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An die Deutsche Forschungsgemeinschaft
Lebenswissenschaften 1: Molekulare und Organismische Biologie
z.Hd. Herr Dr. Thomas Baumgarten

Subject: Statement by the applicants on the expert opinion and on the decision of the review board regarding proposal FI 841/11-1 & STA 1325/3-1

Dear Sir or Madam

We would like to begin by thanking the experts (reviewers) and the members of the Fachkollegium for the work and time they have invested in preparing the expert reports and on the justification of the evaluation. As already mentioned by the Fachkollegium, both reviewers recommend our proposal with highest funding priority. However, there were some concerns by the Fachkollegium which led to the rejection of the application in its submitted form. In this response letter, we address the individual points of criticism in detail below.

Concern 1

The study of macromolecular crowding using deuterated PEG raises questions about how well it mimics actual cellular conditions.

Answer: First of all, macromolecular crowding is only one aspect of "cellular environment mimicking" (the latter typically deals with a much more complex issue). For many questions, it makes sense to focus first on macromolecular crowding by using synthetic or protein-based crowder molecules. Numerous essential properties of proteins (and their interaction partners) such as translational diffusion, conformational dynamics, binding interactions and folding/unfolding transitions have already been analysed very successfully with synthetic macromolecular crowders, such as PEG or Ficoll. For example, in order to understand the physico-chemical principles of macromolecular crowding, it is important to separate entropic from enthalpic effects, which can be done very well by using synthetic crowding agents (*Zosel et al., PNAS, 2020, 117,13480-13489; Stringer et al., J. Phys. Chem. B, 2023,*

127, 5837-5849). Nevertheless, globular proteins are biologically more relevant. After all, cells are crowded with proteins, not PEG, dextran, or Ficoll. But systematic studies of them are more challenging compared to studies using synthetic polymers as crowders, for example due to protein stability at high concentrations or unwanted background signals. In the case of protein crowding, effects of protein–protein interactions have to be considered and chemical interactions may play a role (for more detailed discussion see *Speer et al., Annu. Rev. Biophys. 2022, 51, 267-300*; *Pastore & Temussi, Trends in Biochem. Sci., 2022, 47, 1048-1058*).

As described in section "2.3.2 How IDP conformational dynamics adapt to environmental conditions" the overall aim of the work package is to investigate the different effects of macromolecular crowding by (i) synthetic crowders (linear polymers such as PEG and branched synthetic crowders ficoll and dextran) and make the direct comparison to crowding conditions imposed by (ii) a proteinaceous crowder, namely the maltose binding protein (MaBP). In particular, we want to focus our studies here on the question how crowding by different molecules – (i) synthetic and (ii) protein crowders – affects the structural and dynamic properties of an IDP (in our case the myelin basic protein). Hence, with our proposed experiments we directly address the mentioned criticism and aim to provide a clear answer to the question how well PEG (or the other synthetic molecules dextran and ficoll) can resemble crowding conditions induced by the protein crowder MaBP or alternatively cause different effects. Furthermore, we would like to point out that in cellulo studies are unfortunately not compatible with neutron scattering experiments in this case and in vitro experiments are needed to obtain molecular information under well-defined and controlled experimental conditions.

From a technical point of view MaBP needs to be used in its deuterated form (dMaBP) during the planned neutron scattering experiments to be 'matched out' in 100% D_2O solvent and to be rendered 'invisible' for the neutron beam. For that purpose, one gram of dMaBP has been produced in the meantime by the Deuteration Lab of the Spallation Neutron Source (SNS, Oak Ridge National Lab, USA) under the condition that the SNS instrument suite will be used for the experiments. The match point of the dMaBP has been verified by the SNS Deuteration Lab, and dMaBP was found to be matched out in 100% D_2O which is the prerequisite for our planned neutron scattering experiments. The dMaBP is now ready and can be used for the planned SANS and NSE experiments as described in our DFG & ANR research proposal.

As already mentioned in the application, the measurement with fluorescence-based methods in the presence of crowders is methodologically straight forward, as the crowder molecules are not dye-labelled. Also when using fluorescence techniques, we would first use synthetic crowders (e.g., PEG) and then protein-based ones. For the latter, it has also recently been shown that such smFRET studies are technically feasible (see for example *Galvanetto et al., 2023, Nature, 619, 876-882*). In principle, smFRET could even be used to study cellular crowding behaviour (see for example *König et al., 2021, Angew. Chem. Int. Ed., 60, 10724-10729*), but we are not aiming for such measurements in the context of this proposal.

Concern 2

Additionally, the planned molecular dynamics simulations of intrinsically disordered proteins (IDPs) are somewhat vague and lack necessary detail.

Answer: Concerning the simulation of the partially disordered Myelin Basic Protein, the recent reference by Ramya and Helina Hilda, which is cited in our project description (Ref. [89] ibid), gives a complete description of the protocol, including the generation of starting structures of disordered regions

by the DMRpred software (see Ref. [90]) and details of the following MD simulations. This is certainly a good starting point for the project and shows that the simulation of this protein is not completely new territory. Furthermore, we will test also other approaches for the generation of starting configurations of IDPs, such as the Ensemble Optimization Method (EOM) [1,2] and the Flexible-Meccano approach [3], which have been developed by Svergun, Blackledge, and co-workers to integrate experimental data from SAXS and NMR (see below for all additional references [1-9]). The essential point in this context is to monitor the secondary structure content in the generated starting structures and also in the following MD simulations. For this purpose, we will use our ScrewFit/ScrewFrame algorithms [4,5], which are particularly suited for this purpose since they combine secondary structure description and detection. The folding of any polypeptide chain is here quantified through a succession of screw motions relating residue-fixed coordinate frames and secondary structure elements are visible through repetitions of certain characteristic screw motions. In Ref.[6] the ScrewFit algorithm has been calibrated for secondary structure assignment, using the ASTRAL and SCOP databases for secondary structure elements [7,8]. We think that these analyses will yield useful information on the possible differences between different methods for the generation of starting structures for MD simulations, but also differences resulting from the MD force fields and the simulation algorithm itself. We mention in this context an article by Shrestha, Smith & Petridis [9] who demonstrate by Hamiltonian replica-exchange MD that the structural sampling is enhanced and leads to unbiased and accurate ensembles, which reproduce for three IDPs SAXS and NMR chemical shift experiments.

additional related references

1. G. Tria, H. D. T. Mertens, M. Kachala, and D. I. Svergun, “Advanced ensemble modelling of flexible macromolecules using X-ray solution scattering,” IUCrJ, vol. 2, no. 2, pp. 207–217, 2015.
2. P. Bernado, E. Mylonas, M. V. Petoukhov, M. Blackledge, and D. I. Svergun, “Structural Character- ization of Flexible Proteins Using Small-Angle X-ray Scattering,” Journal of the American Chemical Society, vol. 129, no. 17, pp. 5656–5664, 2007.
3. V. Ozenne, F. Bauer, L. Salmon, J.-R. Huang, M. R. Jensen, S. Segard, P. Bernado, C. Charavay, and M. Blackledge, “Flexible-Meccano: a tool for the generation of explicit ensemble descriptions of intrinsically disordered proteins and their associated experimental observables,” Bioinformatics, vol. 28, no. 11, pp. 1463–1470, 2012.
4. G. R. Kneller and P. Calligari, “Efficient Characterization of Protein Secondary Structure in Terms of Screw Motions,” Acta Crystallogr D, vol. 62, no. 3, pp. 302–311, 2006.
5. G. R. Kneller and K. Hinsen, “Protein Secondary-Structure Description with a Coarse-Grained Model,” Acta Crystallogr D, vol. 71, no. 7, 2015.
6. P. A. Calligari and G. R. Kneller, “ScrewFit: Combining Localization and Description of Protein Sec- ondary Structure,” Acta Crystallogr D, vol. 68, no. 12, pp. 1690–1693, 2012.
7. J.-M. Chandonia, G. Hon, N. S. Walker, L. Lo Conte, P. Koehl, M. Levitt, and S. E. Brenner, “The ASTRAL Compendium in 2004.,” Nucleic Acids Research, vol. 32, no. Database issue, pp. D189–92, 2004.
8. N. K. Fox, S. E. Brenner, and J. M. Chandonia, “SCOPe: Structural Classification of Proteins–extended, Integrating SCOP and ASTRAL Data and Classification of New Structures,” Nucleic Acids Research, vol. 42, no. D1, pp. D304–D309, 2013.
9. U. R. Shrestha, J. C. Smith, and L. Petridis, “Full structural ensembles of intrinsically disordered proteins from unbiased molecular dynamics simulations,” Communications Biology, vol. 4, no. 1, p. 243, 2021.

Concern 3

Lastly, the personnel requested for the project is not fully justified, and a more detailed rationale for the staffing requirements would strengthen the proposal.

Answer: Although the technical expertise for carrying out the experimental work and data analysis is available in the respective working groups, additional personnel are of course required to carry out the extensive work programme in all of our 4 working groups. In addition, it is essential for the success of the cooperation project that communication and cooperation between all four locations of the participating working groups functions well. The employees we are looking for should make a significant contribution to this. Our job description "Doctoral Researcher" may have caused a misunderstanding, we really meant a PhD candidate/student for three of the four positions. In the following, we describe the requirements and specifications of the personnel needed in more detail.

(i) Fitter, Jörg (AG Biophysik, RWTH Aachen)
1 PhD student for 3 years at E 13 TV-L Level (75%): N.N., start as soon as possible
The demanding research programme proposed in this grant application requires a talented PhD student who, in addition to a solid education in physics, has an interest (and if possible prior knowledge) in biological issues. The candidate must not only carry out the numerous fluorescence microscopic and spectroscopic measurements (incl. multi-parameter fluorescence analysis on single molecule level), but also establish some of the latest state-of-the-art analysis methods (for details see *ref. [80],[84]*) in the working group and apply them to the own measurement data.

(ii) Stadler, Andreas (FZ Jülich)
1 PhD student for 3 years at E 13 TV-L Level (75%): N.N., start as soon as possible
The research project requires a very good candidate (physicist or physical chemist) who will perform neutron scattering experiments (incl. suppl. biophysical studies) and data analysis. For neutron scattering experiments best conditions need to be identified and samples have to be pre-characterized using e.g. dynamic light scattering and small-angle scattering before neutron scattering experiments can take place. Analysis of neutron scattering data using polymer-based modelling requires detailed knowledge and skills in scientific programming and data analysis. A certain amount of deuterated MaBP has already been produced by the deuteration lab of the SNS facility (Oak Ridge, USA) under the provision that neutron scattering experiments using deuterated MaBP will be performed at the SNS.

(iii) Peters, Judith (UGA Grenoble)
1 PhD student for 3 years: N.N., start as soon as possible.
Sample production, conducting neutron scattering experiments and data analysis take a lot of time. The PhD student will be in charge of these tasks, will write manuscripts and present the results in international conferences. The scientific subject will enable students to acquire various specialist skills in this multidisciplinary field.

(iv) Kneller, Gerald (Univ. Orleans)
1 Doctoral Researcher for 3 years, start as soon as possible.
The person to be recruited for this task should be a postdoc since some experience with MD simulations, program development, and data analysis is required for a successful accomplishment of the program. The postdoc should hold a PhD in physics or physical chemistry and have deep interest in linking theoretical concepts with experimental data. Ideally he or she should have some experience in programming, data analysis and molecular simulation.

We hope that with this more detailed information we can answer and refute the main criticisms of the review board and **would like now to resubmit our application together with this letter** for a new review, hoping that the project will be funded, as recommended by both referees.

With kind regards

Prof. Dr. J. Fitter & Dr. A. Stadler

also on behalf of our French colleagues Prof. Dr. J. Peters & Prof. Dr. G. Kneller