1st International Electronic Conference on Metabolomics

1-30 November 2016
chaired by Dr. Peter Meikle

IECM

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metabolites

NAD metabolome analysis in cultured human cells using 1H NMR spectroscopy

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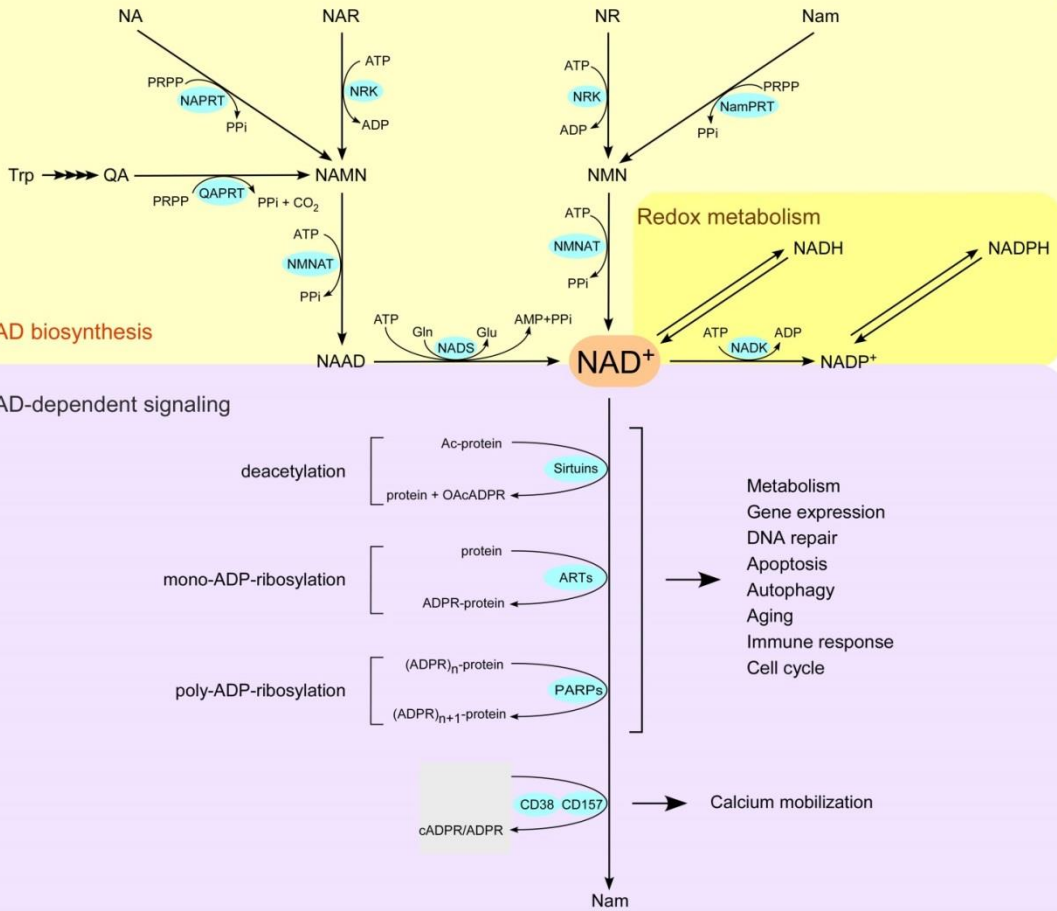* Corresponding author: Shabalin_KA@pnpi.nrcki.ru

Abstract

Nicotinamide adenine dinucleotide (NAD) and its phosphorylated form NADP are the major coenzymes of redox reactions in central metabolic pathways. NAD is also used to generate second messengers (such as cyclic ADP-ribose) and serves as substrate for protein modifications (including ADP-ribosylation and protein deacetylation). The regulation of these metabolic and signaling processes depends on NAD availability. Generally, human cells regulate their NAD supply through biosynthesis using various precursors: nicotinamide (Nam) and nicotinic acid as well as nicotinamide riboside (NR) and nicotinic acid riboside (NAR). These precursors are converted to the corresponding mononucleotides NMN and NAMN, which are then adenylylated to the dinucleotides NAD or NAAD, respectively. Here, we have developed NMR-based experimental approach to identify NAD and its intermediates in culture of human cells. Using this method we have detected and quantified NAD, NADP, NMN and Nam pools in HEK293 cells cultivated in the standard culture medium containing Nam as the only NAD precursor. When cells were grown in the presence of NR and NAR, we have additionally identified intracellular pools of deamidated NAD intermediates (NAR, NAMN and NAAD). Also, we have characterized the potential of different extracellular NAD precursors to maintain the synthesis of intracellular NAD.

Keywords: NMR, NAD metabolome, human cells

Introduction

NAD metabolism in human cells

Nicotinamide adenine dinucleotide (NAD) and its phosphorylated form NADP are the major coenzymes of redox reactions in central metabolic pathways. Besides its central role in cellular metabolism, NAD also serves as substrate of several families of regulatory proteins: protein deacetylases (Sirtuins), ADP-ribosyltransferases (ARTs) and Poly-ADP-ribosyl polymerases (PARPs), which govern vital processes including gene expression, DNA repair, apoptosis, aging, cell cycle progression and many others. NAD is also used by ADP-ribosyl cyclases (CD38, CD157) to produce calcium mobilizing messengers, including ADP-ribose (ADPR) and cyclic ADP-ribose (cADPR). The regulation of these metabolic and signaling processes depends on NAD availability.

Generally, human cells regulate their NAD supply through biosynthesis using various precursors: tryptophan (Trp), the pyridine bases nicotinamide (Nam) and nicotinic acid (NA) or the nucleosides Nam riboside (NR) and NA riboside (NAR). Nam and NA are converted to the corresponding mononucleotides (NMN and NAMN) by nicotinamide phosphoribosyltransferase (NamPRT) and nicotinic acid phosphoribosyltransferase (NAPRT), respectively. NMN and NAMN are also generated through phosphorylation of NR and NAR, respectively, by nicotinamide riboside kinases (NRK). NAMN and NMN are converted to the corresponding dinucleotide (NAAD or NAD+) by NMN adenylyltransferases (NMNAT). NAD synthetase (NADS) amidates NAAD to NAD+ (1).

Here we developed NMR-based experimental approach for quantitative analysis of NAD(P) and its major intermediates in cell extracts and culture medium.

1. Nikiforov A., Kulikova V., and Ziegler M. The human NAD metabolome: Functions, metabolism and compartmentalization. *Critical Reviews in Biochemistry and Molecular Biology*. 2015. 50(4): 284–297.

Experimental procedure

Cell Culture

HEK293 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. NA, NR, or/and NAR were added to the cell culture medium at a concentration of 100 µM. Cells were also treated with FK866 (inhibitor of NamPRT) at a concentration of 1 µM.

NMR sample preparation

100 µM standard solutions of Nam, NA, NR, NAR, NMN, NAMN, NAD, NAAD, NADP, NADH, and NADPH were prepared using DBP buffer: D_2O-based buffer containing 50 mM NaPi (pH 6.5) and 1 mM sucrose as a chemical shift reference (δ(^{1}H), 5.42 ppm) and internal standard for quantification. Samples were stored at −80 °C until NMR analysis.

Culture medium from HEK293 cells was collected and stored at −80 °C. To precipitate proteins, the samples were incubated on ice with 2 volumes of acetonitrile for 30 min and then centrifuged at 15,000 g for 30 min at 4 °C. Supernatants were lyophilized and resuspended in DBP buffer.

10^7 HEK293 cells grown on 100 mm culture dish were washed twice with ice cold PBS, extracted with 67% acetonitrile for 30 min at 4 °C, scraped and centrifuged at 15,000 g for 30 min at 4 °C. The supernatant fraction (extract) was then lyophilized and resuspended in DBP buffer.

Experimental procedure

NMR Analysis

All NMR experiments were performed using a Varian DirectDrive NMR System 700-MHz spectrometer equipped with a 5-mm z-gradient salt-tolerant. VNMRJ 4.2, Mestrelab Mnova 10.0 was used for data analysis.

To optimize parameters of NMR spectra acquisition:

1) Pulse sequence with the suppression of solvent signal was used. This pulse sequence allowed recording the spectra with maximal sensitivity.

2) Relaxation time for each metabolite signal was measured and used to optimize the relaxation delay and other parameters of spectra recording.

3) 1H-spectrum for each metabolite of interest was obtained and several characteristic peaks for the identification and quantification of metabolites were chosen.

4) Number of scans was determined to obtain signal/noise ratio more than 5 for quantification of metabolites.

5) Concentration of metabolite was calculated as a ratio of weighted average intensity of metabolite signals to sucrose signal intensity, where weight equals (1-exp{-(Repetition time)/(Relaxation time)}), Repetition time = Acquisition time + Relaxation delay.

6) Signal intensity was calculated as an integral of multiplet fitted to spectrum.

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Results and discussion

1

The development of NMR-based experimental approach for the quantitative analysis of NAD metabolome in cell extracts and culture medium

Detection of NAD and its intermediates by ^{1}H NMR spectroscopy
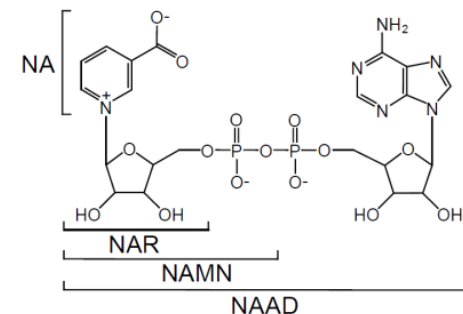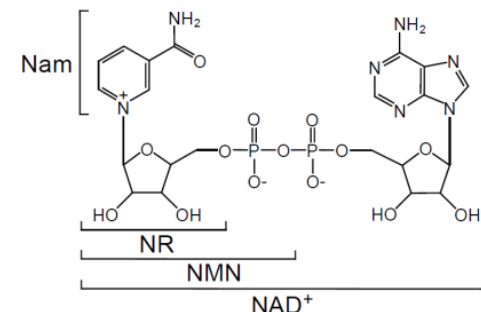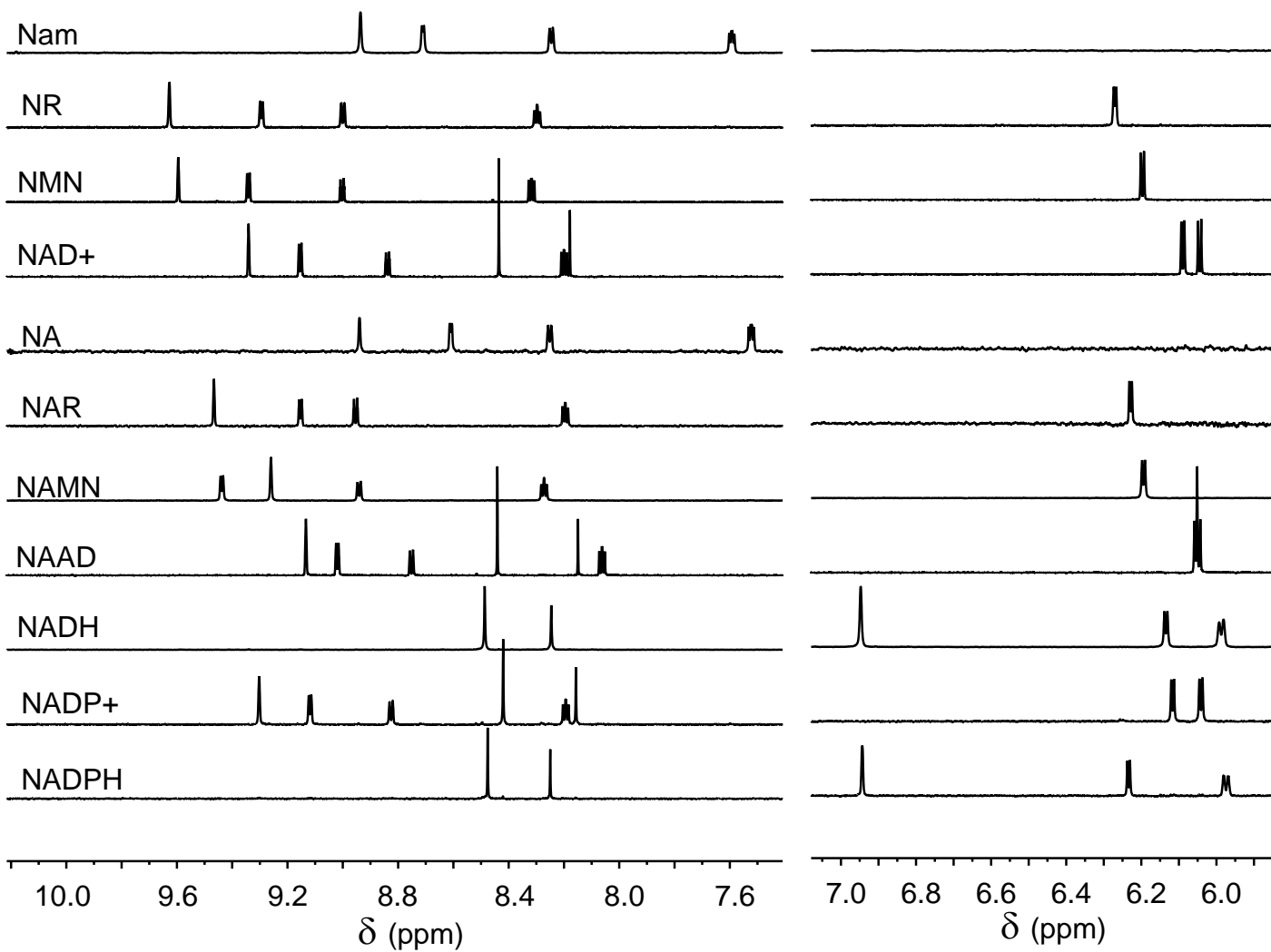
^{1}H NMR spectra of NAD and its major intermediates. Metabolites were dissolved in 50 mM sodium phosphate buffer in D$_2$O (pH 6.5) and analyzed by NMR spectroscopy. The two right panels indicate the structures of NAD and its major intermediates.

^{1}H NMR spectral parameters of NAD and its major intermediates

Metabolite	δ, ppm	M, Class	J's, Hz	T1, sec
Nam	8.94	m		13.73
	8.71	d	5.01	7.97
	8.25	d	7.97	8.52
	7.60	dd	4.93, 7.99	6.29
NR	9.64	s		3.02
	9.31	d	6.18	2.21
	9.02	d	8.03	5.29
	8.31	t	7.11	2.22
	6.29	d	4.39	1.89
NMN	9.60	s		3.16
	9.34	d	6.26	2.56
	9.00	d	8.03	4.68
	8.32	t	7.11	2.97
	6.20	d	5.83	2.27
NAD+	9.34	s		2.32
	9.15	d	6.22	1.76
	8.84	d	8.04	3.99
	8.43	s		2.84
	8.20	t	7.19	2.29
	8.18	s		9.73
	6.09	d	5.44	1.8
	6.04	d	5.94	4.09
NA	8.94	d	2.14	12.23
	8.61	d	4.99	9.41
	8.25	d	7.9	10
	7.52	dd	4.90, 7.91	6.44
NAR	9.47	s		2.93
	9.16	d	6.2	2.06
	8.95	d	7.91	4.92
	8.20	t	7.03	2.38
	6.23	d	4.68	1.89

| Metabolite	δ, ppm	M, Class	J's, Hz	T1, sec
NAMN	9.44	d	6.23	1.92
	9.26	s		2.09
	8.94	d	7.88	4.11
	8.27	t	7.07	2.25
	6.19	d	5.37	1.75
NAAD	9.13	s		2.1
	9.02	d	6.22	1.97
	8.75	d	7.87	3.96
	8.44	s		2.67
	8.15	s		8.99
	8.06	t	7.04	2.18
	6.05	t	5.36	2.92
NADH	8.49	s		2.61
	8.25	s		7.79
	6.95	s		1.79
	6.13	d	5.51	3.78
	5.99	d	8.04	1.50
NADP+	9.30	s		2.4
	9.12	d	6.29	1.82
	8.83	d	8.09	4.09
	8.42	s		2.92
	8.19	m		2.33
	8.16	s		9.6
	6.12	d	5.10	3.95
	6.04	d	5.62	1.75
NADPH	8.48	s		2.84
	8.25	s		8.05
	6.94	d	1.53	1.92
	6.23	d	4.81	3.8
	5.97	d	8.53	1.75

δ – chemical shift (ppm);
M – multiplicity: singlet (s), doublet (d), triplet (t), double doublet (dd);
J's – coupling constants (Hz);
T1 – longitudinal relaxation time (sec).

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We have developed NMR-based experimental approach for the NAD metabolome analysis in various biological samples. This method can be used for the identification and quantification of NAD(P) and its major intermediates at minimal concentration of 0.5 µM with an error no more than 10%. This method is optimized for the quantitative analysis of NAD metabolome in cell extracts and culture medium.

2

Application of ^{1}H NMR spectroscopy to NAD metabolome analysis in cultured human cells

# ^{1}H NMR spectrum of standard cell culture medium
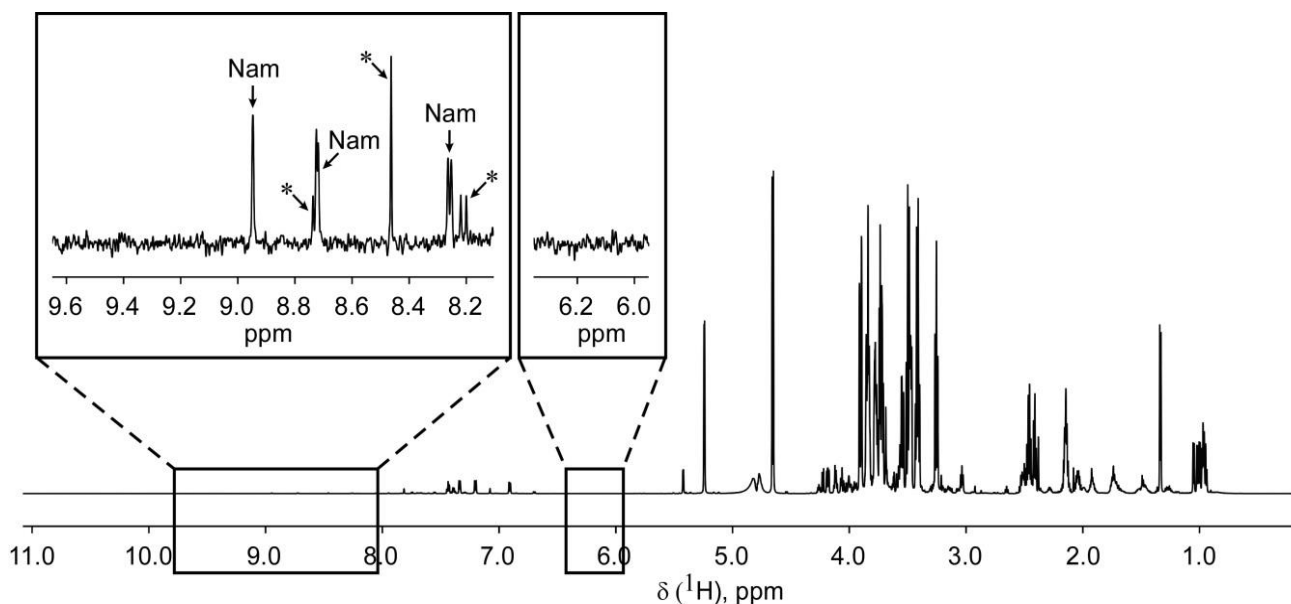
Standard cell culture medium DMEM (Dulbecco's Modified Eagle's medium) contains Nam as the only NAD precursor. Characteristic peaks corresponding to NAD and its intermediates are found in the regions of the NMR spectrum (from 6.0 to 6.3 and from 8.0 to 9.7 ppm) that contain only few signals (designated with asterisk) from components of the cell culture medium.

Characteristic region of ¹H NMR spectrum of cell extract obtained from HEK293 cells cultivated in the presence of Nam
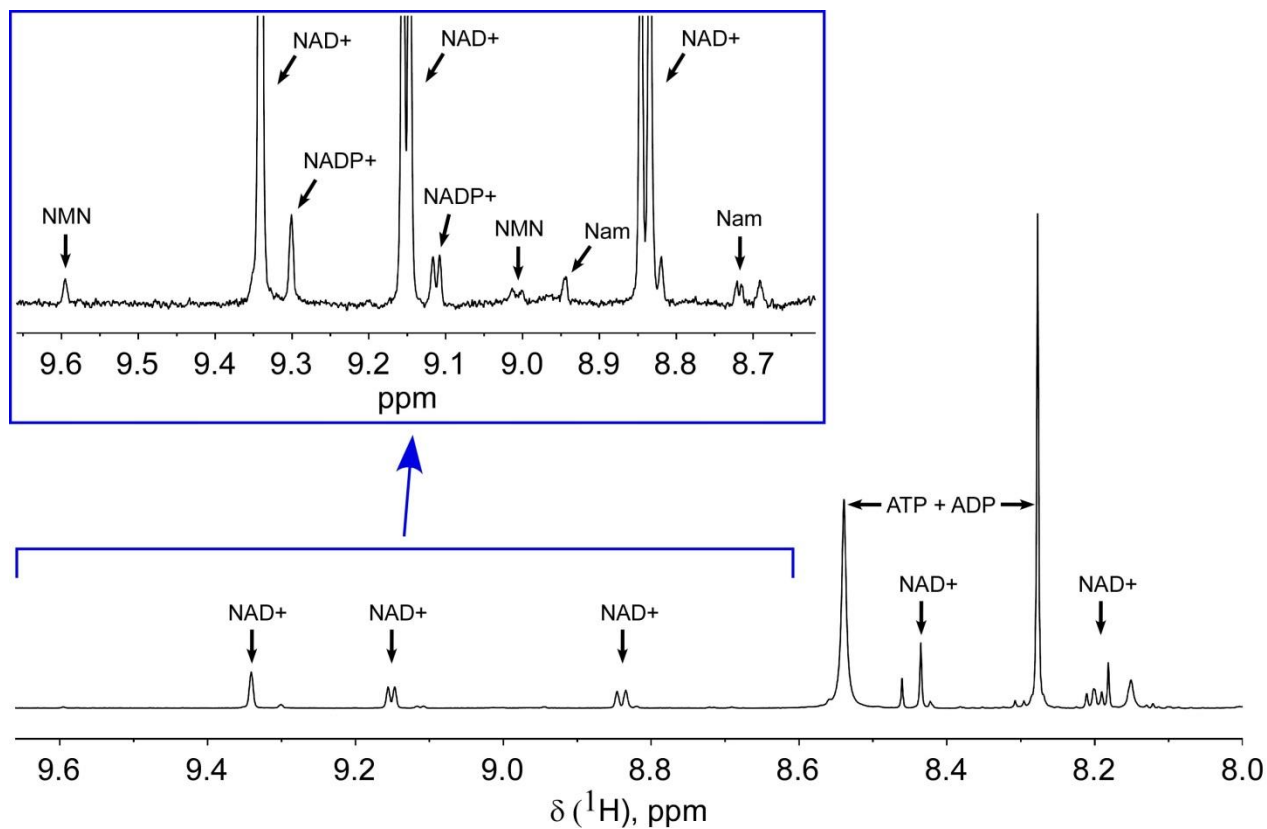
We identified and quantified NAD, NADP, NMN and Nam in cell extract obtained from HEK293 cells cultivated in standard culture medium containing Nam as the only NAD precursor.

Characteristic region of ^{1}H NMR spectrum of cell extract obtained from HEK293 cells cultivated in the presence of Nam, NR and NAR
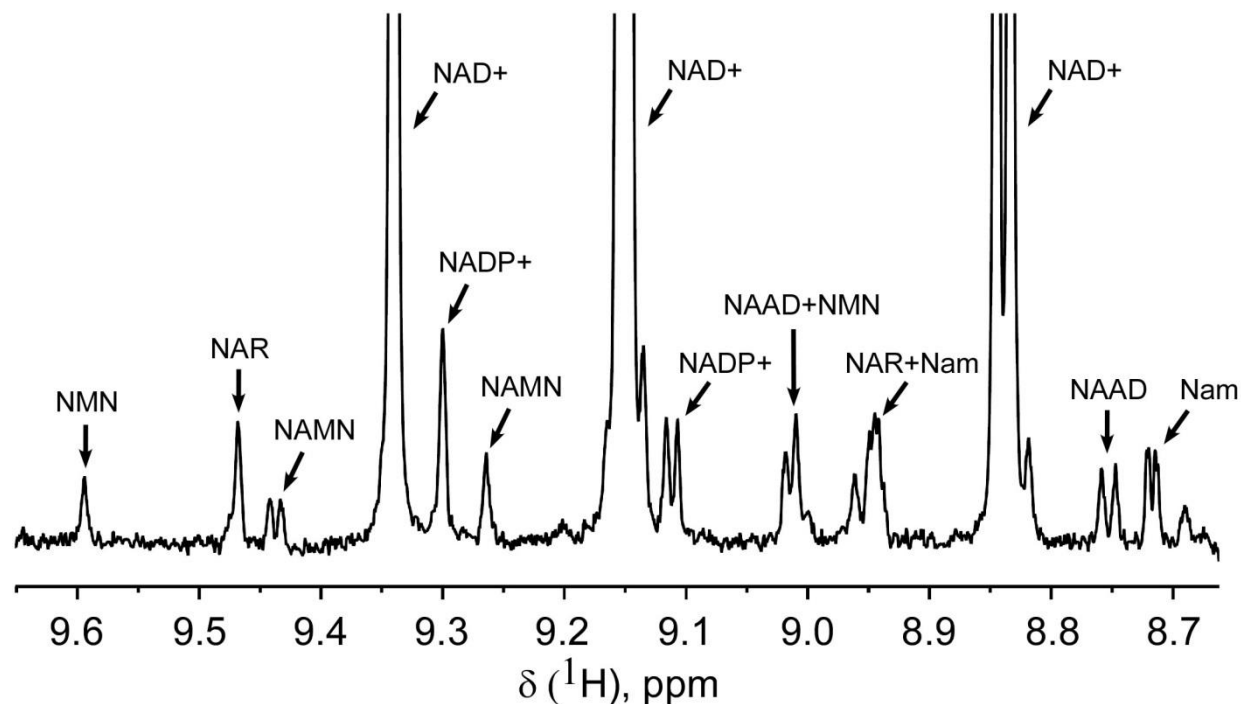

We have identified and quantified NAD, NADP, NMN, Nam and deamidated NAD intermediates: NAR, NAMN and NAAD in the cell extract obtained from HEK293 cells cultivated in the standard culture medium supplemented with NR and NAR as alternative precursors to maintain intracellular NAD pool.

3

Characterization of the potential of different extracellular NAD precursors to maintain the synthesis of intracellular NAD

Efficiency of NAD biosynthesis from its major precursors in HEK293 cells
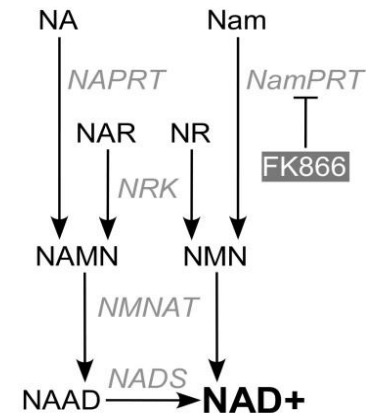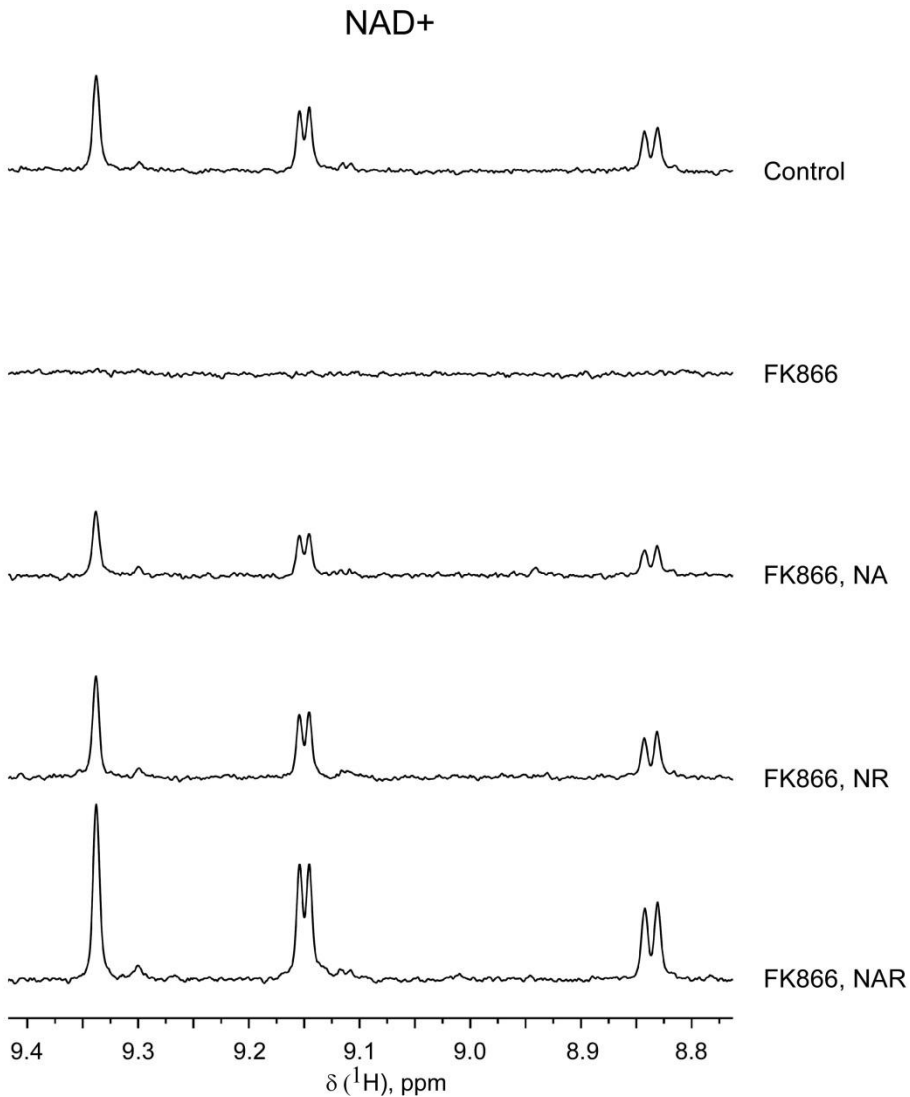
In order to characterize the potential of different extracellular NAD precursors to maintain the synthesis of intracellular NAD, HEK293 cells were cultivated in the standard culture medium in the presence of NA, NR, or NAR. Nam utilization was inhibited by FK866 (NamPRT inhibitor). Cell extracts were analysed by NMR spectroscopy 24 hours after the treatment of cells.

FK866 dramatically reduced intracellular NAD level, which was restored by co-treatment with NA, NR, or NAR. NAR and NA were the most and least efficient NAD precursors, respectively.

Conclusions:

- We have developed NMR-based experimental approach for the NAD metabolome analysis. This method can be used for the identification and quantification of NAD(P) and its major intermediates in cell extracts and culture medium.
- We have identified and quantified NAD, NADP, NMN and Nam in cell extract obtained from HEK293 cells cultivated in standard culture medium containing Nam as the only NAD precursor. When cells were grown in the presence of NR and NAR, we could additionally identify deamidated NAD intermediates (NAR, NAMN and NAAD).
- We have characterized the potential of major extracellular NAD precursors (Nam, NA, NR and NAR) to maintain the synthesis of intracellular NAD in HEK293 cells. NAR and NA are the most and least efficient NAD precursors, respectively.

Acknowledgments