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Report on the doctoral dissertation of Mr. Tomasz Skora entitled "Diffusion and reactions under crowding: Theory and simulations"

The current PhD Thesis consists of seven Chapters (Chapter #1: Introduction, #2: Theoretical background, #3: Methods, #4: Diffusion in crowded media, #5: Enhanced enzyme diffusion, #6: Reactions under crowding, and #7: Conclusions), it contains 5 Appendices, and the Bibliography encompassing 315 References. The Thesis is based on the results published by Mr. Skora [mainly as the first author] as scientific 3 articles in solid peer-reviewed journals (with the impact factors of ca. 3-4, namely in JPCB, PCCP, and Soft Matter), with one more manuscript being reported to be submitted to PRL (and with two more manuscripts in preparation). Mr. Skora coauthored 3 more publications in 2018-2020, making his list of publications very impressive for a PhD candidate.

The main subject of the Thesis (going as a "red thread" through the entire text) is the investigation---based both on the results of extensive computer simulations and theoretical calculations---of the diffusive characteristics and reactions of various tracers in crowded suspensions, often with the target to mimic the conditions in the cytoplasm of living biological cells. Spherical and elongated as well as hard and soft crowders are compared and contrasted, both as hydrodynamics-free systems as well as in the presence of hydrodynamic interactions (HDIs). The reaction and diffusion rates of the tracers are examined in great details, with extensive and well balanced referencing to the existing body of literature, including historical, pioneering, and most recent investigations.

Below, I provide an overview of the main scientific achievements of Mr. Skora in the present Thesis (Chapter by Chapter) and also explicitly list the strengths of the conducted investigations and the applicability of the obtained results.

Chapter 1 starts with a motivation of research on various aspects of the cellular mechanics of proteins and other cell constituents in the presence of macromolecular crowding (MMC). The Section-by-Section overview of the whole work is presented here too. The list of all the abbreviations used is conveniently presented at the start of the text, making the reading of this excellently written and stylistically well-thought dissertation very pleasant and straightforward. This makes me think that the average number of readers of this Thesis might be well above the mentioned ≈1.6.

Chapter 2 describes a number of fundamental theoretical concepts required to understand the results of the current Thesis, including the theory of Brownian motion, the fluctuation-dissipation theorem, some properties of anomalous-diffusion motion, multiple features of MMC, and some details of chemical reactions and reaction equilibria. In this Chapter, the author elegantly presents several physically important concepts and observations in a way easily understandable for an average reader, often with just-right amount of mathematical details. The essential details of all physical concepts [employed later in the core part of the Thesis] are excellently and fully explained in this Chapter, with a proper chronological referencing to the literature sources and with nice historical intermezzos.

In **Chapter 3**, with the preparatory material given in Chapter 2, the candidate presents various computational methods employed to carry out the computer simulations of the Thesis, such as the Brownian and Stokesian Dynamics, as well as the Monte-Carlo-based integration methods. The principles of the explicit or fine-grained all-atom as well as of the coarse-grained simulation methods are described here. The HDIs of different particles diffusing in water---both within the Oseen approximation and via the Rotne-Prager-Yamakawa matrix---are explained in their details here (used in the main part). The latter approach, e.g., gives rise to long-ranged $\propto 1/\text{separation}$-decaying interactions which should be considered in computer simulations, usually performed with the periodic boundary conditions and with the help of the so-called Ewald summation. The latter is explained to the reader in detail in Appendix B. The equations are brilliantly formatted and the notations are kept unified and consistent throughout the entire text of the Thesis. A particular emphasis is set on the Monte-Carlo simulations of various physical systems with excluded-volume interactions (EVIs). The software package *ExVol* developed by Mr. Skora to tackle these challenges is presented in Appendix D.4 and also deposited on *github*.

Chapter 4 is devoted to the consideration of diffusion in crowded media and to a detailed description of different concepts and implications of the MMC, HDIs, and attractive interactions of the tracer particles with the constituents of a [model] biological cell.

This Chapter starts with a structured literature review, opening with an overview of measurements of the implications of MMC both in pro- and eukaryotic cells. Here, the effects of the particle size, the cytoplasm viscosity, etc. are introduced and physically discussed. The mechanisms of diffusion slowing-down due to an increased viscosity, due to EVIs, HDIs, electrostatic interactions (ESIs) as well as other attractive interactions, and, lastly, due to the confinement of a diffusing particle in polymeric networks are systematically overviewed in the following Subsections. Here, certain ambiguities, conflicting findings, and controversies in interpretation of the results of some experimental observations are critically overviewed. This shows not only very solid overall understanding of the field by the candidate, but also an extremely high level of expertise and a wide-field knowledge in a number of specific areas, a prerequisite for a deep and insightful PhD Thesis, like the current one.

In this Chapter, the author overviews the characteristic behaviors of each type of physical interactions (such as EVIs, HDIs, ESIs, etc.) potentially realizable for artificial crowders as well as for real protein molecules (both of spherical and elongated shapes diffusing, i.a., in biological cells). The main target here is to be able later to distinguish the impact of each of these interactions in the data of computer simulations being performed.

In Chapter 4.1 a diffusion slowing-down due to a size-sensitive confinement of the tracer particles in a polymeric network is also discussed, including the resulting transient anomalous (sub-)diffusion. The latter emerges, e.g., due to caging and trapping effects at short times and hopping events of the particle between the cages at long times.

In Chapter 4.2 the first computer simulations of HDIs performed by the author with the help of the Stokesian and Brownian Dynamics are described. A separate piece of software has been developed here to treat the lubrication effects, as presented by Mr. Skora in Appendix D.2. Here, both the short- and long-time diffusion coefficients D are computed---as extracted via fitting the generated time-averaged mean-squared displacements (TAMSD) of ficoll70-like artificial spherical tracers (with the hydrodynamic radii of 5.1 nm) under the MMC-conditions with the occupied fraction of $\phi \approx 5$, 10, and 20 % (both without HDIs as well as with near- and far-field HDIs). Apparent discrepancies between the observed nearly linear decays of D magnitudes with the MMC-fraction ϕ are discussed.

In Chapter 4.3 the author additionally presents the results of the simulations of crowders of elongated shapes---such as, e.g., the MMC-effects induced by about 5-helical-pitch-long double-stranded DNA (ds-DNA) molecules---onto the diffusion of streptavidin tracers. One- and two-component MMC systems are compared and contrasted in this Subsection.

The ds-DNA segments are modeled in a coursed-grained fashion, with 8 beads each containing only 20 % of the "native" ds-DNA charge. The ESIs were thus treated with $\theta \approx 80$ % charge-renormalization fraction due to the condensation of DNA counterions, as theoretically accepted in the solution of a monovalent salt with Manning's fraction (that is $\theta \approx 1\text{-}1.7/7.14 \approx 0.76$ for a ds-DNA in the B-form). For ds-DNA fragments, both translation and rotational diffusivities are computed here and a comparison of the observed trends with the results of simulations from other groups is presented.

All model parameters used in the simulations and the bond potentials are thoroughly described

and critically discussed here too. The statistical uncertainties in determining the *D* values from the observed TAMSD growth are reduced via dividing the set of available *in silico* trajectories of the tracer particles into 5 independent subsets and via averaging among them.

Computer simulations of the tracer diffusion in the solution of elongated molecules are performed here for a more computationally demanding---but at the same time for a much more experimentally relevant--- situation of a realistic 3D system, as compared to some simplistic 2D-based simulations conducted in other groups recently. This more realistic approach is clearly motivated by some experimental datasets, indicating a scientifically "ripe" approach in doing science demonstrated by Mr. Skora.

Realistic parameters of the simulation potentials are chosen and critically assessed here, may be except the Debye screening length λ (that is often taken to be about the Bjerrum length, l_B=0.714 nm, and not λ=2.3 nm). The former value is, however, based on the assumption that all screening effects inside a cell stem from freely diffusing ions of a 1:1 salt at 150 mM concentration that is not the case for, e.g., multivalent ions and [often net-negatively] charged proteins, as in the *E. coli* cell. The Lennard-Jones bond-strength parameters are, e.g., chosen so that the values of the diffusivity of some standard green-fluorescent proteins in the *E. coli* cytoplasm are properly reproduced. It is demonstrated here that elongated ds-DNA rods hinder tracer diffusion much more efficiently than spherical crowders do, due to a larger relative excluded volume (although the fine details of the diffusion slowing-down curve with the MMC-fraction differ somewhat from the experimentally observed trends).

In this Subsection the shape effects emerging for elongated crowders (as compared to spheres) are examined. It is found, e.g., that in the system of both elongated and spherical crowders the excluded-volume argument for predicting the degree of impediment of the tracer diffusion is not sufficient (elongated crowders are more efficient, in agreement with the reports from other groups). The degree of hindrance of rotational diffusion by the MMC is shown here to be weaker than that of translational diffusion.

In Section 4.4 the effects of attractive non-ESIs are reported and discussed, on the example of ds-DNA and streptavidin system. The diffusion coefficients in this situation of attractive interactions between the constituents are carefully computed based on the MSD---and not on the TAMSD---evolution because the aggregation processes taking place in the system are not at equilibrium thus violating the ergodicity hypothesis (that would state that MSD(t)=TAMSD(t) at long times). The impact of mutual attractive interactions in the MMC-system of ds-DNA and ficoll70 molecules with streptavidin tracers are then studied in detail.

In addition to the shape-mediated effects, in Chapter 4.5 one more interesting---and also *in-vivo* important---effect of molecular softness of the crowding molecules is considered by Mr. Skora. For *in-vitro* systems---as well a number of artificial crowders used to mimic the crowded conditions inside biological cells---polymeric and rather soft or floppy crowders---such as the widely used PEG and dextran molecules---are rather common. They are in contrast to, e.g., comparatively hard ficoll molecules. The quantitative understanding of the implications of softness of model crowders is, thus, of paramount theoretical importance because these effects are omnipresent for real crowding molecules in living biological cells.

Here, based on the results of simulated translational diffusion of various tracers in the solutions of soft and hard crowders, the author studied the implications of softness. Both theoretically and in terms of computer simulations, this area is a relatively new field of research, with rather "patchy"/nonsystematic results. The computer code of Mr. Skora---where the effects of softness are separable from other complications (such as nonsphericity of the crowders and their mutual interactions)---is thus a perfect instrument to gain a better understanding of individual contributions to a slowed-down diffusivity of the tracers triggered by MMC at varying volume fractions ϕ.

In this Subsection it is unveiled that for soft spherical crowders the reduction of the diffusivity with the MMC fraction ϕ as about 4 times weaker than that in the solution of hard spherical crowders. Further, the diffusive characteristics of nonspherical tracers (of elongated as well as of Y-shaped form) in the solutions of soft crowders are investigated. An interesting new conclusion is drawn here: "for some macromolecular shapes [of the tracers], the diffusion might be faster in hard crowders".

In **Chapter 5** Mr. Skora progresses from the equilibrium to nonequilibrium considerations and he describes enhanced diffusion of enzymes in the presence of a reaction substrate they can catalyze. Here, i.a., the question of metabolic activity coupled to the macromolecular diffusivity is considered. Again, the Chapter starts with a solid overview of the experimental literature on the enzyme-diffusion enhancement. The effects of enzyme-size reduction

onto the enhanced diffusivity as well as the mechanisms of a self-induced phoresis are discussed.

The model of a fluctuating dumbbell for examining the effects of conformational changes onto the tracer/enzyme diffusivity is introduced and examined here. An entropically favorable state of open dumbbells competes here with a compact state and yields quicker diffusion characteristics. Indeed, in the absence of MMC a reduction of the diffusivity by 15-20 % was observed in simulations. In the presence of MMC by passive spherical crowders, the author studies the behavior of the same fluctuating-dumbbell model. Surprisingly, it is found that the enhancement of the diffusivity increased for the case of enzyme crowding and decreased for sphere-induced crowding.

Moreover, the diffusion of passive spherical tracers in a crowded solution of enzymes (modeled by the same fluctuating dumbbells) is also examined. The conclusion here is that, despite a stronger enhancement of the sphere diffusion with the enzyme concentration, the effect is found to be smaller as compared to the enhanced enzyme diffusion. The studied concentrations of enzymes in these simulations are, however, orders of magnitudes higher than in real experiments, prohibiting a direct comparison of the obtained results.

In Chapter 5.4, the method of H-cell microfluidics is discussed by the author as a robust tool for measuring the diffusion and transport coefficients, as occur in Fick's diffusion law for the space-time evolution of the analyte concentration. The development of the concentration profiles in such fluidic channels is modeled by Mr. Skora via a discretized Euler scheme. The numerical scheme is optimized and applied to the description of the diffusivity-enhancement data of enzymes, highlighting also a need for highly accurate concentration measurements.

Chapter 6 is devoted to the investigation of the shifts of chemical equilibria due to MMC for different reactions taking place in crowded media. In particular, the conformation-changing enzymes and the implications of MMC onto cooperativity of the divalent-binding reactions are considered here. The presentation starts in Section 6.1 with a detailed literature overview of various chemical reactions and transport characteristics in the presence of MMC. Here, the entropic depletion forces as well as more compact conformations of a number of biomolecules being favored in the presence of MMC are discussed. Some enzymatic reactions and protein-folding equilibria are outlined here too, both from the viewpoint of experiments and of simulations, comparing and contrasting the effects induced by small versus large [spherical and inert] crowders.

Essential differences for a "zoo" of biological crowders ubiquitously occurring in the cell cytoplasm---with variability in shape, softness, relative abundances, with heterogeneities of their distributions across the cell, and with a number of (often significant, both specific and nonspecific) interactions present---is then highlighted in the text. This serves as a motivation to study more complex, realistic, and biologically relevant crowders, as performed by Mr. Skora in this PhD Thesis.

In Chapter 6.2 the Michaelis-Menten kinetic model of enzymatic reactions and the enzyme kinetics involving changes in molecular conformations is described by Mr. Skora. An example of a two-state enzymatic system with the open (active) and closed conformations in the presence of MMC is presented. The MMC-induced shifts of the equilibrium are due to the free-energy differences which are computed later within the scope of scaled particle theory. The results of the Brownian-Dynamics simulations within the fluctuating-dumbbell model in the presence of MMC are presented next, as a toy system mimicking the dynamics of size- and conformation-changing enzymes (with both open and closed states). Not surprisingly, the MMC is shown to disfavor open configurations and thus reduce the enzymatic activity. The free energies of enzyme opening and the relative enzymatic rates are systematically computed here as functions of the MMC volume fraction, ϕ.

In Chapter 6.3 the binding of divalent molecules and binding cooperativity in the presence of MMC are treated; these phenomena occur, e.g., upon aggregation of some intrinsically disordered proteins. The latter---serving as one of the motivations for this study---takes place in and causes some neurodegenerative diseases, such as Parkinson and Alzheimer predominantly in cells of older people. With age, the cell-hydration levels are known to become generally lower, strengthening the aggregation effects by MMC and favoring the association reactions of alpha-synuclein and amyloid-beta proteins, respectively.

Regarding the cooperativity, the respective α parameters have been computed both analytically and via computer simulations in the presence of MMC, as compared to those in a crowding-free system. It is demonstrated, for instance, that α becomes progressive more sensitive to MMC for reactive molecules which are larger than the inert crowders. Also, for varying sizes of the reagents and crowders it was found that the magnitude of the

observed changes grows drastically for larger reagent-to-crowder size ratios.

The cooperativity of cyclization and polymerization reactions of polymeric chains consisting of monomeric units in the crowded solution is considered in Chapter 6.3.3 where the results of the Brownian-Dynamics simulations are presented. Here, ficoll70 is used and mimicked in computer simulations, as a typical and widely used artificial crowder, with the volume-occupations fractions from ≈5 % to ≈44 %. The extension of the polymer chain in terms of its gyration radius---both for linear and ring-like molecules---in comparison to that of a self-avoiding walk are systematically investigated here by the means of computer simulations. Several biologically important differences between homo- and heterogeneously crowded (like a realistic cell cytoplasm) media are examined. The relevance of the considered cooperativity effects for the functioning principles of complex biological systems is discussed in the end of this Chapter.

In **Chapter 7** the candidate summarizes the main results of the dissertation and lists its conclusions. Firstly, based on the performed Brownian- and Stokesian-Dynamics simulations, Mr. Skora concludes that considering only long-ranged HDIs is insufficient in highly crowded hard-sphere suspensions, leading to strongly overestimated actual diffusion coefficients (Chapter 4). The importance and the correct description of the near-field HDIs in these systems are pointed out.

Secondly, Mr. Skora concludes (based on the results of Chapter 4) that elongated crowding particles are more efficient in slowing down the tracer diffusion, as compared to spherical crowders [at the same volume-occupation fraction]. It is hypothesized that---despite this trend being consistent with some fluorescence-correlation-spectroscopy (FCS) measurements---in order to correctly predict the magnitude of the observed effects some unknown attractive interactions between the tracers and the crowders used in the experiments need to be taken into account.

It is pointed out that accounting simultaneously for shape effects of the crowders, for softness of their structure, with a proper treatment of the near- and far-field HDIs, as well as including in the theory and in simulations the accurate magnitudes of the tracer-crowder interactions are all of vital importance for constructing the correct and universal/(most general) theory of the MMC effects in real biological cells. In the latter, all these ingredients of MMC contribute with yet-to-be-found "weighting factors", likely specific for each tracer type.

The third conclusion is that, generally, soft crowders slow down the diffusion of the tracers less effectively, as compared to the effects of hard crowders, but it is not the case for elongated crowders (as shown in Chapter 4). In mixtures of hard and soft spherical crowders with variable proportions soft particles diffuse quicker and hinder diffusion to a lesser extent. In contrast, as Mr. Skora demonstrated, highly elongated tracers---such as ds-DNA segments---diffuse faster in suspensions of hard crowders. Quantifying the effects of shape and softness of crowders and tracers universally is, thus, (again) a challenging task for predicting the actual magnitude and the direction of the effects of MMC in the cytoplasm of real cells where none of the constituents is ideally spherical, infinitely hard, and perfectly inert with respect to its interaction or contact partners.

Fourthly, as discussed in Chapter 5, Mr. Skora states that the activity-induced enhancement of the enzyme diffusion depends critically on the crowder type. For those enzymes reducing their dimensions upon binding to a substrate it is found that a certain enhancement of diffusion takes place, in agreement with experimental evidence. In crowded systems this enhancement is unveiled to be sensitive to the type of crowders, being, e.g., weaker if MMC is due to passive crowders. Again, these conclusions as well as further developments along these new research directions are vital to properly understand the operational principles of biological enzymes in crowded cellular environments.

The fifth conclusion (based on the results of Chapter 5) is devoted to testing the predictions regarding the enhancement of the diffusion coefficients of proteins via precise enough H-cell-based measurements.

The sixth conclusion of the current PhD Thesis (based on the results of Chapter 6) is about the interpretation of the results of chemical-kinetics measurements in MMC-systems featuring microscopic conformational changes of enzymes in the course of the reaction.

The seventh conclusion (as discussed in the results of Chapter 6) is that MMC can trigger enhanced cooperativity of multivalent-binding reactions, in applications to chemical reactions in bio-cells. It is shown, e.g., that MMC can induce cooperativity even in non-cooperative systems. For multi-step reactions it is concluded that the direction of the effect depends on the actual sequence of changes (expansion or contraction) of macromolecular conformations during the process. Lastly, Mr. Skora unveils the properties of folding-unfolding, polyme-

rization, and ring-formation reactions in the presence of MMC, with a number of biologically relevant conclusions.

The eighth (and the last) conclusion is the list of open-source software packages developed by Mr. Skora (*pyBrown* and *ExVol*) and used by him to perform a large portion of computer simulations of the current PhD Thesis. These packages are made freely available via the *github* platform: this fact will certainly facilitate further developments and generalizations as well as including these software packages into large packages or networks by the MMC-simulation community.

Finally, Appendices A, B, C, D, and E contain all necessary technical details regarding implementation of the computational methods used, the applied software packages, and some experimental setups. The Appendices are as follows: #A: Brownian Dynamics implementation details, #B: Ewald summation of the Rotne-Prager-Yamakawa diffusion matrix, #C: Resistance matrix scalar functions, #D: Software, and #E: Fluorescence-correlation spectroscopy (FCS).

Specifically, in Appendix A Mr. Skora describes the forward-Euler-, the Ermak-McCammon-, and the midpoint-based schemes, the potentials of repulsive and attractive interactions are presented, the ESIs and the harmonic potentials are overviewed, as well as the TAMSD-based methods of the trajectory analysis applied to quantify the properties of translational and rotational diffusion are presented.

In Appendix D the software packages developed by Mr. Skora are described in detail (*pyBrown* and *ExVol*); the customized versions of other software packages are discussed as well (such as *BD_BOX* and *PyGRPY*). The simulations codes are uploaded to and made public via the standard *github* web-page: the broad community of specialists can thus access the source scripts and further develop on the subject.

Finally, in Appendix E the experimental and mathematical details of the FCS-based methods for measuring the diffusion coefficients and for describing the dynamics of macromolecules *in vivo* and *in vitro* are presented to the reader.

Summing up, the doctoral Thesis of Mr. Skora is of extremely high quality and without doubts it satisfies all formal and scientific requirements. The dissertation meets the conditions specified in Article 187 of the Act of July 20, 2018 Law on Higher Education and Science (Journal of Laws of 2022, item 574, as amended), and---in my opinion---the candidate should proceed to the next stage of the examination process and admitted for a public defense.

The excellence of the reported material, the rigorous and well-structured way in presenting the results, the methodologically well-thought arrangement of the findings, and the extremely solid record of publications accompanying the current PhD work (with 3 published papers on the subject of the Thesis, with 1 more submitted, and with 2 more manuscripts in preparation) certainly highlight the exceptional performance by Mr. Skora on the scope of the Thesis. Of additional value are the novelty of the research area developed and a promising future scientific growth of the candidate.

In the course of this PhD study, Mr. Skora has not only become an expert in high-level extensive computer simulations of various complex crowded biologically relevant systems, he also has reached high standards in analytical calculations. The knowledge of the subject demonstrated by Mr. Skora in this PhD Thesis is remarkable: this fact not only truly designates a highly talented individual, but it also highlights endurance/persistence in achieving scientific goals and a true passion in doing science.

For all these reasons, I recommend the doctoral dissertation of Mr. Skora to the Council of the Institute and the Evaluation Committee for a **distinction** as an outstanding PhD Thesis.

Potsdam, 11.02.2023 Dr. A. Cherstvy