using Graphpad Prism. Each data point corresponds to n=3 independent experiments **(D)** HPLC chromatograms of MhNuc reaction towards NMN before (I) and after (II) incubation. **(E)** MS-MS analysis of enzymatic NR confirming the expected mass of m/z = 255.098. **(F)** HPLC chromatograms of MhNuc activity towards NaMN before (I) and after (II) incubation. **(G)** MS-MS analysis of enzymatic NaR confirming the expected mass of m/z = 256.082. **(H)** HPLC chromatograms of MhNuc activity towards NMNH before (I) and after (II) incubation, showing the characteristic absorbance of the compounds at 340 nm. **(I)** MS-MS analysis of enzymatically produced NRH confirming the expected mass of m/z = 257.113.

The letters above each peak correspond to the structures represented in Supplementary Table 2 (NR, NaR and NRH fragmentation analysis).

Figure 4. *In vitro* validation of the NAD^+-enhancing activity of the enzymatic NAD^+ precursors and identification of NaMN as a new NAD^+ enhancer. (A) AML12 cells were supplemented with vehicle (PBS) or 500 µM of the different NAD^+ precursors for 16 hours and collected in 2 M $HClO_4$ for NAD^+ determination. (B) AML12 cells were supplemented with increasing concentrations of NaMN for 16 hours and collected in 2M $HClO_4$ for NAD^+ measurements. (C) HeLa cells, skin fibroblasts or HepG2 hepatocytes were supplemented with vehicle (PBS) or 500 µM NaMN for 16 hours and collected in 2 M $HClO_4$ for NAD^+ determination.

Values are expressed as mean ± SEM of n=4 independent experiments. * indicates a statistical difference of $p < 0.05$ of treatment vs control.

737 **Table 1. Kinetic parameters of RaNADD and MhNuc**

	RaNADD		MhNuc		
	NAD^+	NADH	NMN	NaMN	NMNH
K_M (mM)	0.51 ± 0.05	0.14 ± 0.03	0.99 ± 0.19	2.89 ± 0.31	0.26 ± 0.03
V_{max} (μM/min)	28.84 ± 1.02	65.55 ± 3.04	11.46 ± 0.84	3.4 ± 0.15	6.87 ± 0.26
k_{cat} (s^{-1})	2.85	32.33	6.82×10^{-2}	2.24×10^{-3}	0.69
k_{cat}/K_M ($s^{-1} \cdot mM^{-1}$)	5.58	224.38	6.88×10^{-2}	7.74×10^{-4}	2.71

738

739

740

FIGURE 1

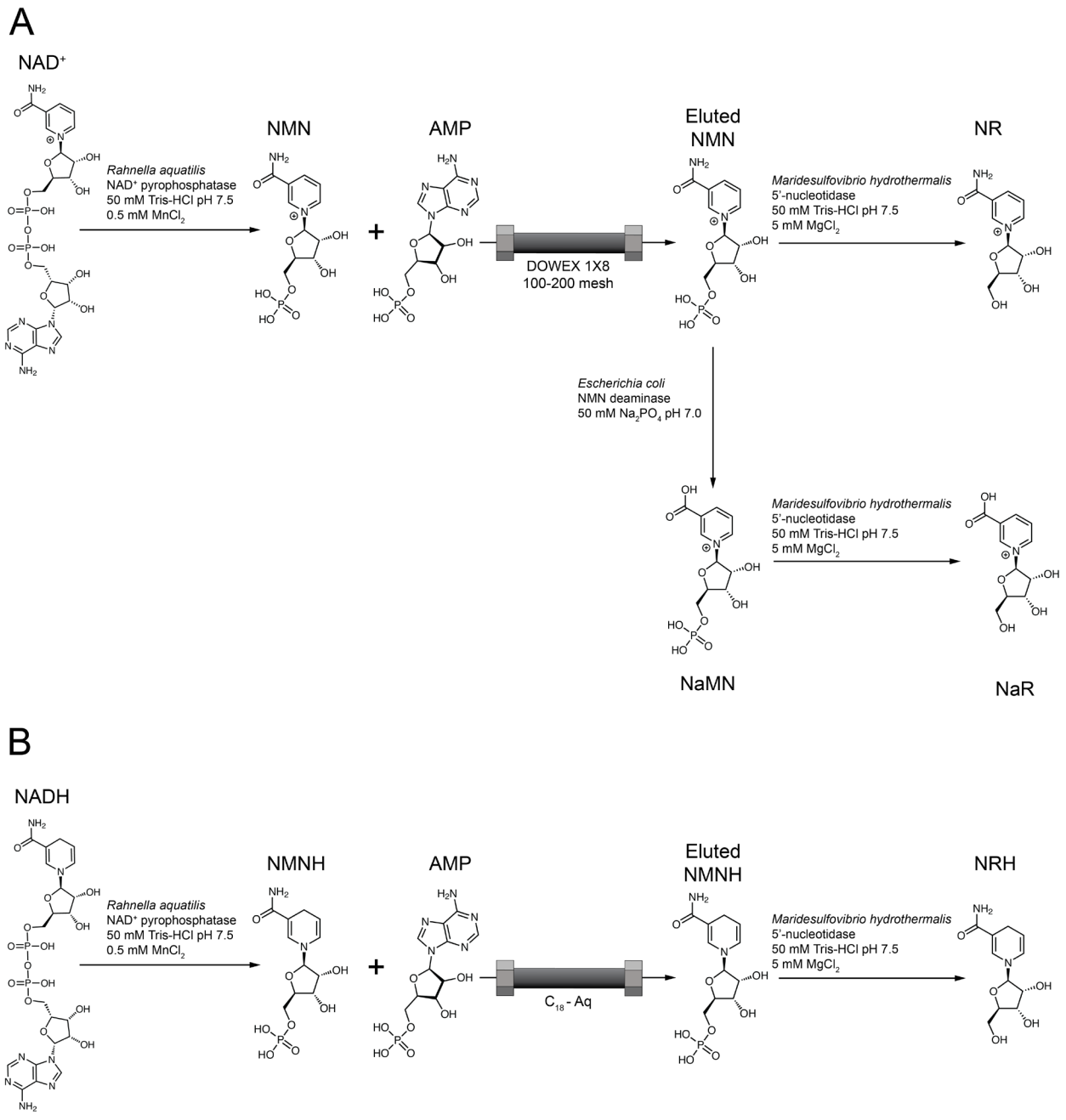

A
NAD+
Rahnella aquatilis
NAD+ pyrophosphatase
50 mM Tris-HCl pH 7.5
0.5 mM MnCl2
NMN
AMP
DOWEX 1X8
100-200 mesh
Eluted
NMN
Maridesulfovibrio hydrothermalis
5'-nucleotidase
50 mM Tris-HCl pH 7.5
5 mM MgCl2
NR
Escherichia coli
NMN deaminase
50 mM Na2PO4 pH 7.0
NaMN
NaR
B
NADH
Rahnella aquatilis
NAD+ pyrophosphatase
50 mM Tris-HCl pH 7.5
0.5 mM MnCl2
NMNH
AMP
C18 - Aq
Eluted
NMNH
Maridesulfovibrio hydrothermalis
5'-nucleotidase
50 mM Tris-HCl pH 7.5
5 mM MgCl2
NRH

FIGURE 2

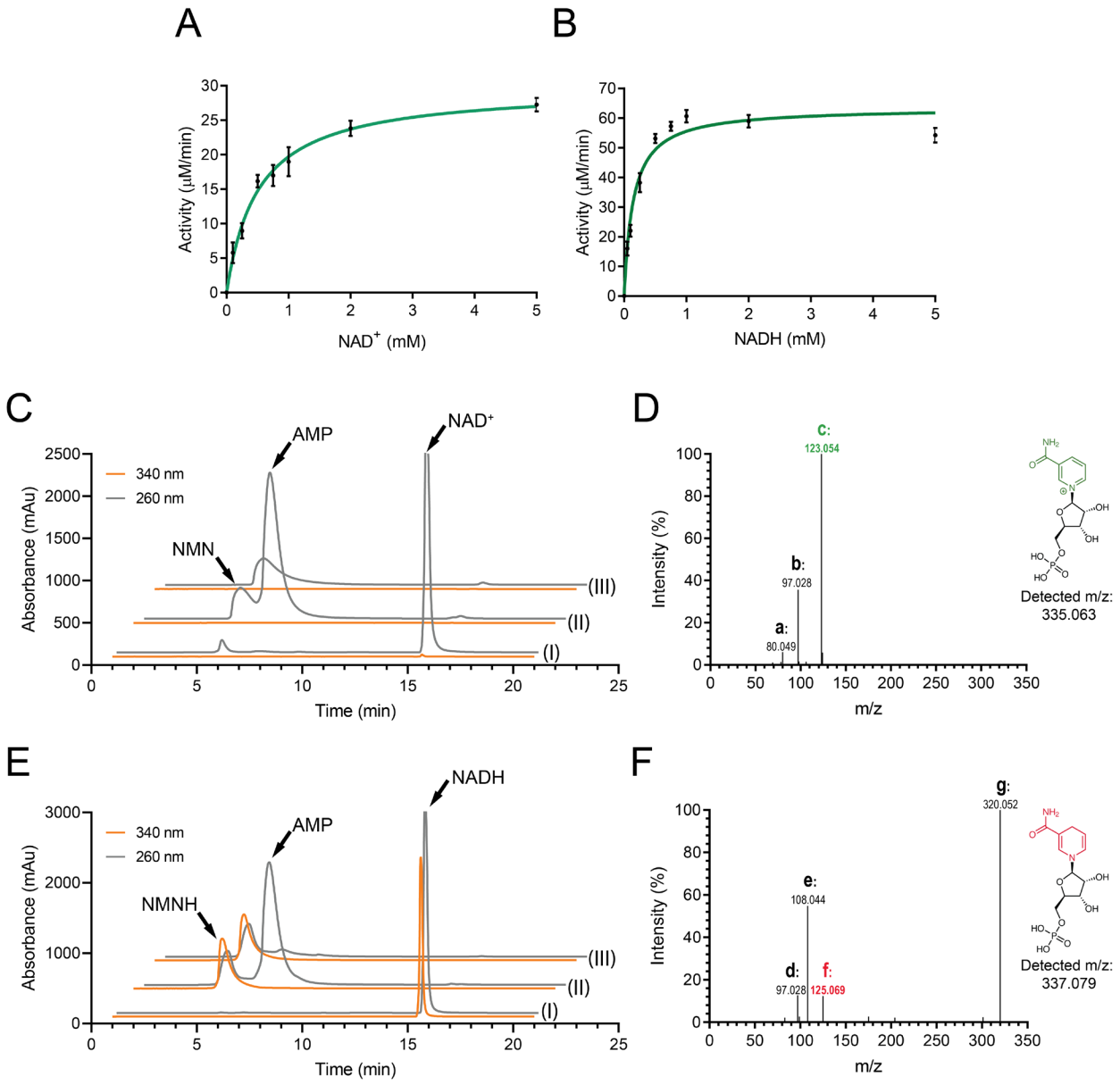

A
B
Activity (μM/min)
NAD+ (mM)
NADH (mM)
C
D
AMP
NAD+
NMN
340 nm
260 nm
(I)
(II)
(III)
Absorbance (mAu)
Time (min)
Intensity (%)
m/z
c:
123.054
b:
97.028
a:
80.049
Detected m/z:
335.063
E
F
NADH
NMNH
g:
320.052
e:
108.044
d:
97.028
f:
125.069
Detected m/z:
337.079

FIGURE 3

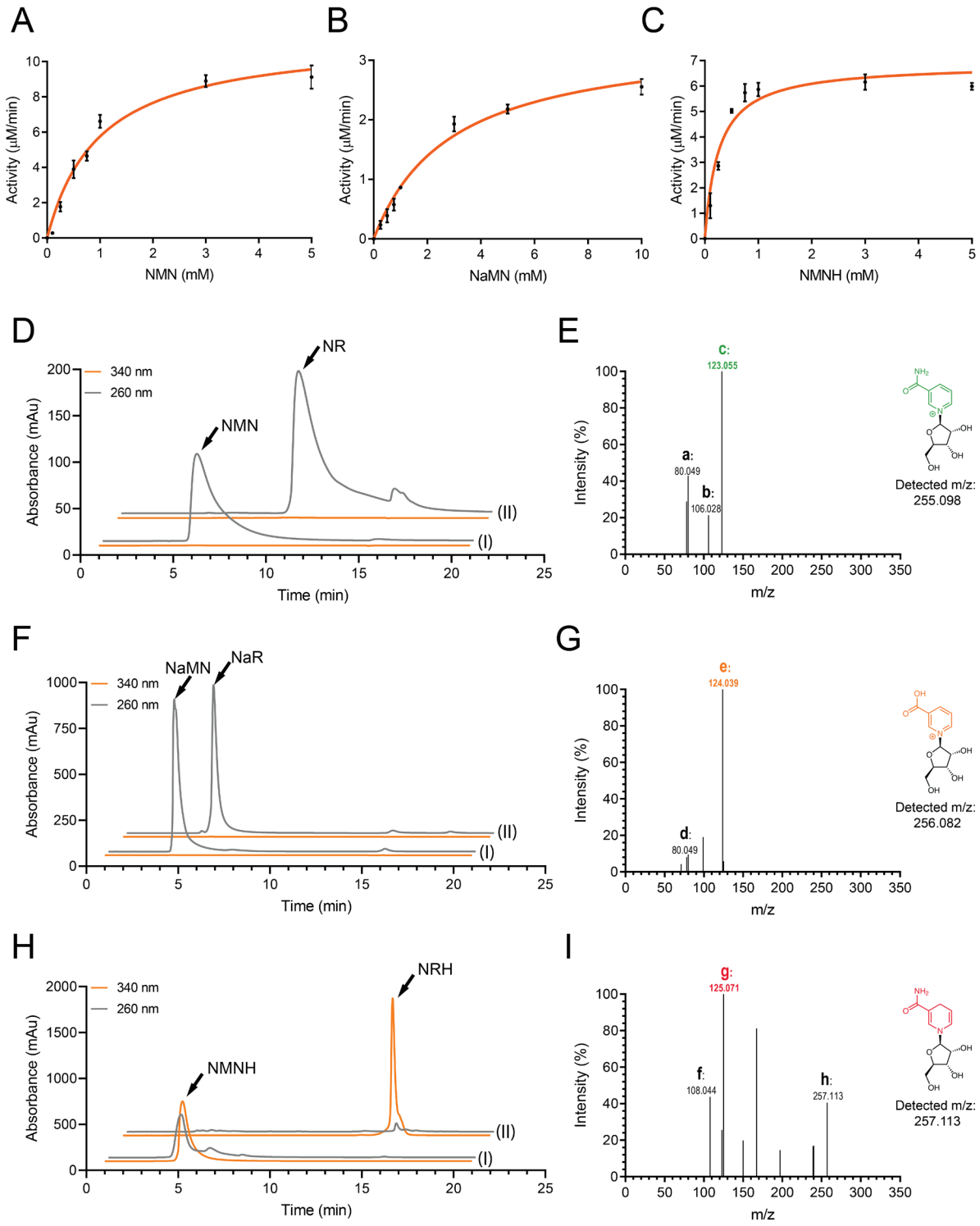
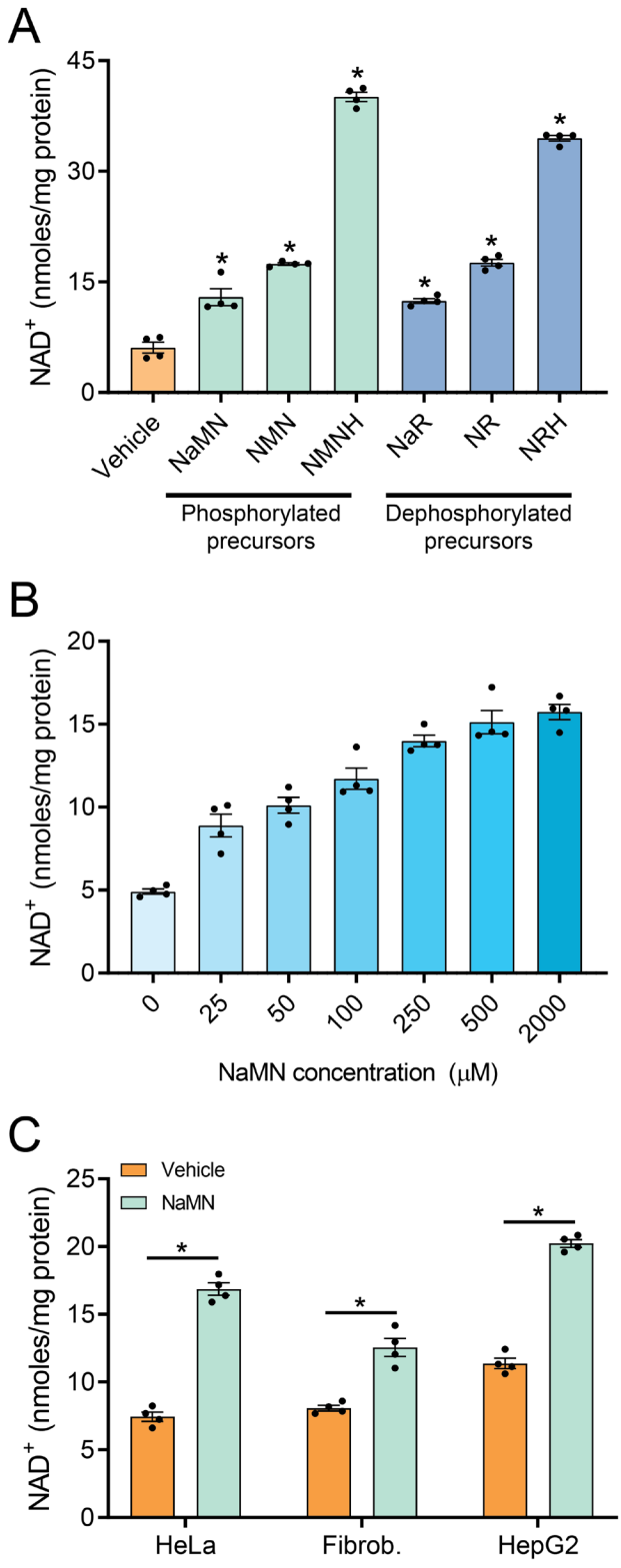

A
Activity (μM/min)
NMN (mM)
B
Activity (μM/min)
NaMN (mM)
C
Activity (μM/min)
NMNH (mM)
D
340 nm
260 nm
NR
NMN
(II)
(I)
Absorbance (mAu)
Time (min)
E
c:
123.055
a:
80.049
b:
106.028
Intensity (%)
m/z
Detected m/z:
255.098
F
340 nm
260 nm
NaMN
NaR
(II)
(I)
Absorbance (mAu)
Time (min)
G
e:
124.039
d:
80.049
Intensity (%)
m/z
Detected m/z:
256.082
H
340 nm
260 nm
NRH
NMNH
(II)
(I)
Absorbance (mAu)
Time (min)
I
g:
125.071
f:
108.044
h:
257.113
Intensity (%)
m/z
Detected m/z:
257.113

FIGURE 4


A
NAD+ (nmoles/mg protein)
45
30
15
0
Vehicle
NaMN
NMN
NMNH
NaR
NR
NRH
Phosphorylated precursors
Dephosphorylated precursors
B
NAD+ (nmoles/mg protein)
20
15
10
5
0
0
25
50
100
250
500
2000
NaMN concentration (μM)
C
Vehicle
NaMN
NAD+ (nmoles/mg protein)
25
20
15
10
5
0
HeLa
Fibrob.
HepG2