Short thesis for the degree of doctor of philosophy (Ph.D.)

Understanding structure and dynamics with advanced NMR and *in silico* methods

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I. Introduction and objectives

1. The biological function and activity of proteins are strongly related to their three-dimensional structure. However, due to the conformationally diverse nature of protein chains, these native structures are not static, they exhibit fluctuations over time and might change upon interaction with other molecules. It is crucial to consider the contribution of protein dynamics when elucidating biological mechanisms, especially in the case of the unique family of intrinsically disordered proteins, drawing significant scientific attention in the past decades.

The amino acid sequence is changing over time through evolution often resulting in altered structure and dynamics. Gene technology allows us to „accelerate" evolution via targeted mutations to synthesize proteins with better properties. During my doctoral years, we were studying the MEF2D transcription factor with mutations designed to modify its dynamical nature. The MEF2 protein family is playing role in complex cellular processes via gene regulation. In muscular and nervous tissues, they participate in cell proliferation, embryonic development and apoptosis. The MEF2D isoform is crucial in muscle cell differentiation processes, it was shown that MEF2D knock-out mouses suffer from cardiac dysfunction. A conserved β-domain regulated by alternative splicing is proved to be crucial for the maximum transcriptional activity via its role in transactivation. This short motif (286-292, *TEDHLDL*) is located in the disordered C-terminal region, a site for binding partner proteins and transcriptional co-regulators.

In collaboration with the Fuxreiter group, we hypothesized that without a well-defined structure, the biological function of the MEF2D β-domain is mainly determined by protein dynamics. We have introduced directed mutations to perturb the dynamical profile of the β-domain and the flanking residues and we were following the corresponding biological responses. During the doctoral work, we planned a detailed study of the structure and dynamics of β-domain mutant MEF2D mini-proteins with promising preliminary biological responses and bioinformatical scores.

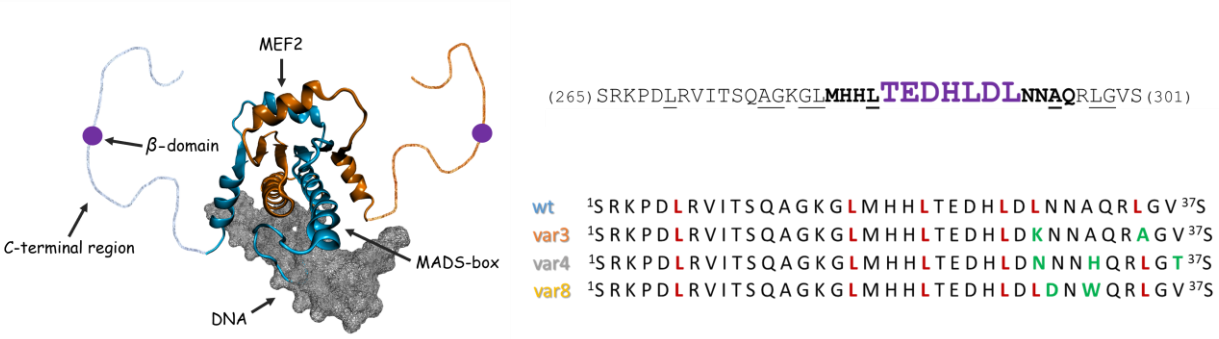

Figure 1. *Left: the solution-state NMR structure of the MEF2D-dimer (blue/orange) binding core domain with an oligonucleotide (grey). The hand-drawn part represents the unstructured C-terminal region of the protein incorporating the β-domain with transactivation roles. Right (top): the sequence of the β-domain (purple) and the flanking residues. Right (bottom): the sequence of the studied 37aa mini-proteins. ^{15}N-labeled leucines are red, while the mutations are labeled with green.*

2. NMR spectroscopy is a widely used, interdisciplinary technique and an invaluable tool for investigating biologically relevant compounds as well. The measurement techniques are being constantly developed with advancements in hardware (e.g., larger magnets, cryoprobes), pulse program design and data collection methods (e.g., non-uniform sampling, NUS). Our group is working on the development of NOAH type (NMR by Ordered Acquisition using ^{1}H-detection) NMR super sequences allowing the concatenation of different experiments with reduced measurement time and more spectral information. During my Ph.D., our objective was to combine NMR experiments useful for complete resonance assignment of small- and medium-size molecules. Another goal was to significantly reduce the corresponding measurement time with an efficient pulse sequence design. The main starting point was to reduce the relaxation time, a long delay component crucial for the magnetization recovery between the repetition of experiments. Secondly, we also aimed to eliminate the recovery delay with the Ernst-angle concept, unique excitation techniques and pulse sequence design strategy (NO Relaxation Delay Spectroscopy, *Figure 2*).

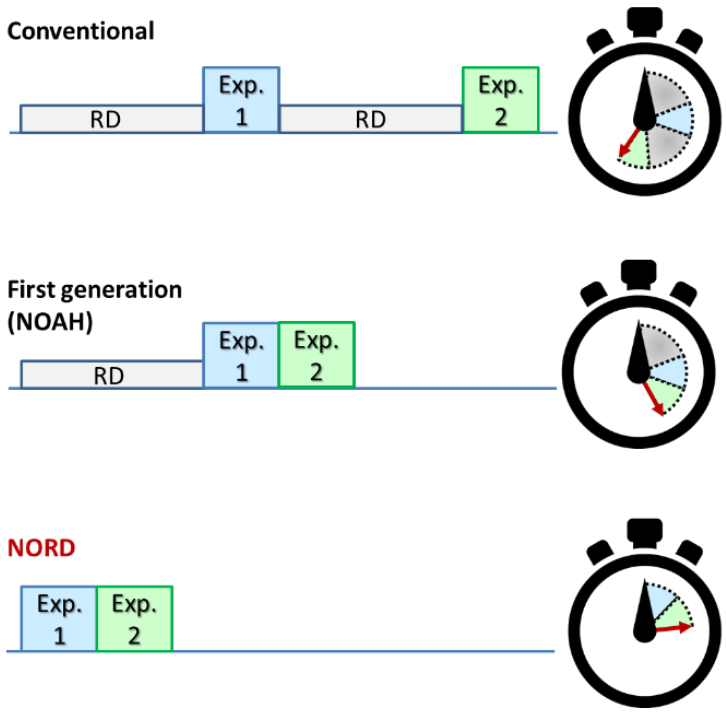

Figure 2. *The length of the relaxation delay (typically between 0.5 - 3 s) significantly determines the overall measurement time. Conventional pulse sequences require a delay before the repetition of experiments. The first generation of concatenated (NOAH) experiments efficiently share the available magnetization between modules, therefore only one delay time is needed at the beginning of the sequence. The NORD pulse sequence design strategy presented in the dissertation eliminates the relaxation delay providing extremely rapid measurements.*

II. Methodology

NMR measurements were performed on a Bruker Avance Neo 700 MHz spectrometer equipped with Prodigy TCI cryoprobe. Data processing and analysis were carried out with the TopSpin 3.0 software. Molecular dynamics calculations ran with the AMBER16 simulation engine on the Leo (GPU) unit of the high performance computing center of the University of Debrecen. The simulation data was processed with the AmberTools software package and in-house python and shell scripts. Biological experiments (e.g., microscopy, Luciferase activity assay) on the MEF2D mutants were carried out by the Fuxreiter group using HEK293 and C2C12 cell lines. NanoDSF measurements were performed by the Erdődi group using a Prometheus NT.48 machine.

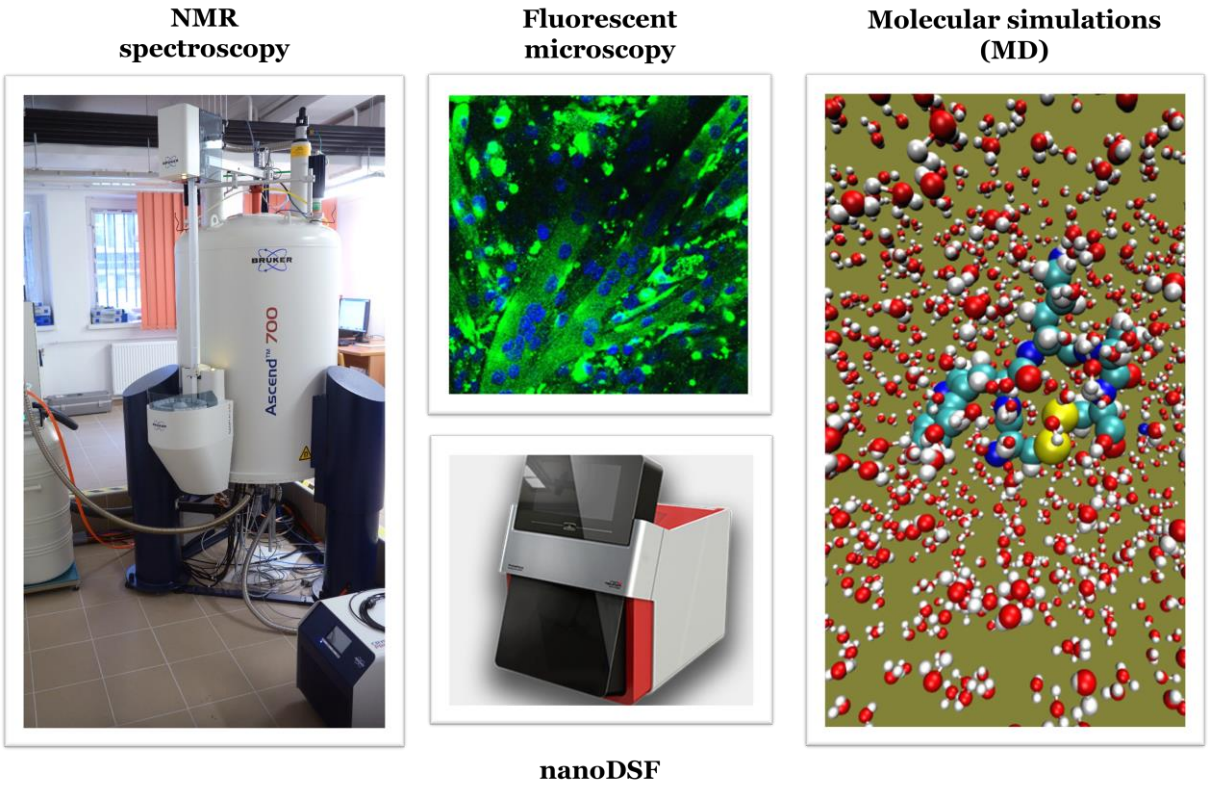

Figure 3. *Experimental and computational methods utilized in the doctoral work.*

III. New scientific results

1. The structural dynamics of the MEF2D β-domain fine-tunes the transcriptional activity in muscle cell differentiation processes.

In preliminary experiments, the Fuxreiter group studied biological responses by transfecting different MEF2D β-domain mutants into cell lines. Following the muscle cell differentiation with transmission and fluorescent microscopy, the fusion process was perturbed: cells expressing enhanced dynamical MEF2D variants showed slower differentiation. On the other side, the differentiation speed was enhanced with transfecting more rigid variants (*Figure 4A*). These findings were also confirmed by the transcriptional activity measurements on MEF2D variants (*Figure 4B*) and by following the target gene and regulatory factor expression levels during differentiation.

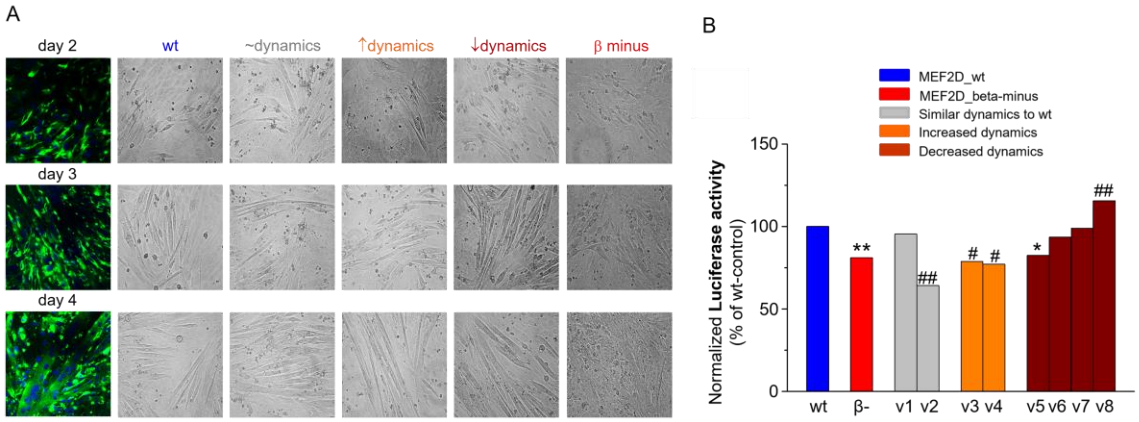

Figure 4. *A) Following C2C12 muscle cell differentiation with transmission and fluorescent microscopy. The wild-type (wt) MEF2D, its dynamical variants and one mutant without the β-domain were transfected. B) Normalized MEF2D transcriptional activity in HEK293 cells with the same MEF2D mutants transfected.*

Our results show that mutations decreased the backbone mobility and the degree of disorder in *var8* peptide with point mutations N29D and A31W, evidenced by various parameters compared to the wild type. On the experimental side, enhanced translational diffusion (*Figure 5A*) and residue-wise backbone order parameters (S^2, *Figure 5C*) were shown by NMR spectroscopy compared to the other variants. On the computational side, MD simulations provided further details at atomic-level and supported the experimental observations. The increased molecular compactness of *var8* was confirmed by the MD-derived order parameters (*Figure 5D*) as well as by the smallest radius of gyration and the larger propensity for secondary structures (*Figure 5E*). Intramolecular structure stabilizing interactions were also verified, including a network of H-bonds, salt bridges, and cation-π effects (*Figure 5B*). A different dynamical picture was obtained for proteins *var3* and *var4* where increased flexibility was evidenced by both experimental and theoretical data. Less compact conformations were indicated by slower translational diffusion and larger molecular radius. Furthermore, these proteins also feature a higher fraction of unclustered structures, and the populations of clustered conformations are distributed to multiple, similar-sized groups, indicating more structural diversity (*Figure 5F*).

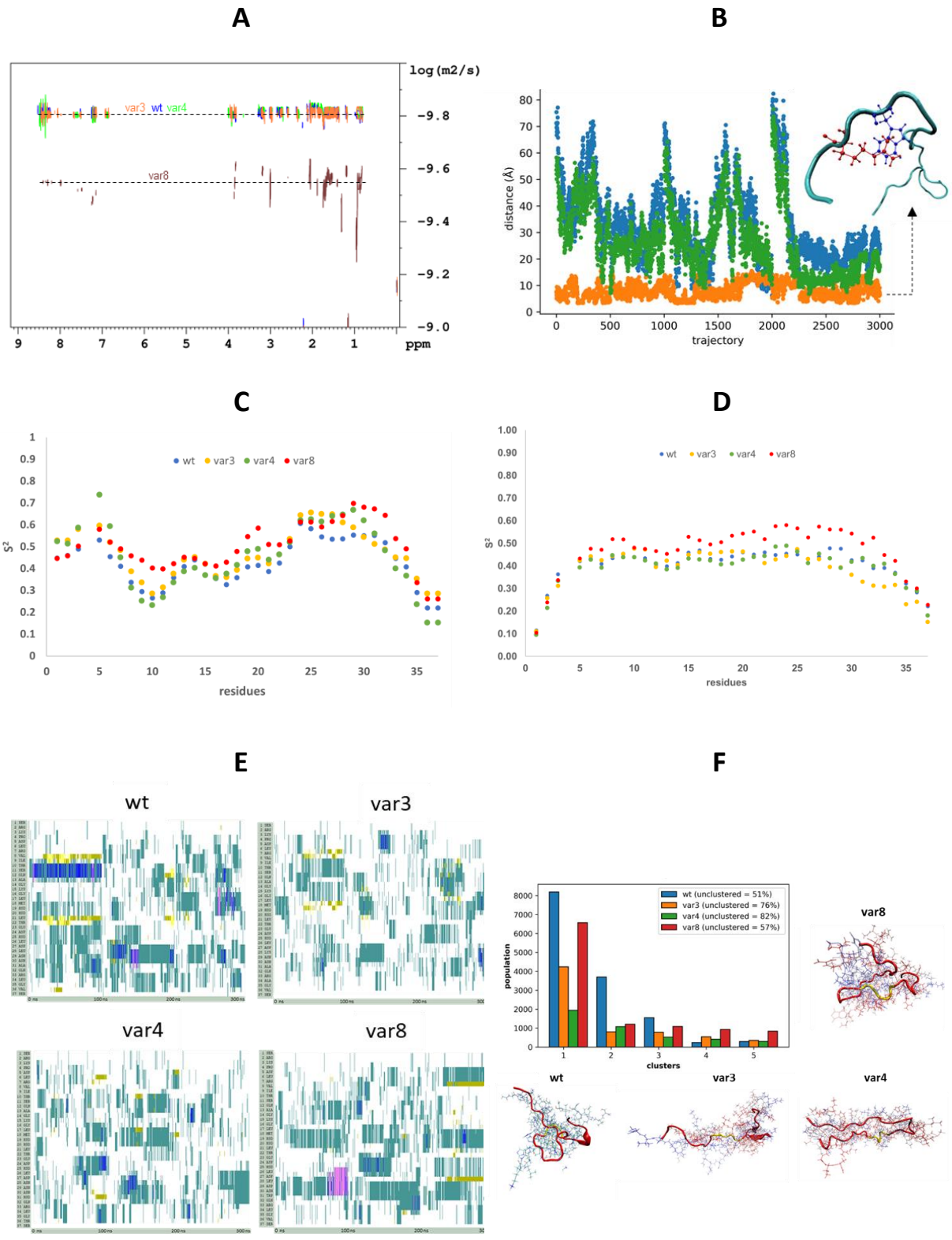

Figure 5. *A) Diffusion (DOSY) NMR spectra. B) A cation-π interaction between W31-R33 is stabilizing the structure of var8 (MD) C) S^2 calculated from NMR chemical shifts (Wishart method). D) S^2 calculated from MD trajectories (IRED analysis). E) The occurrence of secondary structures in MD trajectories of the mutants. F) The distribution and sizes of the cluster groups and representative conformers.*

The atomic-level structural and dynamical data obtained on the mini-protein variants together with the cell culture experiments on differentiation confirmed the role of the MEF2D β-domain in myogenesis. We have shown that the dynamical properties of this motif are also important in fine-tuning the biological activity possibly via rewiring the protein interaction network. In the case of MEF2D *var8*, when targeted point mutations stabilize the intrinsically disordered β-domain, the transcriptional activity is enhanced at the early stage of muscle cell differentiation. On the other side, myoblast fusion is slower if higher structural dynamics is introduced (*var3, var4*) in comparison to the wild-type MEF2D.

2. We proved that the MEF2D var8 mini-protein mutant forms oligomers.

Additionally, diffusion and relaxation data (*Figure 6A*) from NMR and independent NanoDSF (*Figure 6B*) measurements identified an oligomerization equilibrium in *var8* solution, which was confirmed and studied in detail by MD simulations. Only this slightly more rigid variant established higher-order protein assemblies stabilized via intermolecular interactions, such as cation-π electrostatic bonding and the formation of hydrophobic cores (*Figure 6C*). With the raised local protein concentration in transcription factor hubs or protein droplets, the transcription efficiency is facilitated and this mechanism is very likely exploited by MEF2D *var8*. However, further experiments are required to elaborate the biological background.

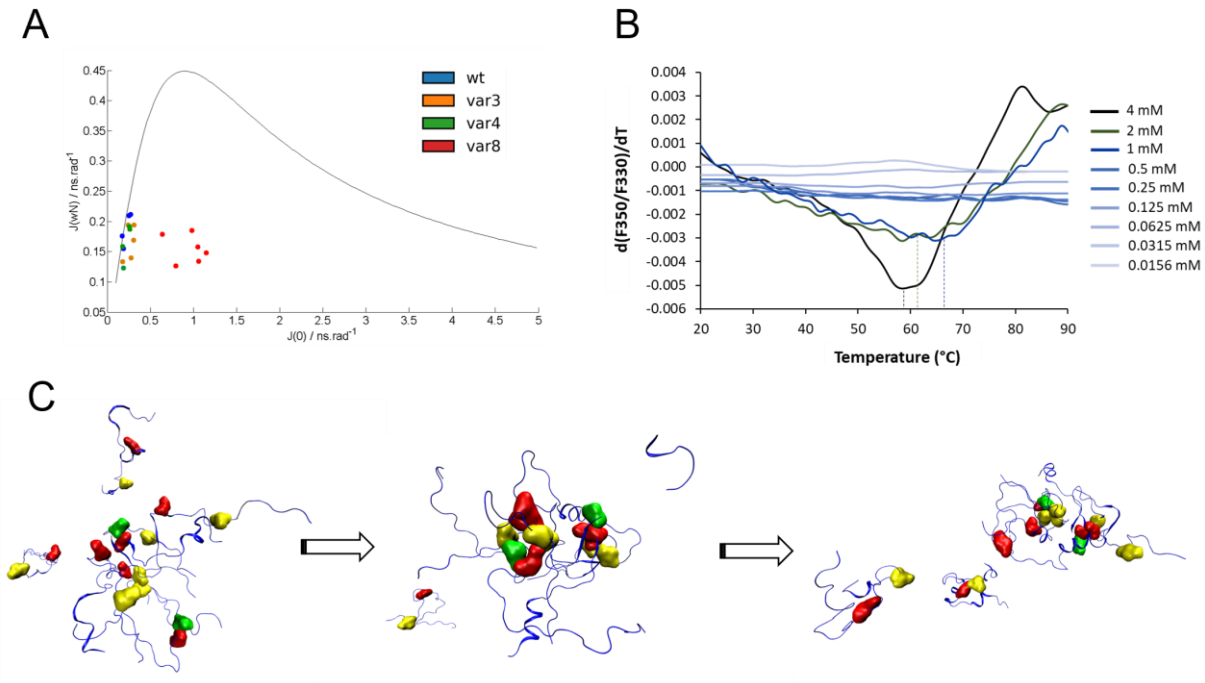

Figure 6. *A) Reduced spectral density mapping: there is an exchange contribution in var8 relaxation (oligomerization) B) The concentration dependence of the fluorescent effects (NanoDSF): the oligomerization is reversible, complexes dissociate with dilution. C.) The formation and dissociation of hydrophobic cores in a var8 MD trajectory (10, 60, 90 ns from left to right).*

3. We have developed efficient, NOAH-type combined NMR experiments with the incorporation of an isotope-selective BANGO excitation.

In the NOAH-type measurements, efficient spin manipulations were achieved by replacing the relevant non-selective pulses with the BANGO isotope-selective excitation, splitting the available ^1H magnetization and distributing them between the experiments. With only one recovery delay at the beginning of the series of the concatenated modules, BANGO {SEA XLOC}-{H2OBC} was performed on an ibuprofen sample in 42 minutes. 59 % of instrumental time was saved, when compared to the standalone SEA XLOC and H2OBC measurements (102 minutes together). The same improvement was shown with BANGO {HMBC}-{H2OBC} (*Figure 7, A,B*), correlation maps were obtained within 11 minutes, by replacing SEA XLOC with the more sensitive HMBC. 5 minutes were required to record BANGO {HMBC}-{H2OBC} on a trisaccharide sample with non-uniform sampling (NUS) (*Figure 7, C*).

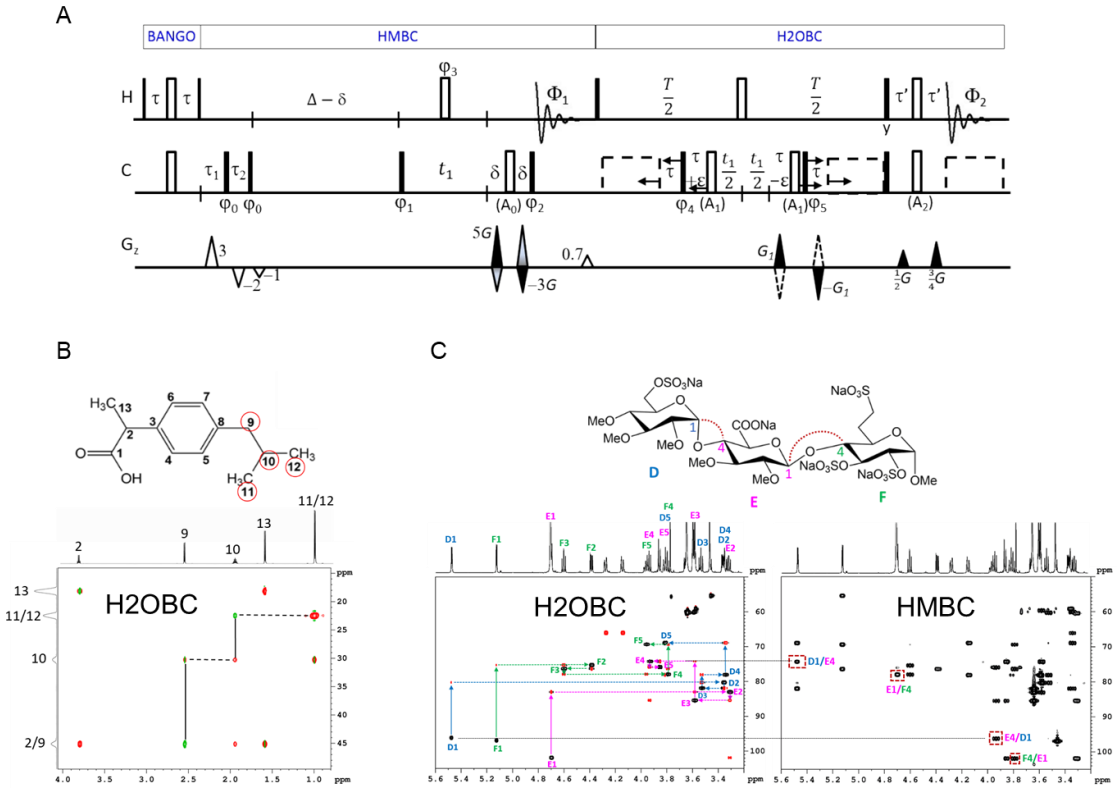

Figure 7. *A) The pulse sequence of the two-module BANGO {HMBC}-{H2OBC} experiment. B) H2OBC spectrum extracted from BANGO {HMBC}-{H2OBC} measurement on ibuprofen showing the C9-C10-C11/12 spin system. C) The „assignment walk" demonstrated on H2OBC and HMBC spectra on a trisaccharide. H2OBC shows correlations within the spin systems of the sugar residues, while HMBC peaks determine the connectivities between them.*

4. We have designed concatenated, three-module NMR experiments with double BANGO excitation.

Further improvement was achieved with the double BANGO pulse schemes with more efficient magnetization usage, allowing the introduction of a third experimental module in the sequence. The novel BANGO {SEA XLOC}-{HMBC}-{H2OBC} (*Figure 8, 1*) and BANGO {SEA XLOC(ZQ)}-{SEA XLOC(2Q)}-{H2OBC} experiments utilized the isotope selective BANGO excitation pulses that were optimized based on the 'Ernst-angle principle'. The first two modules share the ^{13}C - ^{12}C - H magnetization pool, while the third one exploits the ^{13}C - H pool. For complete magnetization recovery, only one relaxation delay is required at the beginning of the pulse sequence resulting in reduced measurement times. The new experiments were tested on ibuprofen and prednisolone compounds. The resonance assignment of prednisolone is challenging due to severe signal overlaps, therefore we have developed a method to distinguish between correlations of different carbon-multiplicity and correlations of different type (one- vs. multiple-bond) with phase editing and subsequent linear combination of the resulting subspectra (*Figure 8, 2*).

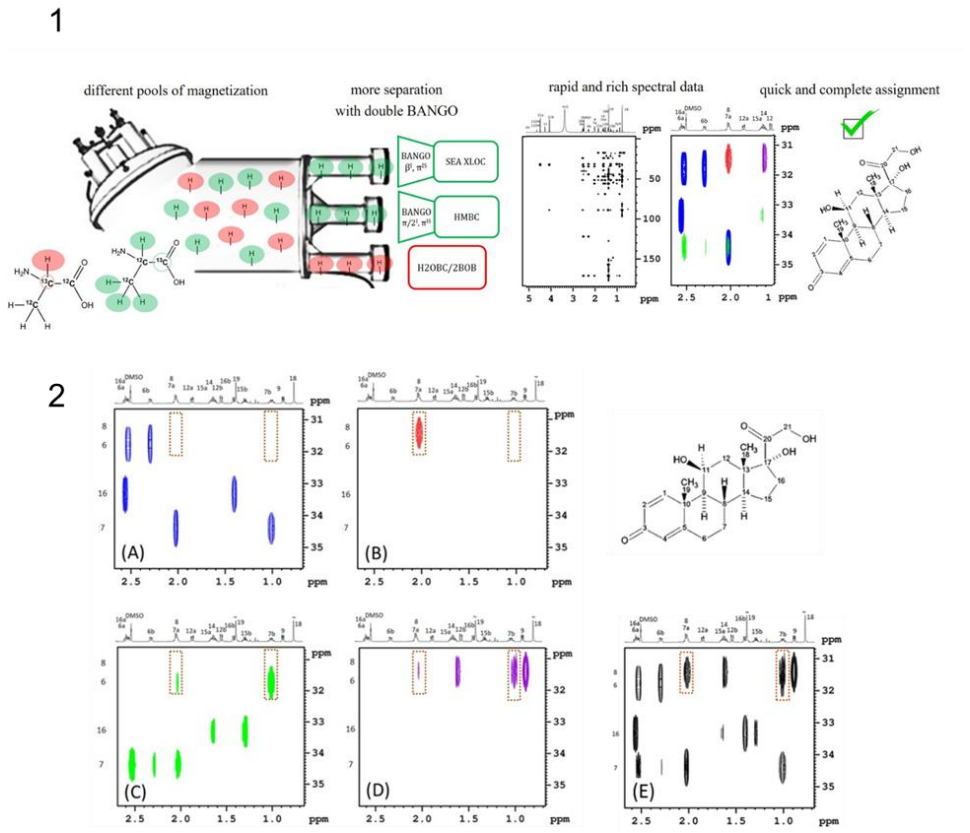

Figure 8. 1) *The three-module BANGO {SEA XLOC}-{HMBC}-{H2OBC} experiment exploits different magnetization reservoirs (red: ^{13}C-H and green: ^{13}C-^{12}C-H protons). The complete homo- and heteronuclear resonance assignment is feasible after the extraction of spectra from the complex dataset. 2) Prednisolone signal* overlaps *were resolved with the linear combinations of the edited spectra. The one- and two-bond correlations of C6, C7, C16 (A,C, even multiplicity) and the one- and two-bond correlations of C8 (B,D, odd multiplicity) are displayed. All correlations are shown in the H2OBC spectrum (E) without editing.*

5. We have developed a novel pulse sequence design strategy yielding extremely rapid combined NMR measurements without relaxation delay (NORD: NO Relaxation Delay spectroscopy).

The selective handling of different magnetization components was carried out by the BANGO (arbitrary excitation angles) and the BIG-BIRD (arbitrary phase and excitation angle manipulations) sequences. After the 'Ernst-concept' based optimization of pulse angles, phases and sequential ordering of the individual experiments, complex and time-efficient combined pulse sequences were established without recovery delays. Since the magnetization share is highly efficient, the 'non-active' components have enough time to recover while the active experiment is running (*Figure 9A*). NORD {HMBC}-{H2OBC} with NUS was measured on trisaccharide and pentasaccharide samples in less than 2 and 6 minutes, respectively. The three-module NORD {SEA XLOC}-{HMBC}-{2BOB} was acquired in 42 minutes on hydrocortisone, providing sufficient data for the complete NMR assignment of the molecule (*Figure 9B,C*).

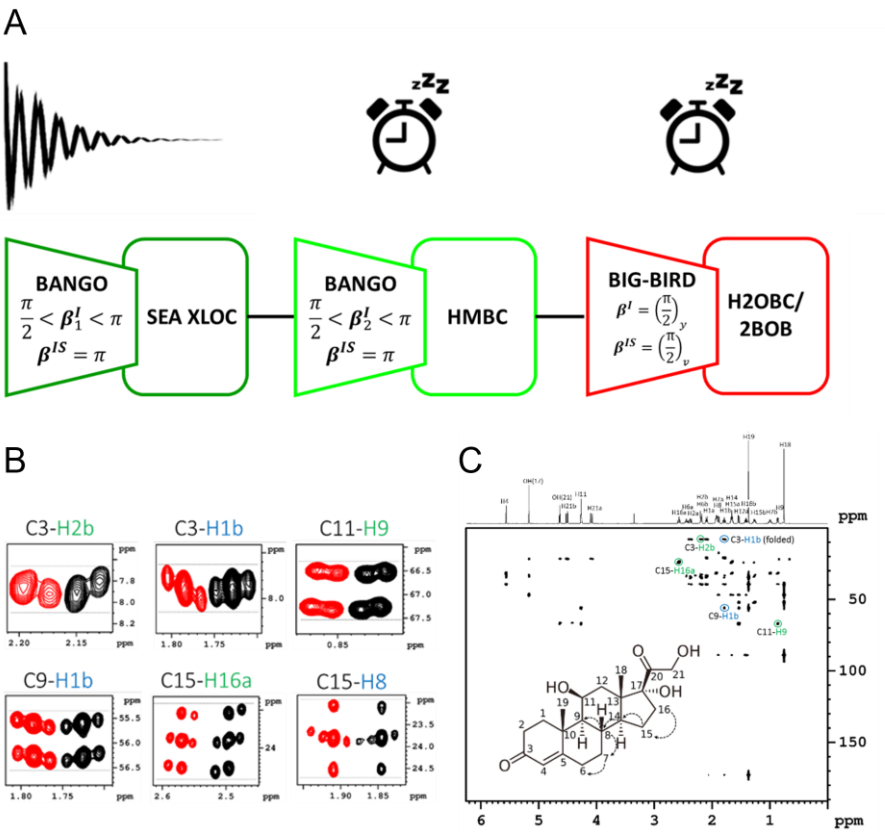

Figure 9. *A) The simplifed scheme of the NORD {SEA XLOC}-{HMBC}-{H2OBC} experiment. The magnetization reservoir exploited by the third module is relaxing towards equilibrium while the first two experiments are running. B) Two- and three-bond ^{1}H - ^{13}C correlations in hydrocortisone are distinguished with the SEA XLOC measurement based on peak widths (in F1) comparison of ZQ (red) and 2Q (black) subspectra. C) HMBC spectrum of hydrocortisone used for resonance assignment.*

IV. Potential applications of the results

The results of this study contribute to the structural and dynamical understanding of the underlying molecular regulation mechanisms behind muscle cell differentiation. We have shown an approach how the transcriptional activity of the MEF2D can be fine-tuned. The role of protein dynamics is also highlighted which must be considered in structure-activity relationships, especially when working with intrinsically disordered proteins. Moreover, the rich atomic-level data obtained might be used in a structure-based drug design project targeting MEF2D in the future.

The new, combined NMR experiments contribute to the method development efforts of the NMR community. The time efficiency and the obtained diverse set of spectral data makes the BANGO and double BANGO sequences good candidates for routine measurements in rapid, automated workflows for the structure elucidation of small- and medium-sized molecules in the pharmaceutical industry.

Finally, the NORD approach provides a pulse sequence design framework for a new family of rapid NMR measurements. To date, NORD experiments are one of the fastest ways to obtain ^{13}C-^{1}H and ^{1}H-^{1}H correlation information useful for the resonance assignment of small- and medium-sized molecules. However, it is worth noting that this pulse sequence design strategy can be easily extended and adapted to further multinuclear and multidimensional NMR methods on a broader scale. More work is going on in our lab to introduce additional experiments and measure new NMR-active nuclei.

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Subject: PhD Publication List

Candidate: Tamás Milán Nagy
Doctoral School: Doctoral School of Chemistry
MTMT ID: 10057983

List of publications related to the dissertation

Foreign language scientific articles in international journals (3)

1. **Nagy, T. M.**, Kövér, K. E., Sørensen, O. W.: NORD: NO Relaxation Delay NMR Spectroscopy. *Angew. Chem.-Int. Edit. 60* (24), 13587-13590, 2021. ISSN: 1433-7851. DOI: http://dx.doi.org/10.1002/anie.202102487 IF: 16.823

2. **Nagy, T. M.**, Kövér, K. E., Sørensen, O. W.: Double and adiabatic BANGO for concatenating two NMR experiments relying on the same pool of magnetization. *J. Magn. Reson. 316*, 1-4, 2020. ISSN: 1090-7807. DOI: http://dx.doi.org/10.1016/j.jmr.2020.106767 IF: 2.229

3. **Nagy, T. M.**, Gyöngyösi, T., Kövér, K. E., Sørensen, O. W.: BANGO SEA XLOC/HMBC-H2OBC: complete heteronuclear correlation within minutes from one NMR pulse sequence. *Chem. Commun. 55* (81), 12208-12211, 2019. ISSN: 1359-7345. DOI: http://dx.doi.org/10.1039/C9CC06253J IF: 5.996

List of other publications

Hungarian scientific articles in Hungarian journals (1)

4. Gróf, P., Knapp, K., Schlosser, G., **Nagy, T. M.**, Timári, I., Borics, A., Kövér, K. E., Csík, G., Májer, Z.: Diszulfidhidat tartalmazó ciklikus peptidek UV-besugárzásának hatására keletkező szabadgyökök és szulfhidril-csoportok detektálása. *Magyar Tud. 177* (1), 50-54, 2016. ISSN: 0025-0325.

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Foreign language scientific articles in international journals (4)

5. Timári, I., **Nagy, T. M.**, Kövér, K. E., Sørensen, O. W.: Synergy and sensitivity-balance in concatenating experiments in NO relaxation delay NMR (NORD).
Chem. Commun. 58 (15), 2516-2519, 2022. ISSN: 1359-7345.
DOI: http://dx.doi.org/10.1039/D1CC06663C
IF: 6.065 (2021)

6. Bereczki, I., Szűcs, Z., Batta, G., **Nagy, T. M.**, Ostorházi, E., Kövér, K. E., Borbás, A., Herczegh, P.: The First Dimeric Derivatives of the Glycopeptide Antibiotic Teicoplanin.
Pharmaceuticals (Basel). 15 (1), 1-17, 2022. EISSN: 1424-8247.
DOI: http://dx.doi.org/10.3390/ph15010077
IF: 5.215 (2021)

7. Gyöngyösi, T., **Nagy, T. M.**, Kövér, K. E., Sørensen, O. W.: Distinguishing between two- and three-bond correlations for all 13C multiplicities in heteronuclear NMR spectroscopy.
Chem. Commun. 54 (70), 9781-9784, 2018. ISSN: 1359-7345.
DOI: http://dx.doi.org/10.1039/C8CC05156A
IF: 6.164

8. **Nagy, T. M.**, Knapp, K., Illyés, E., Timári, I., Schlosser, G., Csík, G., Borics, A., Májer, Z., Kövér, K. E.: Photochemical and structural studies on cyclic peptide models.
Molecules. 23 (9), 1-20, 2018. EISSN: 1420-3049.
DOI: https://doi.org/10.3390/molecules23092196
IF: 3.06

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V. Conference presentations related to the dissertation

1.) Nagy, T. M., E. Kövér, K., Sørensen O. W: **NORD spektroszkópia: mérések holtidő nélkül!** *NMR Working Committee Meeting, 09.09.2021, Mád, Hungary.*

2.) Nagy, T.M., Gönczi, M., Fehér, K., Bécsi, B., Erdődi, F., E. Kövér, K., Fuxreiter, M.: **Az aggregáció szerepe a rendezetlen fehérjék működésében: NMR módszerfejlesztés és számításos kémiai vizsgálatok.** *New National Excellence Program conference, 14.06.2021, online.*

3.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **A dinamika és az oligomerizáció szerepe a MEF2D rendezetlen fehérje működésében.** *NMR Working Committee Meeting, 13.10.2020, online.*

4.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **Rendezetlen fehérjék dinamikai vizsgálata és NMR-kísérletek fejlesztése.** *New National Excellence Program conference, 15.06.2020, online.*

5.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **Rendezetlen fehérjemodellek NMR és számításos vizsgálata.** *Applied quantum chemistry and molecular dynamics group, 12.03.2020, Debrecen, Hungary.*

6.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **A MEF2D β-domén bolyhossága és szerepe a biológiai aktivitásban: NMR és számításos vizsgálatok.** *I. Young Chemist's Symposium, 03.04.2019, Debrecen, Hungary.*

7.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **Fuzziness of MEF2D β-domain and its role in biological activity: NMR and in-silico studies** *NMR Working Committee Meeting, 17.05.2019, Balatonszemes, Hungary.*

8.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **Determining fuzziness of the Mef2D β-domain by NMR experiments and MD-calculations.** *9th Chemistry towards Biology Conference, 24.09.2018, Budapest, Hungary.*

9.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **Rendezetlen peptidmodellek dinamikájának vizsgálata NMR-rel és számításos módszerekkel.** *NMR Working Comittee Meeting, 16.05.2018, Balatonszemes, Hungary.*

VI. Posters related to the dissertation

1.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **Dynamical studies of the Mef2D β-domain by NMR and computational methods.** *European Magnetic Resonance Meeting, 26.08.2018, Nantes, France.*

2.) Nagy, T.M., Gönczi, M., Fehér, K., Fuxreiter, M., E. Kövér, K.: **The fuzzy β-domain of MEF2D: NMR and computational studies.** *European Magnetic Resonance Meeting, 27.08.2019, Berlin, Germany.*