VĚDECKÉ SPISY VYSOKÉHO UČENÍ TECHNICKÉHO V BRNĚ
Edice Habilitační a inaugurační spisy, sv. 731
ISSN 1213-418X

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BIOPHYSICAL CHEMISTRY – PHYSICAL CHEMISTRY IN SERVICE OF THE LIFE SCIENCES

BRNO UNIVERSITY OF TECHNOLOGY
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BIOPHYSICAL CHEMISTRY – PHYSICAL CHEMISTRY IN SERVICE OF THE LIFE SCIENCES

BIOFYZIKÁLNÍ CHEMIE – FYZIKÁLNÍ CHEMIE VE SLUŽBÁCH PŘÍRODNÍCH VĚD

SHORT VERSION OF HABILITATION THESIS

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ
NAKLADATELSTVÍ VUTIUM

BRNO 2022

KEYWORDS
biophysical chemistry; microbiology; humic substances; polyhydroxyalkanoates; hydrogels

KLÍČOVÁ SLOVA
biofyzikální chemie; mikrobiologie; huminové sloučeniny; polyhydroxyalkanoáty; hydrogely

PLACE OF STORAGE
Printed version of the full-length version of the habilitation thesis is stored at Faculty of chemistry, Brno, University of Technology.

MÍSTO ULOŽENÍ PRÁCE
FKompletní verze habilitační práce je uložena na Fakultě chemické Vysokého učení technického v Brně.

ISBN 978-80-214-6085-0
ISSN 1213-418X

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Author's Introduction

I was born in 1983 in Nové Město na Moravě. The story of my fateful relationship with the Brno University of Technology (BUT) began immediately after graduation from grammar school (Gymnázium Tišnov) in the fall of 2001, when I started my studies at the Faculty of Chemistry. Although originally enrolled to study of material chemistry, I fell in love at first sight with physical chemistry and completed my Master's degree in this field in 2006. Then I continued to study for a doctoral degree in physical chemistry at the same institution and obtained my Ph.D. in 2009 by defending the thesis called "*Hydrogels of Humic Acids – Experimental Model and Application Form*".

My academic work at the Institute of Physical and Applied Chemistry of the Faculty of Chemistry started in 2010, i.e. at the time when the efforts to innovate the Consumer Chemistry study program were at their peak so that it better resonated with the current state of the chemical industry in the Czech Republic. I participated in this innovation (in addition to teaching basic physicochemical courses) by introducing a new course Modern Fiber Materials and Sorbents (replacing the previously taught Textile Chemistry) and a laboratory exercise for this course. Soon I also took over under my guarantee and at the same time innovated the content of the Supramolecular Chemistry course. As part of the teaching of these subjects and my scientific research activities, I further deepened my interest in the application of basic physical-chemical concepts in various material areas.

An important milestone for my further scientific and pedagogical development came in 2015 when I was invited to participate in the implementation of a research project aimed at clarifying the complex biological and evolutionary role of microbial polyesters. My role in the newly built interdisciplinary team was to evaluate the applicability of physicochemical approaches and techniques in the analysis of microorganisms. The obvious analogy between the gel environment, with the analysis of which I had accumulated enough experience in previous years, and the intracellular space of bacterial cells, as well as the fascination arising from working with living organisms, led to the fact that I have remained involved in this interdisciplinary (and interinstitutional) collaboration until today. The previously unknown horizons that opened up to me in connection with the application of physical chemistry to living organisms also inspired and motivated me to introduce and gradually intensify the teaching of biophysical chemistry at the Faculty of Chemistry (today, the Biophysical Chemistry course is already taught within three follow-up master's study programs and is now supplemented with computational exercises and laboratory practice).

Currently, at the Faculty of Chemistry, I participate in guaranteeing and teaching several applied physical-chemical subjects (in addition to the above, for example, Physical Chemistry of Dispersions), as well as in individual teaching (since 2011, I have been the supervisor of 30 diploma and 37 bachelor's theses). In both my teaching and research activities I have always been trying to initiate and personally contribute to making Biophysical Chemistry a unique direction at all levels of teaching as well as within the scientific and research focus of the faculty. I believe that the message of BUT's main motto (*Sapere aude*. Dare to know.) can be well illustrated by the example of this direction, which has an extraordinary ability to blur the boundaries between scientific disciplines as easily as between the involved faculty workplaces.

Preface

The habilitation thesis presents my personal view of biophysical chemistry as a modern, distinctive chemical discipline. In the full-length version of the thesis, I introduce my perspective on the current state of the field, in the context of historical moments, which in my opinion have contributed to the formation of biophysical chemistry as an independent scientific discipline. Furthermore, I also provide a subjective view of the main challenges that this discipline currently faces, as well as the opportunities in which I see the main merit of biophysical chemistry in the near future. In this short version, I focus more on the other content of the thesis – on my personal contribution to the development of the state of knowledge in the field. However, let me first spend a few words on a brief introduction to the field of biophysical chemistry.

Biophysical chemistry is a branch of science where biology, physics and chemistry meet. It is a broad discipline with a very wide scope delimited by the range of biological systems that are subject to its interest. Who, then, are the biophysical chemists among whom I dare to rank myself? In my view, they are scientists whose subjects of research interest are biological systems, who focus on the chemical components and chemical processes that form these systems, and who apply physical laws to understand how the systems behave. Inevitably, they also need to use math to express the problems and find solutions. In short, they are scientists who every day ask the essential question: "What is life?", or, more specifically, "What is the physical chemistry of life?". They take inspiration from many great personalities who had been asking the same questions before. They realize the urgent challenges of life science that should be dealt and understand that on the quest to reach these noble goals, they can contribute by a series of tiny steps provided by solving the partial topics of their interest.

The unifying physico-chemical concept I have been using in the research introduced in the following chapters is based on efforts to understand how the molecular thermodynamics (mainly the interactivity of molecular principal components) of the particular system –regardless of its degree of complexity – rules its structural (e.g. supramolecular architecture) and material (deformation, transport) behavior and how this relationship manifests itself in the environmental, ecological or biological functioning of the system. Also, the methodology used in these studies was largely the same regardless of the specific system studied. It combines methods of structural analysis, morphological tests involving techniques of advanced microscopy, and both routinely used (e.g. thermal analysis or rheometry) and originally developed (e.g. diffusion-based) techniques providing further material and physicochemical characterization of the studied system. Aside from providing a complex overview of different aspects of system performance, the methodology was also designed to combine the macro-and micro-scale perspective on the system properties.

Furthermore, in all the contributed topics, I have tried to combine a quest for understanding how the natural systems work with an application of this fundamental knowledge in applied research and development of new functional bio-based materials. Hence the studies on the structure of humic substances and on the barrier and controlled release role they play in natural ecosystems were accompanied by the analyses of bioabsorption of these substances from artificial biostimulants and the development of original humics-based soil amendments. Similarly, fundamental research projects focused on the evolutionary and ecological role of polyhydroxyalkanoates went hand in hand with applying the revealed knowledge on how these materials affect the stress robustness of microbial cells in defining novel trends in their biotechnological production and also in the development of their application forms with tunable material properties.

If I was asked to characterize my scientific career so far in a single slogan, I would probably use "standing astride". For the whole time, I have been standing astride borderlines between apparently diverse scientific topics, between fundamental and applied research projects, and sometimes also with each leg embedded in a different research team. Probably, this may seem to someone like a lack of a firm thematic anchoring or even like scientific volatility. Nevertheless, as every colloidal chemist can confirm, it is exactly the boundary separating different phases where the most exciting phenomena take place. It was perhaps the main goal of the habilitation thesis to demonstrate that this "standing astride" position may be in a fact surprisingly beneficial in science.

Chapter 1:
Molecular interactions in biopolymers and natural organic matter: from structural principles to macro-scale effects

„He who loves practice without theory is like a seafarer who boards ship without wheel or compass and knows not wither he travels."
(*Leonardo da Vinci*)

At first glance, biopolymers and humus, respectively, may be considered just two different stages of the life cycle of carbonaceous organic substances, differing fundamentally in their structure as well as their environmental fate. Nevertheless, in a more careful view, a surprising similarity between these two families of natural compounds becomes evident. In a fact, it can even be said that biopolymers mean the same for living organisms as humics do for the non-living part of nature. Based on their wide supply of chemical functionalities, they both play a variety of structural, nutritional and functional roles where they are irreplaceable by other components of their natural environs. In all these functions, the performance of both types of compounds lies primarily in the way they interact with other polymers and low-molecular components of the system. In our research studies, we have focused on humic acids (HAs) as the key constituent of natural organic matter (NOM) regarding its interactivity in condensed natural environments, and to polyhydroxyalkanoates (PHAs) as the family of microbial polyesters with a complex biological role and great potential in replacing petroleum-based polymers in the production of plastics.

1.1 Humic substances: native transport systems & promising artificial carriers

Natural organic matter provides a remarkably complex pool of organic compounds. Among its various constituents, the stable organic fraction called *humus* (the latin expression for "earth, soil", originating probably from *humi, "on the ground"*) attracts special attention mainly as the major cause of fertility of soils. This vital ecological merit of humus arises from the finely tuned interplay between various chemical, physical and biological mechanisms of action. On the one hand, humus represents a storehouse for essential soil nutrients and minerals, on the other, it supports soil structure and improves physical parameters such as porosity, thus improving soil aeration as well as water absorbency and drainage. Moreover, humus supports the biological activity of soils, not only indirectly by serving as an energy source for soil microorganisms, but their decomposition products can also selectively inhibit or stimulate the growth of soil microbiota or, in some cases (e.g. via the production of auxins) it directly chemically promotes growth of higher plants.

1.1.1 Development of novel methodology for interaction studies based on diffusion processes

One of the most inspiring scientific events that I attended during my career was IHSS-14, the 14th International Meeting of International Humic Substances Society, which took place aboard a ship sailing from Moscow to St. Petersburg in 2008. Besides the non-traditional venue, this meeting was exceptional also in a rare effort of its scientific committee to conclude the scientific program with a tangible formulation of the state-of-the-art of humic substances (HS) research and definition of the main gaps in knowledge on HS. As a part of this effort, nine specific research priorities for HS were identified. Among them, a systematic reactivity and interactivity mapping of HS was urged in order to reveal how the exact chemical structure and particular chemical functionalities of HS contribute to their inherent environmental performance and also to their value for individual application fields.

At the time of the IHSS-14 conference, we were already working on the development of an unconventional methodology based on diffusion experiments in hydrogels made from HS. Diffusion, as a process that results from a dynamic behavior on the level of individual molecules but its manifestations can be easily observed on the macro-scale level, represents a smart solution for the sought experimental design. As far as it is directly influenced by all chemical and physical interactions that the molecules of the diffusing compound undergo, it allows investigation of the molecular binding in a quantitative way, and, at the same

time, an effect of this binding on the macro-scale system dynamics is directly demonstrated. Another advantage of the diffusion-based methods lies in a broad variety of setups that can be used according to the specific requirements regarding the volume of the analyzed sample, the concentration of the tested solute, time duration of the experiment, etc. For all these experimental designs, corresponding mathematical solutions of Fickian diffusion equations are available. This allows determining the effective diffusion coefficient as a primary experimental outcome by which the interactions in the studied system may be quantitatively and reproducibly characterized.

Initially, we adopted the diffusion-in-gel method in an investigation of heavy metal transport in humus-containing systems [I]. Copper was used as a model metal mainly because of its outstanding affinity towards humic substances and also because of its easy spectroscopic quantification. As an experimental gel matrix, we used humic acid hydrogels that were prepared by controlled coagulation of humic acids by acidification of their alkaline solution. The gel (obtained by centrifugation and removal of supernatant) was filled into a container (usually a plastic or glass tube with well-defined internal dimensions) and brought into contact with a source of the diffusing solute (cupric ions). At the given time(s), we then determined the concentration of the solute at different positions of the gel sample by the manual slicing of the gel and gel-liquid extraction of the solute. This study confirmed that the diffusion-in-gel method represents a valuable option for reactivity and permeability mapping studies on both natural and artificial matrices containing humic substances. All tested methods proved themselves experimentally feasible and allowed easy and relatively quick determination of the diffusion coefficient as a parameter that characterizes and quantifies dynamic effects of HA-solute binding. The same conclusions have been reached also in a parallel study, in which the diffusion from the source solution with time-variable concentration[II]. In this work, we have also determined (effective) partition coefficient as an additional parameter that characterizes the macroscopic manifestation of HA-solute binding on the preferential absorption of the solute by the gel at the solution-gel interface. The basic methodology developed and successfully utilized in these initial works was supplemented with fractionation of the diffused copper ions according to the strength of its binding in the humic gel in the subsequent study[III]. The results of this study showed that the distribution of diffusing copper ions among the determined fractions (freely moving, weakly, and strongly bound) stayed constant after passing a certain time. This indicates the existence of a binding equilibrium which is created and maintained as the diffusion of the ions proceeds.

The above-mentioned works represented an important first step in the involvement of diffusion assays in an investigation of solute binding on HS and how it is reflected in the dynamics (e.g. barrier behavior) of the HS-containing systems. Nevertheless, we were well aware that this simple method suffered also some severe limitations. Above all, the procedure for the humic hydrogel preparations did not allow control of the relative content of HS in the gel. The low internal pH of the gel, induced by the acid-induced coagulation, also suppresses the dissociation of weakly acidic groups of HS and reduces the electrostatic binding of cationic solutes. Moreover, the aggregation behavior of HS is influenced by their molecular structure and it is, therefore, uncertain how the solute diffusion process would be affected by inevitable changes in the internal (physical) structure of the gel brought by the use of different HS (e.g. HS from different sources or with various chemical modifications). Therefore, we launched parallel research aiming at the development of an alternative methodology where these drawbacks would be overcome. This research effort resulted in the design of novel method, that was first introduced in two subsequent papers published in the journal "Reactive and Functional Polymers" (the experimental framework is schematically shown in Fig. 1). The main innovation lies in the use of a carrier gel matrix, without any specific binding affinity towards the diffusing solute, in which the solute-binding humic component is physically trapped. Agarose was used as

[I] Sedláček, P. and Klučáková, M. Diffusion experiments as a new approach to the evaluation of copper transport in humics-containing systems. *Collection of Czechoslovak Chemical Communications.* 2009, 74, 1323–1340.

[II] Sedláček, P. and Klučáková, M. Simple diffusion method applied in evaluation of metal transport in model humic matrices. *Geoderma.* 2009, 153, 11–17.

[III] Kalina, M., Klučáková, M., and Sedláček, P. Utilization of fractional extraction for characterization of the interactions between humic acids and metals. *Geoderma.* 2013, 207–208, 92–98.

the inert gel-forming polymer and humic acids (HA) were penetrated in the physically crosslinked agarose matrix during the cooling of the agarose-HA solution [IV,V]. By this procedure, both the agarose and HA content in the gel can easily be manipulated. Furthermore, because the whole volume of the solution gelatinizes, gel samples of variable shapes and sizes can be prepared directly during the gelation in the suitable container. The combination of steady-state and non-stationary diffusion experiments provides a reasonably complex overview of the transport properties of a studied system. While the experiments in diffusion cells focus on the steady-state stage of the diffusion process and illustrate primarily the barrier properties of a material after its penetration by the solute, the transient stage of the process is described by the non-stationary experiments more elaborately.

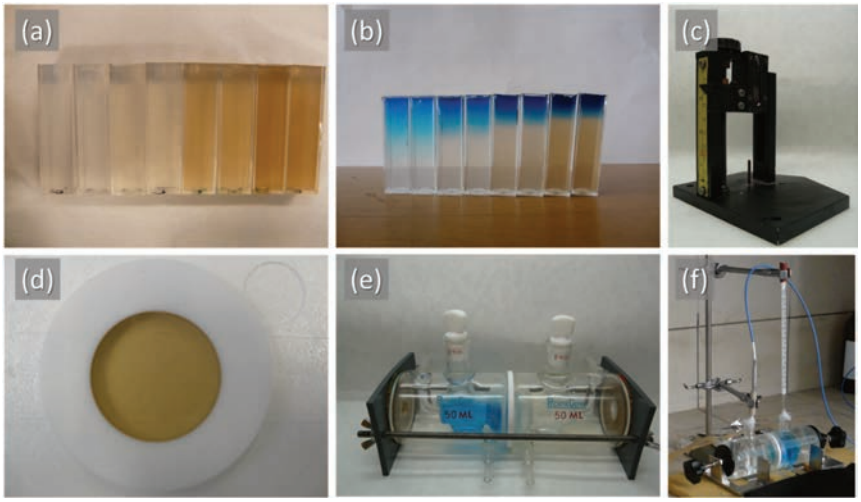

Fig. 1 Developed methodology for solute binding studies based on diffusion studies in agarose gels. Equipment for non –stationary (*a – c*) *and steady-state* (*d – f*) *diffusion experiments.* (*a*) *Cuvettes with various concentrations of humic acids immobilized in agarose hydrogel,* (*b*) *the same cuvettes after 48 hours of contact with a solution of methylene blue.* (*c*) *Original accessory for measurement of UV-VIS spectra at different positions from the solution-gel interface.* (*d*) *Gel-holding insert for the diffusion cell apparatus.* (*e*) *Side-by-side diffusion cell setup with* (*f*) *online monitoring of solute concentration in the acceptor compartment by fiber optic spectrophotometry.*

The usability of all the diffusion-in-gel techniques is limited to the systems, where the reactive component (HA) is capable of being transformed into the gel form. But what if this is not the case? We, therefore, searched for an alternative diffusion-based experimental technique also for these systems and found the solution in dialysis experiments[VI]. These experiments can be easily performed in the diffusion cells apparatus used also for the through-diffusion assays. In the dialysis binding study, diffusion cells, initially filled with solutions of HS and low-molecular solute, respectively, are separated by a dialysis membrane that is permeable only for the later solute. During the experiment, the decrease of the concentration of the permeant solute in its source compartment is monitored in time until the equilibrium is reached. Hereby, both the kinetics of the solute diffusion and its equilibrium distribution between bound and unbound forms can be investigated.

[IV] Sedláček, P., Smilek, J., and Klučáková, M. How the interactions with humic acids affect the mobility of ionic dyes in hydrogels – Results from diffusion cells. *Reactive and Functional Polymers.* 2013, 73, 1500–1509.

[V] Sedláček, P., Smilek, J., and Klučáková, M. How the interactions with humic acids affect the mobility of ionic dyes in hydrogels – 2. Non-stationary diffusion experiments. *Reactive and Functional Polymers.* 2014, 75, 41–50.

[VI] Rybárik, J., Sedláček, P., and Klučáková, M. Transport of Organic Dyes in Systems Containing Humic Acids. *Inżynieria Mineralna.* 2019, 21.

1.1.2 On the role of carboxylic groups in the binding of amphiphilic substances by humics

In the preliminary binding studies, summarized in the previous section, the newly developed diffusion methodology was used in experiments that aimed primarily at verifying that the solute-HS interaction occurs and that it is effectively demonstrated in the solute diffusion in the system. To provide a more detailed understanding of the mechanism of these interactions, a follow-up study was designed to focus more on the structural aspects of the solute binding.

HS possess a complex amphipathic (supra)molecular structure that provides the universal binding ability. The irreplaceable role of polar structural moieties (mainly carboxyls and alcoholic/phenolic hydroxyls) in binding the hydrophilic solutes and surfaces [1] has been widely reported. Similarly, sorption capacity for non-polar compounds such as polycyclic aromatic hydrocarbons has usually been attributed to the content of hydrophobic moieties including aromatic rings [2] and aliphatic carbon groups [3]. We decided to focus our research on the role of HA's carboxylic groups in binding hydrophilic and amphiphilic solutes. For this purpose, we were looking for the way how the content of –COOH binding sites in the structure of studied HS may be controlled. Based on our cooperation with research group of Prof. Laurent Grasset from University of Poitiers, we have implemented the method of selective methylation of HA's carboxyls in order to block these binding sites; among the published methylation procedures, we have chosen exposure of HA to TMS-diazomethane in methanol [4]. High effectivity of this substitution procedure was repeatedly confirmed spectroscopically (13C NMR, FTIR) as well as by means of potentiometric titration.

At first, we implemented the use of methylated lignitic HA into the original methodology developed for the study of copper-binding in coagulated HA hydrogels[VII]. Using the technique of instantaneous planar source, we monitored the diffusion of copper ions in hydrogels prepared from mixtures with different relative content of original and methylated HA. As expected, the relative content of methylated humic acids affected the rate of the transport of copper in the gel. Nevertheless, from the more detailed evaluation of the diffusion data, we have found that this effect originates from the structural changes in the gels rather than from the affected solute binding. This confirmed the expected drawbacks of the coagulated humic gels – the internal structure of these gels can be severely altered when the chemical structure of the HS is changed. Therefore, further experiments were performed with HA (original and the methylated ones) interpenetrated in the supporting agarose gels. In these experiments, we have also turned our attention from metal ion solutes to cationic organic dyes. The first reason for this switch comes from the experimental constraints – the higher absorption coefficient of the organic dye allows for analyzing diffusion and binding of the solute at lower concentrations as compared to the inorganic ions. Nevertheless, the main motivation for the involvement of this type of organic solutes lies in their environmental relevance – the combination of aromatic and/or aliphatic structural backbone with a positively charged polar group(s) is common for numerous compounds of considerable ecological importance like pesticides, growth retardants, and industrial waste materials (e.g. surfactants, disinfectants or industrial dyes).

We have published the results of a comprehensive study on the role of –COOH groups in the binding of charged organic solutes (Methylene blue and Rhodamine 6G) on lignite HA in the journal "Chemosphere"[VIII]. In this study, we analyzed HA entrapped in agarose gels via steady-state through-diffusion experiments, transient in-diffusion study and also an equilibrium partitioning assay. In this complex transport-mapping study, we have revealed surprisingly similar effects of original (HA) and methylated (mHA) humic acids on the partitioning and rate of transport of both tested solutes in the humics-interpenetrated agarose gels. Because the methylated carboxyls in mHA are unable to dissociate, this has indicated the simple electrostatic attraction between the positive charge on the solute and the negative charge brought by the HA's carboxyls contributes to the binding of the charged organic solutes less than expected. We proposed in the paper that the interactions between aromatic moieties in the molecular structure of the solute and

[VII] Klučáková, M., Kalina, M., Sedláček, P., and Grasset, L. Reactivity and transport mapping of Cu(II) ions in humic hydrogels. *Journal of Soils and Sediments.* 2014, 14, 368–376.

[VIII] Smilek, J., Sedláček, P., Kalina, M., and Klučáková, M. On the role of humic acids' carboxyl groups in the binding of charged organic compounds. *Chemosphere.* 2015, 138, 503–510.

humic substances play a crucial role. Moreover, we have complemented this diffusion study also with conventional batch-sorption experiments in the suspension of HA. On the significant discrepancy that we found between the sorption and diffusion results, we have experimentally demonstrated the previously proposed benefits of our methodology – we have shown how methylation of HA changes the textural properties of solid HA and hence also their sorption performance. Unlike the suspension, in the form of gel-immobilized hydrated chains, HA's binding performance is driven primarily by the molecular structure and the structural aspects of the interactions are hence monitored much more reasonably.

In the follow-up study, we have subjected original and methylated lignite HA also to the dialysis experiments described in details elsewhere[VI]. From the collection of the data obtained by the diffusion and dialysis experiments, it can be clearly illustrated that a wide range of solute-to-HA concentration settings can be covered by different setups in the proposed methodology. From all the diffusion-mapping experiments, values of apparent binding constants of the solute (MB) are provided for interactions with HA (original lignitic and methylated lignitic, respectively). In a fact, this constant represents the effective equilibrium constant of the supposed transition between free and bound solute

$$K_{app} = \frac{[\mathrm{MB}_{\mathrm{bound}}]}{[\mathrm{MB}_{\mathrm{free}}]},$$

where $[\mathrm{MB}_{\mathrm{bound}}]$ and $[\mathrm{MB}_{\mathrm{free}}]$ represent the equilibrium activity (that equals dimensionless concentration for the diluted systems) of the bound and free solute, respectively. We have discussed in detail in our published works[I,III-V,VII] how the value of the apparent binding constant can be calculated from the experimentally determined diffusion coefficients. In the dialysis study, the constant is determined directly from the distribution of a solute between the two chambers (equilibrium constraint of the same activity of the free form of the solute is assumed). We have found that the particular value of the binding constant varies in the orders of magnitude not only with the change in solute-to-HA concentration but also when the constraints of the transport phenomenon or the colloidal form of HA are altered. Once again, this illustrates that the manifestations of the binding of the solute on its transport in a system are strongly conditions-specific. On the other hand, it also shows that the proposed methodology is outstandingly robust – selection of an appropriate experimental technique enables investigation of the binding under particular required circumstances (transient, steady-state or equilibrium). Last but not least, a comparison of K_{app} values of original HA and the methylated ones show that the contribution of –COOH groups to the solute binding depends strongly on the solute-to-HA concentration and the mode of transport. It can be seen that under some circumstances, the binding of MB on methylated HA is apparently stronger as compared to the original HA. This further indicates an essential role of other than carboxylic structural motifs in the binding of positively charged organic compounds.

It should be emphasized that this phenomenon seems to be general for the binding of various charged organic compounds on diverse humic acids. Aside from the lignitic HA, we have confirmed the similar binding behavior of original and methylated humic acids also for IHSS reference humic material[IX]. Furthermore, we have also found similar binding behavior for the binding of cationic surfactants (Septonex in particular) – similarly to the aromatic organic cations, also these aliphatic charged structures are significantly bound to humic acids and this binding is only slightly suppressed when the HA's carboxyl groups are masked with selective methylation (data not published yet). Hence, I can summarize a great deal of experimental work that we have devoted to understanding the binding of charge organic compounds to humic substances as follows: unlike the binding of strictly hydrophilic (e.g. metal ions or polar mineral surfaces) or hydrophobic (e.g. polyaromatic hydrocarbons) materials that can be easily attributed to distinct binding regions in the supramolecular conformation of humics (hydrophobic core in the latter case, polar surface in the former one), in the binding of amphiphilic solutes both HA's regions participate on the solute binding with the relative contributions depending on the particular binding conditions (the correspondingly updated general concept of HA binding is provided in Fig. 2).

[IX] Smilek, J., Sedlacek, P., Lastuvkova, M., Kalina, M., and Klucakova, M. Transport of Organic Compounds Through Porous Systems Containing Humic Acids. *Bulletin of Environmental Contamination and Toxicology.* 2017, 98, 373–377.

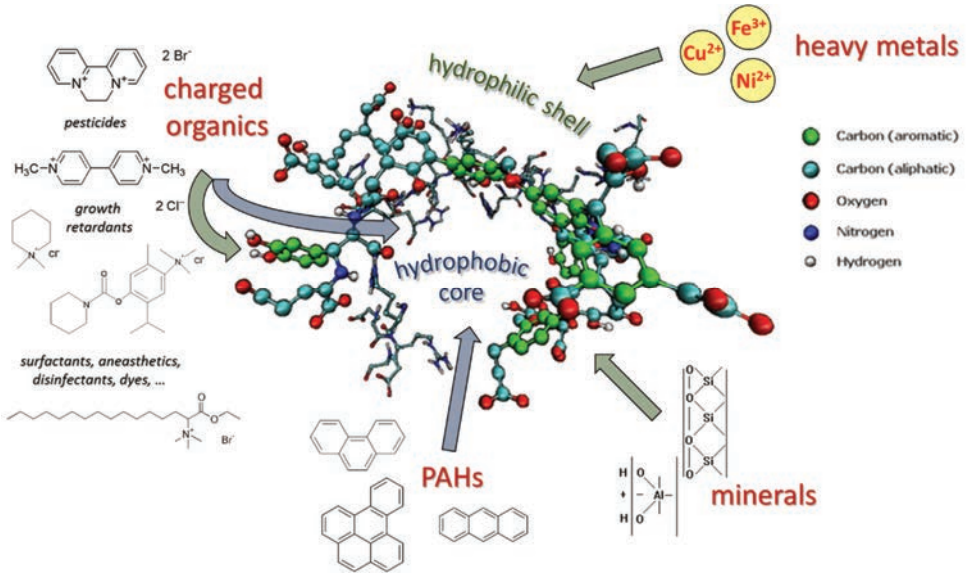

Fig. 2 Supramolecular architecture of humic acids providing different binding preferences according to the sorbate molecular structure. The concept of binding charged organic compounds was updated based on our experimental study.

1.1.3 Transport of humic substances through skin and plant cuticle

It is not only the intrinsic environmental and ecological role that they play in their natural habitats but also their outstanding application potential, that makes these substances so attractive scientifically. Among the wide range of proposed application fields, utilization of biological effects of humic substances in the production of plant growth stimulants, veterinary medicine and also human health-care is currently gaining ground. Figuratively speaking, the scientific attention is slowly but surely shifting from the effect of HS on environmental health to the effect on the health of individuals (plants, animals and humans).

We therefore paid our attention to the penetration of liquid HS through plant cuticles. This was motivated by the growing interest – not just scientific, but also commercial – in the utilization of liquid HS in the production of plant biostimulants (mainly for foliar application). On the other hand, a debate has recently arisen about the real efficiency and economical feasibility of the application of humates in agriculture. It was stressed by some authors that the actual data on the benefits of the use of humates is rather ambiguous or inconsistent [5-7] and that a comprehensive physico-chemical characterization and a careful assessment of the mechanism of their foliar action are still needed [7]. Considering the absorption of humate by the plant organism as a critical step preceding the biological action, and taking into account our previous experience with diffusion-in-gel techniques, we, therefore, focused on the development of experimental methodology for assessment of the rate of trans-cuticular transport of humates into plants. The method that we have developed (see Fig. 3) is based on spectrophotometric monitoring of the humate diffusion through an isolated plant cuticle that is placed between two agarose hydrogels forming the common diffusion couple arrangement. We published results of the pilot study[X] on the usability of the technique where we had used artificial lignohumate and the cuticles obtained from the leaves of *Prunus laurocerasus* plant via chemical and enzymatic isolation procedure, respectively.

In the study, we have demonstrated how the rate of lignohumate penetration differs for the cuticles isolated in a chemical and enzymatic way. Furthermore, we have also confirmed that barrier properties of

[X] Smilkova, M., Smilek, J., Kalina, M., Klucakova, M., Pekar, M., and Sedlacek, P. A simple technique for assessing the cuticular diffusion of humic acid biostimulants. *Plant Methods.* 2019, 15, 83.

the stomatous and astomatous sides of a leaf differ significantly. Aside from the particular results on the transport of used lignohumate through the tested cuticles, we have provided a general discussion of the pros and cons of the proposed technique in the paper. In general, the proposed diffusion technique represents an easy and cheap tool for an in-vitro experimental assessment of the transcuticular penetration ability of humates from liquid agrochemical preparations. It has no ambition to simulate the real conditions during the foliar feeding process. Rather, it aims to answer general research questions concerning the penetration of humic-based substances into leaves, such as how the permeability of cuticles for humates varies among either various plant species or diverse humic materials.

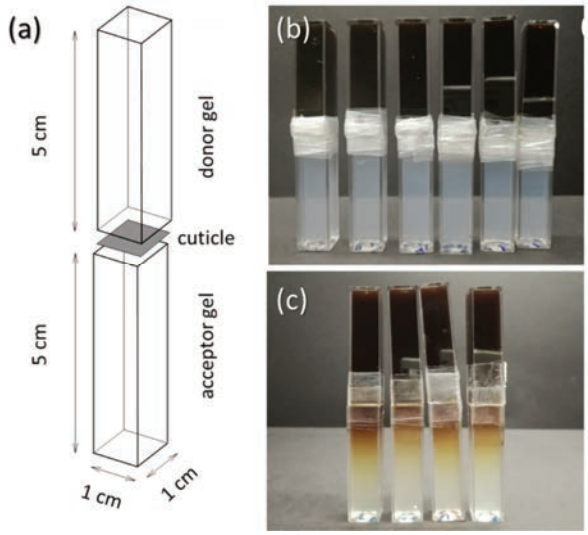

Fig. 3 Experimental study of the transport of humate-based biostimulants through plant cuticles. (*a*) *Schematic diagram of the experimental setup – diffusion couple of donor* (*i.e. humate containing*) *and acceptor agarose gels separated by isolated plant leaf cuticle.* (*b*) *Diffusion couples at the start of the experiment.* (*c*) *Diffusion couples after 7 days of the experiment* (*chemically isolated abaxial cuticle*).

1.1.4 From understanding the natural function to the development of novel application forms

As I already pointed out in the preface, I have always put a special effort into implementing the fundamental knowledge gained from the basic phenomenological investigations into an applied research attitude to the topic. In course of the study on interactions of humic substances, we have practically experienced how the binding ability of HS comes to the fore when the humics are incorporated in a hydrogel medium. This could be employed not only in a reasonable simulation of the binding performance of natural HS but also in designing and developing new application forms of HS. Taking into account the high apparent molecular weight of HS and the proven ability to coagulate HS from solution by non-covalent crosslinking, their universal binding capability and complex inherent biological activity and also the demonstrated ability of some humic fractions to penetrate the surface of the plant (1.1.3) and even human body (discussed in the full-length version of the thesis), development of hydrogels with incorporated HS seem exceptionally promising for the application fields ranging from agriculture to human medicine. Humic component of the gels could provide numerous functions according to the specific requirements of the application [8]. In soil amendment preparations, HS are primarily intended as an active ingredient that aims at improving the quality and content of soil humus, whereby the gel form can help to assure a controlled release of the humic component to increase the effectivity of its fixation in soil and prevent unwanted losses e.g. by rainwater washout. The proven ability of lower-molecular-weight HS to penetrate plant cuticles[XI] also rationalizes the

[XI] Smilkova, M., Smilek, J., Kalina, M., Klucakova, M., Pekar, M., and Sedlacek, P. A simple technique for assessing the cuticular diffusion of humic acid biostimulants. *Plant Methods.* 2019, 15, 83.

idea that the humic substances released from the gel carrier into the soil could also provide direct bio-stimulating effects on plants [9]. Moreover, their widely described binding ability towards diverse solutes also determines HS as the potent colloidal carriers, that can control the soil concentration level and mediate the bioabsorption of various compounds, ranging from in-/organic nutrients [10], through growth enhancers and retardants, to pesticides [11, 12]. Similarly, also in the applications focused on the care of human health and wellness, the combination of the gel form and a humic ingredient as either active (providing direct medicinal or other biological effects) or auxiliary (utilized in order to solubilize and/or improve the absorption of other bioactive compounds) ingredient [13, 14] seems increasingly attractive. Furthermore, either in agricultural or topical health-care applications, the gel form provides also one additional significant benefit – it manages the water content at the place and prevents its drying out. Naturally, this capability is of similar importance for agriculture (e.g. prevention of rainwater run-off and keeping the soil moist) as for medical or cosmetic applications (e.g. wound healing, skin hydration, etc.). We have therefore paid attention to both types of potential applications of HS prepared and characterized some original hydrogel compositions with HS incorporated in them.

Regarding the design of humics-containing gels intended for use as soil amendments, we have followed two basic preparation routines. The first path that we followed in the development of HS-containing hydrogels for agricultural uses led us to superabsorbent hydrogels, where the humic component is chemically trapped in the poly(acrylic acid) polymer network. These materials, designed in order to provide a combined controlled release of HS and mineral nutrients together with extremely high water-holding capacity, will be discussed in detail in section 3.2.1. The second way of the applied research was focused on the electrostatic cross-linking of humic acids with the use of oppositely charged biopolymer chitosan (see Fig. 4). The main findings of the pilot study dealing with this original gelation approach, based on the combination of two abundant and renewable natural materials, were described in detail in a book chapter[XII]. The formed polyelectrolyte complexes (the concept will be further discussed in 3.2.2) provided hydrogels, whose properties – such as dry matter content, swelling behavior, mechanical properties of both swelled and dried material, but also sorption performance – can be significantly influenced by the preparation procedure (concentration of the gelling component, pH and ionic strength in the HA and chitosan solutions, addition of low-molecular electrostatic crosslinker, temperature, etc.). To sum up, for both HS-gelation strategies that we followed, we have demonstrated that the particular material properties of the gels - including the possibility of managing them through the preparation process - are extraordinary promising, and I believe that our contribution in this field may open the door to new, unconventional trends in the design of HS-based formulations for the use in agricultural and environmental technology.

Apart from the design, preparation and characterization of the HS-containing gels intended for agricultural use, we have also dealt with the potential utilization of HS in gels for cosmetic and pharmaceutical applications. We have demonstrated how the ability of HS to enter the body through the skin can elucidate the biological effects of topical applications of HS *per se*. Furthermore, we also considered whether and how the presence of HS affects the transdermal absorption of other active pharmaceutical ingredients (APIs). We have therefore complemented skin-penetration experiments on HS in Franz cells with a screening study where an API was contained in the hydrogel with or without HS (lignohumate in particular). In both experimental studies, we tested various compositions of hydrogel carriers (several carbomers and xanthan were used as thickeners) and we focused on penetration of lignohumate and/or API (diclofenac, ketoprofen and salicylic acid were used) through the synthetic porous membrane and the model skin (porcine ear skin). Surprisingly, we have revealed reproducible enhancement of the skin-penetration rate for all tested APIs when the lignohumate was present. Our results hence strongly supports the idea that the solubilizing ability, caused by their complex amphiphilic molecular structure, provides HS with a great potential for use in topical preparations as a skin absorption enhancer. We have also found out that our finding is consistent with several studies provided recently by Mirza [15–17] who demonstrated how complexation with HS enhances a pharmacokinetic profile of several drugs in oral delivery.

[XII] Klučáková, M.; Sedláček, P.; and Ondruch, P. Preparation and characterization of new application forms of humic acids. *In Recent Research Developments in Materials Science*; Research Signpost: Kerala, 2009; pp. 59–80.

Altogether, both the new insights into the structural aspects and binding behavior of HS, uncovered during our extensive fundamental research on these compounds, and the preliminary suggestions on their possible application merits, resulting from the applied research studies, led us to the strong conviction that humic substances represent a clear example of how fascinating materials, multi-faceted in their natural roles, and at the same time beneficial and inspiring in their artificial use, nature has created and offers us to explore.

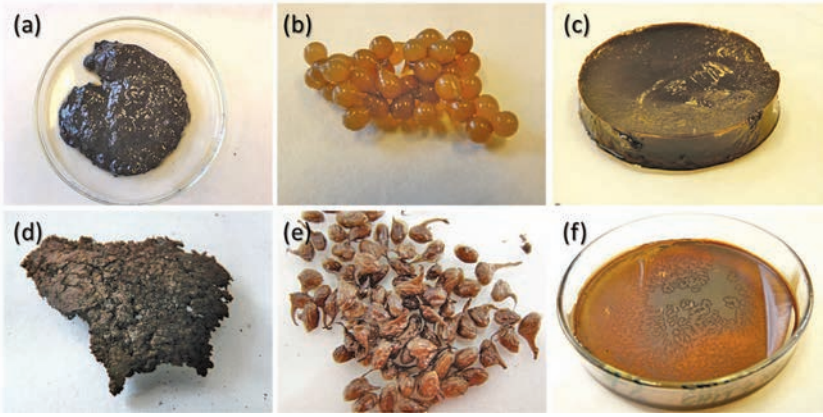

Fig. 4 Swelled (*a-c*) *and the corresponding dried* (*d-f*) *forms of HA-chitosan hydrogels.* (*a,d*) *Macrogel prepared by fusion of chitosan and HA solutions.* (*b,e*) *Hydrogel beads prepared by adding a viscous chitosan solution dropwise into HA solution.* (*c, f*) *Whole-volume gelation via alkalization of chitosan/HA mixture by ammonia vapors. Drying of the gel* (*c*) *results in film* (*f*) *with a high swelling ability.*

1.2 Microbial polyesters: what makes their structure in cells so special?

Obviously, HS represent just one of many examples of fascinating natural materials that have long been and still are attracting the attention of scientists worldwide. The number of choices for a curious material scientist is increased vastly when shifting his/her attention from the non-living to the living nature. Let me highlight one special example from the large family of biopolymers. Polyhydroxyalkanoates (PHA, chemical structure is shown in Fig. 5) has continuously been attracting a great and still growing deal of scientific interest since they were discovered by Lemoigne in 1926. PHA are microbial polyesters accumulated by numerous prokaryotes in form of intracellular granules (see Fig. 6), primarily as a carbon and energy storage material. Poly(3-hydroxybutyrate), or PHB, the homopolymer of 3-hydroxybutyric acid, is the most widespread natural representative of these polymers, however, it was reported that there are more than 150 hydroxyalkanoic acids that can be introduced into the polymer chain by various microbes [18], PHA in a fact offer a great variety of structural functionalities and hence cover a wide range of material properties. According to the particular monomers contained, PHA are referred to as short-chain-length (scl-PHA) when they are composed of hydroxyacids with 3 – 5 carbon atoms, whereas medium-chain-length PHA (mcl-PHA) contain monomers with 6 – 14 carbon atoms.

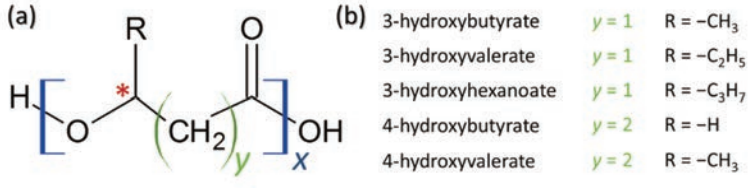

Fig. 5 (*a*) *Schematic representation of the molecular structure of PHA.* (*b*) *The most common PHA monomers. The main chiral center of the structure is marked with an asterisk.*

Although the purpose for PHA accumulation had long been seen just in the energy metabolism, recent research findings (including the results of our research projects introduced in Chapter 2 of this thesis) revealed the biological (or even the evolutionary) role of PHA is in fact much more complex. The specific role of PHA in protection against and adaptation to diverse stress factors will be discussed thoroughly later. Apart from that, it has also been described that PHA contributes to the establishment of symbiosis between prokaryotes and plants [19, 20] or insects [21], and participates in other survival strategies such as endospore formation in Bacilli and related species [22].

Analogically to HS, PHA as well play not only an irreplaceable natural role but they are also considered to be auspicious materials for artificial uses. Being produced from renewable resources by approaches of microbial biotechnology, and offering highly esteemed application- and lifecycle-related features such as biocompatibility, biodegradability and compostability, PHA represent highly promising "green" candidates for replacement of petrochemical polymers on the way toward sustainable production of plastics. An involvement of biopolymers in the production of bioplastics represents one of the most topical R&D issues. Current global production of bioplastics (2.11 million tons in 2020, [23]) is expected to increase by more than 35% by 2025, still representing less than 1% of the total annual production of plastics (368 million tons in 2020 according to Bioplastics market data, 2020). Among the bioplastics currently on the market, PHA yet represent a minor contributor (1.7% of the total amount of bioplastics produced in 2020); nevertheless, the market share is foreseen to increase significantly to 11.5% by 2025.

One reason for this expected growth certainly lies in the aforementioned versatility of composition that can be provided by various members of PHA family. However, there are two constraints that need to be overcome to open the door for a broader expansion of PHA on market. First, a complex understanding of how the application performance of PHA products can be tailored by chemical structure (i.e. monomer composition and polymerization degree) and physical conformation (i.e. degree of crystallinity) is needed. Second, technologically feasible and economically competitive processes for the biosynthesis of PHA with adjustable composition and molecular weight must be developed.

In our research study on the material aspects of PHA, we first performed an *in vivo* investigation in that we focused on the native structure of PHA in bacterial cells (1.2.1). Then, we have developed a biotechnological process, that allows the preparation of PHA materials with structure and application properties that can easily be manipulated by the cultivation conditions (described in the full-length version of the thesis).

1.2.1 Polyhydroxyalkanoates *in vivo*: staying unfavorable

At first glance, it could seem that structural biologists must find PHA molecules absolutely boring. Their primary chemical structure does not compare to the complexity of other fascinating biopolymers such as proteins or nucleic acid. Nevertheless, a closer look at structural aspects of PHA can bring an unexpected attractiveness for biologists and also at least one surprising similarity with the above-mentioned biopolymer family stars: the fact that we can distinguish a native and denatured (i.e. the biologically active and inactivated, respectively) form of the compound and that transformation between the two forms can be induced easily (at least in one direction).

There are in fact two especially fascinating structural aspects of the intracellular PHA. First, the way how the hydrophobic polymer is made compatible with the aqueous cell cytoplasm and compartmentalized there in a form capable of metabolic utilization. Second, the fundamental difference between basic material (mainly mechanical) properties of PHA inside and after isolation from the bacterial cells. It is well known that isolated PHB, the most common member of PHA family, constitutes a brittle material with an elongation to break as low as a few percent. This deformation behavior is attributed to the high crystallinity of the polymer. Nevertheless, it is well evidenced that inside the bacterial cells, the same polymer shows astonishing flexibility. It was repeatedly demonstrated by visualization of PHA accumulating bacteria with scanning electron cryomicroscopy (cryoSEM) that the cells, fixed and fractured at temperatures below -100 °C, show a specific plastic deformation of PHA granules, manifested by characteristic needle-type deformation artifacts where the granule stretches by more than 100% its original size (see Fig. 7b,d).

Although the first reports on this unusual deformation behavior of cellular PHA date back to the 1960s, little attention was paid to the obvious discrepancy with the highly crystalline nature of the isolated polymer back then.

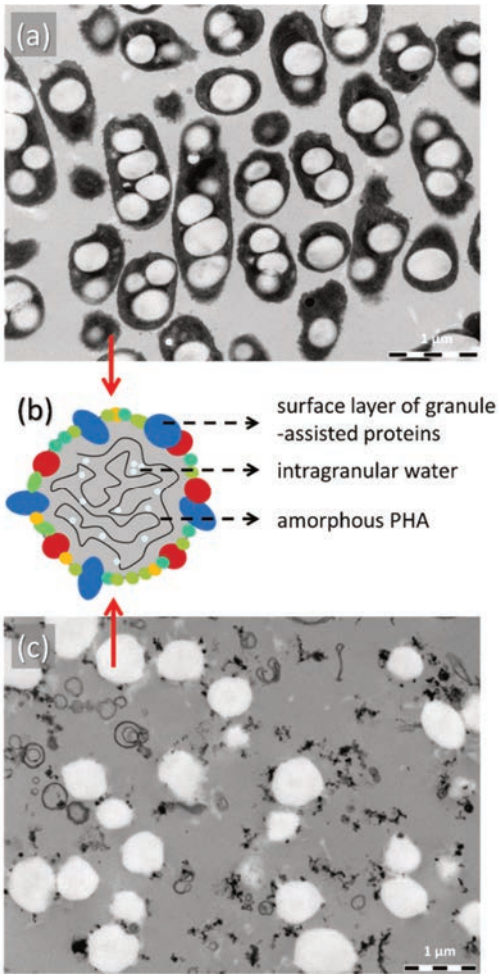

Fig. 6 Morphology of PHA granules in PHA-producing bacteria. (*a*) *PHA granules in the cells of the mesophilic PHA producer Cupriavidus necator* H16. (*b*) *Schematic representation of fundamental structural features of PHA granules.* (*c*) *Isolated PHA granules.*

We have provided an in-depth review the history of structural studies on the structure and supramolecular architecture of PHA granules *in vivo* in our review article[XIII] where we have also summarized our original contribution to the topic. We have described how the view on the mechanism of PHA solubilization in the cell shifted from the original idea of the polymer granule core covered by a layer of phospholipids [24] to the currently accepted notion of the surface layer of so-called PHA granule-associated proteins (PGAPs), that include PHA synthase, PHA depolymerase, functionally multifaceted surface proteins so-called phasins, and regulatory proteins [25]. We have also described how this supramolecular protein-

[XIII] Obruca, S., Sedlacek, P., Slaninova, E., Fritz, I., Daffert, C., Meixner, K., Sedrlova, Z., and Koller, M. Novel unexpected functions of PHA granules. *Applied Microbiology and Biotechnology.* 2020, 104, 4795–4810.

polyester composite is involved in the metabolism of PHA. Nevertheless, in our research, we have paid our attention mainly to the second interesting attribute of the native PHA – their strictly amorphous nature.

Speaking in exaggeration, the discovery of the inherent amorphousness of intracellular PHA was brought about more by scientific ignorance than by intent. It was in the 1980s, when the research group of Sanders measured ^{13}C NMR spectra of intact PHA-containing cells. The authors later conceded that they had ignored the conventional wisdom prejudging NMR to fail in analyzing cellular PHA, and therefore succeeded to record spectra of PHA in the resolution unattainable for a crystalline polymer. Later, they supported their NMR results with X-ray diffractometry and proved the absence of a substantial quantity of crystalline PHA in the cells. Since then, the biological importance of the amorphous structure of intracellular PHA has continuously been debated as well as a mechanism by which this thermodynamically unfavorable state of the polymer is maintained. The mechanism was expected to be simple, universally available and undemanding for any specific chemical or biological mediator because it was soon revealed that the transfer of genes related to PHA synthase enabled the formation of apparently fully biologically functional PHA granules not only in PHA non-accumulating bacteria [26], but also in green plants [27].

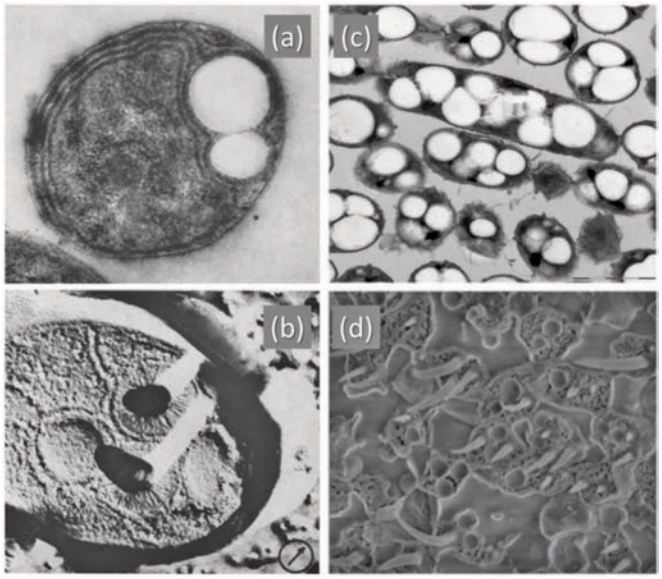

Fig. 7 Characteristic plastic deformation of PHA granules during cryoSEM imaging of PHA accumulating cells. (*a, b*) *First report on the needle-type deformation published for Nitrobacter cells by Van Gool [*122*] in 1969.* (*c, d*) *Similar deformation behavior of C. necator H16 revealed in our study*[XIX]. (*Results of cryoSEM* (*b, d*) *and TEM* (*a, c*) *visualization of the cells are compared for clarity.*)

Two main plasticizing effects has been proposed (see Fig. 8). The first one calls for the presence of an anonymous substance that acts as a plasticizer in the granule – it restricts the PHA chains from the molecular reorientation necessary for the crystallites to be formed. It is well known that despite its hydrophobic nature, PHA granules contain a significant amount of water inside (up to 10 wt. % according to Lauzier [28]).

Therefore, since the very beginning of these considerations, speculations on the role of water in this effect have been arising repeatedly (as reviewed in[XIII]), however, without any experimental evidence. The alternative mechanism for PHA plasticization was proposed by those who had evidenced the need for it – by the group of Sanders. Their explanation follows the laws of chemical kinetics and suggests that the crystallization of native PHA is restricted by the extremely low volume of the polymer granule where the rate of nucleation converges to zero [25, 29]. The authors also supported their theory with an experimental study on the rate of crystallization of artificially synthesized amorphous PHA of the submicron size.

Nevertheless, even this explanation was not fully consistent with all experimental observations (e.g. with the crystalline-shell/amorphous-core PHA granules described by Lauzier [28]).

When reviewing the literature sources on this topic, our attention was caught by frequent reports on how various chemical and physical treatments "denature" PHA – i.e. alter the structure of PHA into the form not utilizable by the cells (again, the summary of these works is provided in our paper[XIII]). At the time, we had already been fully engaged in the topic of the involvement of PHA in stress survival and robustness of bacteria (discussed in detail in 2.2). Therefore, we were inspired to design a systematic study relating stress exposure of PHA-containing cells, structural and morphological changes induced on PHA granules by the stress, and the impacts of these changes on the survival and cultivability of the cells.

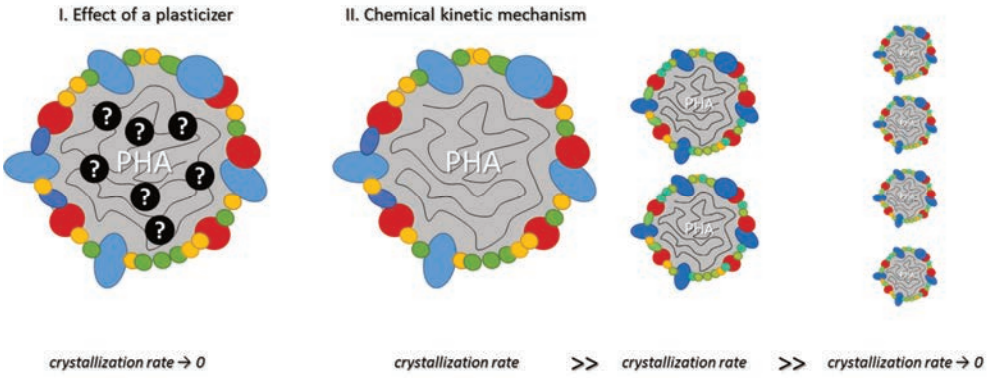

Fig. 8 Two formerly proposed theories on the mechanism of PHA plasticization in vivo – I. effect of a molecular plasticizer, and II. chemico-kinetic model based on limited volume of the granules.

In our study[XIV], we exposed bacterial cultures of a PHA producer (*Cupriavidus necator* H16) with high content of accumulated PHA in the cells (above 70% of cell dry weight) to various stress conditions (elevated temperature, freezing/thawing cycles, high salinity, acidic pH). With TEM, we determined changes caused in the cellular morphology and ultrastructure, and with time-resolved ATR (*attenuated total reflection*) FTIR spectroscopy, we monitored changes in PHA crystallinity in the cultures as they were freely dried on the ATR crystal. We have confirmed by the combination of these two assays that only those stress factors that caused the aggregation of intracellular granules induced also the crystallization of PHA. With respect to the significant spectral signs of protein denaturation in these samples, we inferred that the aggregation process was initiated by stress-induced structural changes in the granule-associated proteins inactivating their surfactant role on the granule-cytoplasm interface. The fatal impact of granule aggregation on cell physiology was also confirmed in our study by the cell cultivation test. This finding that granule aggregation is a necessary condition for PHA crystallization could in itself be considered strong evidence for the correctness of the chemical kinetic explanation of the amorphousness of intracellular PHA proposed by Lauzier. Nevertheless, we have also revealed that in the otherwise intact cells with the stress-induced aggregation of PHA granules, the crystallization never followed the stress exposure immediately. Instead, it always occurred with a significant time delay after the cell culture got dried. Obviously, the increased volume of coalesced granules was not by itself able to initiate the crystallization process. The importance of the cell drying hence brought the plasticizing role of water back to the scene.

Based on these experimental results, we proposed an updated view on the way of PHA plasticization *in vivo* that assumes concurrency and synergism of both previously considered mechanisms (see the schematics of our idea on the mechanism of stress-induced PHA crystallization *in vivo* in Fig. 9). We consider

[XIV] Sedlacek, P., Slaninova, E., Enev, V., Koller, M., Nebesarova, J., Marova, I., Hrubanova, K., Krzyzanek, V., Samek, O., and Obruca, S. What keeps polyhydroxyalkanoates in bacterial cells amorphous? A derivation from stress exposure experiments. *Applied Microbiology and Biotechnology.* 2019, 103, 1905–1917.

the metastable amorphous state of PHA as a result of the well-tuned interplay of kinetic effects resulting from the sub-micron volume of the granules and the plasticizing effect of intragranular water. Anyway, the physical state of cellular PHA is another obvious example of a topic where physical chemistry and biology shake their hands. The long-known inability of intracellular depolymerases to cleave crystalline PHA has led to the current use of the terms “native” and “denatured” PHA as synonyms for amorphous and crystalline PHA, respectively [30]. Furthermore, I will discuss in the next chapter that recent studies (including ours) have shown that the biological consequences of the unique physical state of native PHA are not limited to the metabolism of this compound and are in fact much more complex. To conclude, this particular research topic can hence be used as another example of the general message repeatedly emphasized in this thesis – the fact that the (molecular) interactions build up the specific supramolecular architecture of the system, which in turn rules its material properties and – in this case – also the biological performance. Designing and performing the experimental studies in order to uncover this relationship for diverse ecological and biological systems is where I see the exclusive and irreplaceable role of biophysical chemistry.

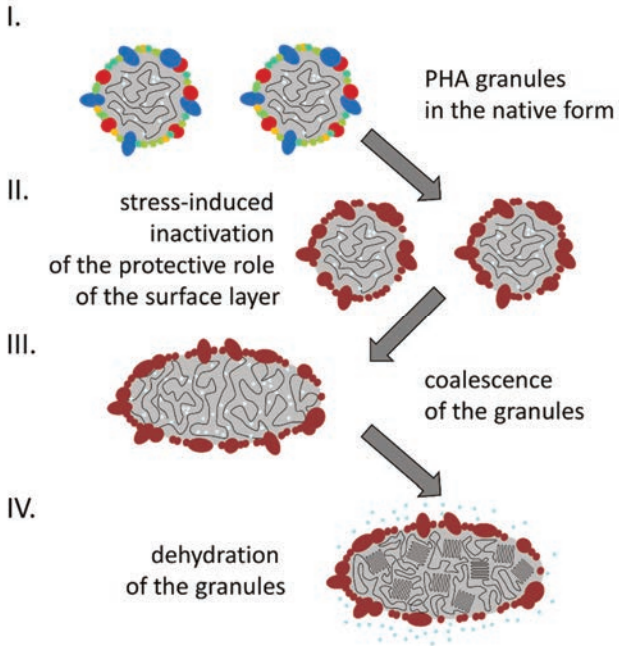

Fig. 9 Stress-induced crystallization of PHA in the cells as explained by the synergy of chemical-kinetic and water-mediated effects proposed as the updated view on PHA plasticization in vivo[XIII,XIV].

1.2.2 Development of nature-inspired bioplastics with tailorable properties

Similar to our research interests in humic substances, also in the case of PHA we have put an effort into utilizing the fundamental knowledge gained on the native behavior of the material also in its applied research. The application potential of PHA is outstanding. Over the past years, the range of end uses that PHA has been proposed or tested for, as well as the list of PHA producing and/or researching companies has steadily grown. In the current commercial uses of - as well as in the applied research on - PHA, PHB still represents the main subject of interest. Nevertheless, the inherent crystallinity and the associated brittleness and rigidity of PHB represent a severe limitation of the material, especially in the film-forming segment, e.g. in the packaging industry. Therefore, special attention has recently been paid to alternative PHA materials – mainly copolymeric and mcl-PHA, and also the PHA-containing polymer blends or resins [31–33]. Furthermore, regardless of the relative growth of the segment of PHA bioplastics in recent years, incomparably high current production costs compared to other (bio)plastics still represent the severe

limitation hindering the further expansion of PHA in the market. Therefore, strong efforts are currently targeted also on searching for novel trends in the biotechnological production of PHA that would increase the economic feasibility and competitiveness of PHA uses.

In our applied research interest in PHA materials, we have been trying to aim at both above-mentioned targets. We have developed and published several biotechnological processes for microbial production of PHA copolymers or terpolymers[XV,XVI]. The cost-effectiveness and competitiveness of the technological process were improved by the involvement of thermophilic producers with respect to the recently proposed concept of Next-Generation Industrial Biotechnologies. We have confirmed that changing the monomer composition of PHA by varying cultivation conditions enables manipulation with the crystallinity of the material in a wide range. For example, relative content of 3HV in P(3HB-*co*-HV) polymers biosynthesized by *Schlegelella thermodepolymerans*[XV] influences crystallinity markers obtained by DSC and FTIR analysis. Evidently, with the single bacterial producer cultivated under different conditions, it is possible to obtain copolymers with crystallinity ranging from about 60 % (P(3HB)) to apparently zero (P(3HB-*co*-33% 3HV)). In the subsequent study[XVI] we evaluated our own thermophilic isolate as a candidate producer of PHA copolymers. This bacterium, isolated from the urban composting plant located in Brno, was taxonomically classified as a member of the genus *Aneurinibacillus*, designated as *Aneurinibacillus* sp. H1 and deposited in the Czech Collection of Microorganisms. In the pilot assessment of its biotechnological potential, we have revealed its extraordinary ability to synthesize PHA copolymers and terpolymers containing high molar fractions of 3HV and 4HB subunits when valerate and/or 1,4-butanediol are used as 3HV and 4HB precursors, respectively.

The study was followed by a thorough material analysis of the produced polymers in form of the solvent (chloroform) casted films[XVII]. Furthermore, we have also launched a follow-up in-depth study on the application-relevant properties of the produced PHA copolymers produced by *Aneurinibacillus* sp. H1, where we have been focusing mainly on the deformation behavior of the films, their surface properties, transport properties (i.e. release kinetics of hydrophilic and hydrophobic solutes from the polymer films) and barrier performance (gas permeability of the films). Considering also potential biomedical uses of the polymers, we have assayed also the biodegradation of the films in model body fluids. Preliminary results confirm that all these properties, expectedly dependent on the crystallinity of the material, can be tailored via adjustment of the monomer composition of PHA copolymers by controlling the conditions of the biotechnological process.

To conclude, the degree of the molecular order in the architecture of PHA systems is not only of great biological importance in the natural intracellular PHA granules but also of extraordinary significance in the development of reasonable PHA materials for artificial uses. As nature has found ways of how dealing with PHA crystallinity in the living cells, we also need to find our options on how to manipulate this essential, performance-ruling property of PHA utilized technologically. In our work, we have experienced how beneficial the synergy between bio(techno)logy and material (or physical) chemistry might be in taking this challenge.

[XV] Kourilova, X., Pernicova, I., Sedlar, K., Musilova, J., Sedlacek, P., Kalina, M., Koller, M., and Obruca, S. Production of polyhydroxyalkanoates (PHA) by a thermophilic strain of Schlegelella thermodepolymerans from xylose rich substrates. *Bioresource Technology.* 2020, 315, 123885.

[XVI] Pernicova, I., Novackova, I., Sedlacek, P., Kourilova, X., Kalina, M., Kovalcik, A., Koller, M., Nebesarova, J., Krzyzanek, V., Hrubanova, K., Masilko, J., Slaninova, E., and Obruca, S. Introducing the Newly Isolated Bacterium Aneurinibacillus sp. H1 as an Auspicious Thermophilic Producer of Various Polyhydroxyalkanoates (PHA) Copolymers–1. Isolation and Characterization of the Bacterium. *Polymers.* 2020, 12, 1235.

[XVII] Sedlacek, P., Pernicova, I., Novackova, I., Kourilova, X., Kalina, M., Kovalcik, A., Koller, M., Nebesarova, J., Krzyzanek, V., Hrubanova, K., Masilko, J., Slaninova, E., Trudicova, M., and Obruca, S. Introducing the Newly Isolated Bacterium Aneurinibacillus sp. H1 as an Auspicious Thermophilic Producer of Various Polyhydroxyalkanoates (PHA) Copolymers–2. Material Study on the Produced Copolymers. *Polymers.* 2020, 12, 1298.

Chapter 2:
"Physical microbiology": physicochemical support for biotechnology and microbiology

"Gentlemen, it is the microbes who have the last word"
(*Louis Pasteur*)

In the next chapter, we will unzoom our focus from individual chemical components (such as the biocompounds discussed previously) to whole organisms – although perhaps the simplest ones. My personal scientific interest in microbes began in 2015 when I was invited to join a research project dealing with PHA-producing bacteria. Before I had considered bacteria as something that no physical chemist would intentionally keep company with and if someone accidentally met it, after all, he or she would care only about getting himself/herself prescribed antibiotics. Very soon, I realized how silly my original idea of bacteria was.

Bacteria are the oldest known and the most widespread inhabitants of the planet Earth. We can find some of the approximately five million trillion trillion (no, the second trillion is not there by mistake!) of them literally everywhere – from deep thermal vents to clouds in the atmosphere, to the inside of our body (in there, the number of bacterial cells outnumbers the human body cells by tens of percent). Lined up end to end, they would stretch some 10 billion light-years — literally from here to the edge of the visible universe. Among these huge numbers, the vast majority of them is harmless or even helpful for humans. And, furthermore, these "simple" organisms are outstandingly attractive for scientists. Not just because most of them are yet to be identified, but also the old acquaintances are still full of surprise, as we have experienced in our research.

2.1 Being stuffed with plastics: polyhydroxyalkanoates in bacteria

Nowadays, when plastic pollution becomes one of the most pressing environmental issues, we may easily have the plastics up to our necks. But what if this is not just figuratively speaking? How would it feel to have our bodies stuffed with plastic? Terrible vision, isn't it? Surprisingly, we know organisms that experience exactly this feeling and, moreover, it seems that in fact, they enjoy it very much.

Again, we are of course talking about bacteria. More specifically, about some of the polyhydroxyalkanoates (PHA) accumulating bacteria. These organisms literally stuff their cells with PHA in form of cellular inclusions called carbonosomes or PHA granules.

Originally, it was assumed that the role of PHA is solely in the storage of energy and carbon for the situations when their other sources are depleted. Nevertheless, at the time when I entered the topic of PHA, more and more suggestions began to appear that the ecological role of these polymers is in fact much more complex. At that time, great attention was paid to the biotechnological production of PHA at Faculty of Chemistry, BUT, and the involved researchers came up with various reports on how exposition of bacterial culture to controlled stress dose might be beneficial in terms of providing enhanced accumulation of PHA in bacterial producers [34–36]. Moreover, several other authors had reported that some PHA accumulating strains revealed higher stress resistance against various environmental stress factors than mutants unable of PHAs production or degradation [37–42]. Among them, Kadouri proposed that a detailed understanding of the role of PHA in stress resistance and adaptation represented a great challenge to the microbial ecology [42]. Altogether, this motivated the establishment of a new interdisciplinary research group at Faculty of Chemistry, BUT, that decided to accept this challenge and perform a complex study on how the PHA presence affects the biochemical and biophysical state of bacterial cells and what are the particular mechanisms of PHA involvement in stress robustness of bacteria.

I have joined this research group to provide methodological and descriptive approaches of physical chemistry, that I had been using by that time mainly in soft matter analysis. In the full-length version of the habilitation thesis, I summarize how these attitudes, rather unconventional concerning the study of living

organisms, were found beneficial in providing original information on the effects of PHA on the chemical and physical structure of the cells and their morphology and physiological state. In this short version, I will focus just on the biophysical consequences of PHA presence in the cell, and also on how the knowledge on the complex effects of PHA was transformed into understanding the interconnection between PHA and stress resistance and adaptation of microbial cells.

2.1.1 Biophysical consequences of PHA in cells

A comprehensive understanding of the physiological state of a cell always involves putting together numerous pieces of a puzzle, that are brought from different angles of analytical perspective. Aside from metabolic, morphological or chemical analyses, the determination of various physicochemical parameters of the cellular space (such as pH, redox potential, viscosity and many others) has long been recognized as especially contributive. Therefore, a number of biophysical techniques providing these parameters have already become a standard part of an analytical toolbox for biologists. Definitely, fluorescence spectroscopy represents a great example. With a plethora of fluorescent probes with specific affinity to individual cell components or sensitivity to particular physicochemical conditions, and in combination with advanced microscopy or microfluidics instrumentation, fluorescence methods become an outstandingly powerful approach covering a wide range of applications, from a routine cell viability testing to study of dynamic cellular processes and events. Not surprisingly, we have involved a battery of techniques of fluorescence spectroscopy also in our research on PHA-producing bacteria. In particular, intracellular pH, microviscosity, and the presence of reactive oxygen species were analyzed during the stress-exposure experiments by means of fluorescence techniques. Nevertheless, because this methodology was covered by another part of our team, I will not deal with this method in this text. Instead, I am going to focus more on the methods whose previous application in (micro)biology was rather scarce.

First of all, let's get straight to another of the basic spectroscopic methods. We have demonstrated how powerful a combination of two simple spectrophotometry approaches – light scattering measurement and diffuse transmittance spectrophotometry – can be in understanding how the accumulation of PHA granules affects the radiation exposition behavior of bacterial cells. In the study[XVIII] where we focused on the evaluation of the protection role of PHA granules against the harm brought by UV radiation to bacterial cells, we investigated PHA accumulating (*C. necator* H16) and non-accumulating (*C. necator* PHB^{-4}) cultures by the combination of standard spectrophotometric (direct transmittance), nephelometric (measurement of scattered light intensity at 90°) and diffuse transmittance analyzes. The illustrative results of this analysis are shown in Fig. 10.

Evidently, PHA accumulating cells shown much more intensive light scattering in Vis region (see Fig. 10 a,b). Taking into account the similar size and shape of both strains, it is obvious that the extra scattering (the most clearly represented by the increased slope of the dependence shown in Fig. 10b) is provided by the cell ultrastructure – i.e. by the PHA granules. Not surprisingly, no significant light absorption was found neither for PHA positive nor negative culture in the Vis region confirming that no pigments or other cell components photoactive in this spectral region are present (Fig. 10c). On the other hand, specific absorption in UV region (absorption band at about 254 nm can be attributed to light absorption by nucleic acids, DNA in particular) was found to be significantly reduced (by around one-third) in the PHA accumulating culture. In the paper, we attributed this reduced absorption of harmful UV radiation to the protective shielding effect of PHA granules resulting from their great light-scattering ability (note that PHA granules are not randomly distributed in bacterial cells, but they are attached to DNA via specific proteins). These assumptions were also supported by the results of nephelometric analysis where the relative efficiency of light scattering by the PHA producer as compared to the mutant strain increases significantly in the UV-region (Fig. 10e). We have also experimentally proved the biological consequences brought by this protective effect on the survival of the cells exposed to UV radiation in the study. In this study, we have

[XVIII] Slaninova, E., Sedlacek, P., Mravec, F., Mullerova, L., Samek, O., Koller, M., Hesko, O., Kucera, D., Marova, I., and Obruca, S. Light scattering on PHA granules protects bacterial cells against the harmful effects of UV radiation. *Applied Microbiology and Biotechnology.* 2018, 102, 1923–1931.

demonstrated that utilization of unusual, yet simple and widely available, spectroscopic approaches may be surprisingly beneficial in understanding even such complex biological phenomena as the resistance of bacterial cells against UV irradiation.

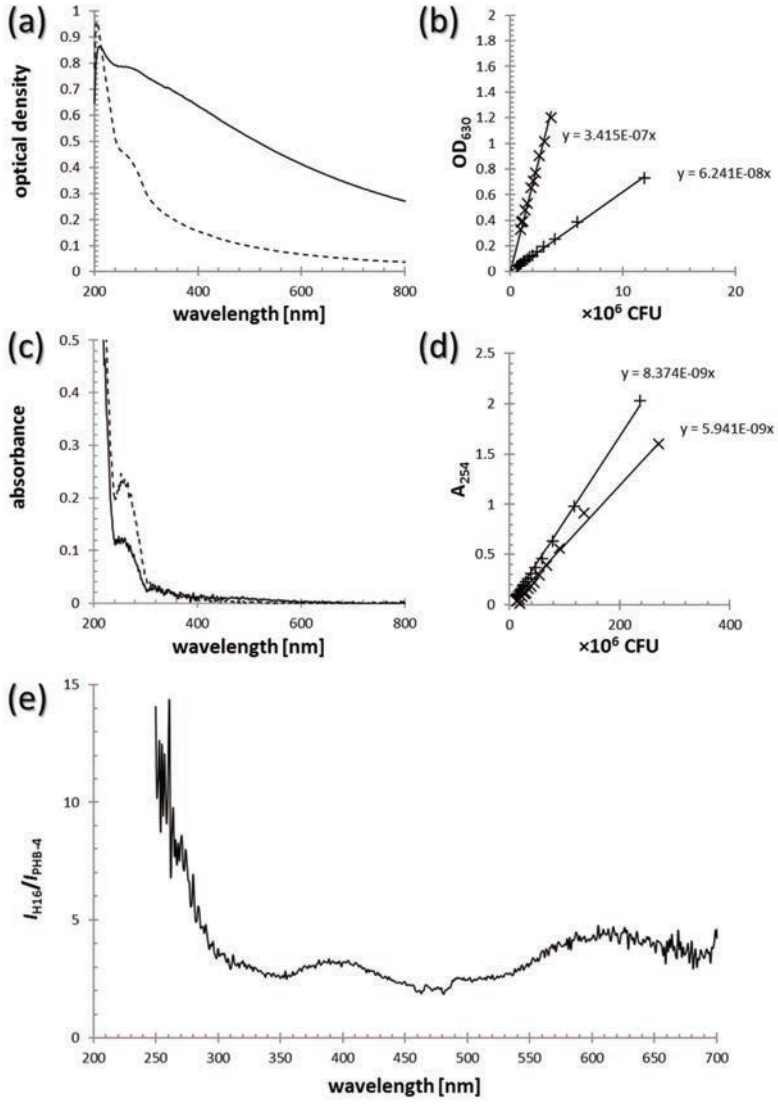

Fig. 10 Spectral characteristics of PHA accumulating (*C. necator* H16) *and non-accumulating* (*C. necator* PHB–4) *cultures.* (*a*,*c*) *Spectra of C. necator H16* (*solid*) *and C. necator PHB–4* (*dashed*) *with the same cell density* (*1×10⁶ CFU*) *obtained by direct transmittance measurement* (*a*)*, and by diffuse transmittance measurement* (*c*)*, respectively.* (*b*) *Optical density at 630 nm, determined by direct transmittance measurement as a marker of total light scattering, as a function of cell density of C. necator* H16 (×) *and* PHB^{-4} (+)*, respectively.* (*d*) *Absorbance at 254 nm, determined by diffuse transmittance measurement as a marker of specific UV absorption by DNA, as a function of cell density of C. necator* H16 (×) *and* PHB^{-4} (+)*, respectively.* (*e*) *Ratio of the normalized intensities of scattered light for C. necator* H16 *and* PHB^{-4} *at 90° as determined by nephelometry. Data were taken from publication*[XVII].

Moreover, we have also utilized various methods of thermal analysis primarily to reveal how a behavior of cellular water is influenced by the presence of PHA. For instance, in our study[XIX] we utilized thermogravimetry as a method of determination of the intracellular water content. We have optimized the technique originally proposed by Uribelarrea [43] in which the intracellular and extracellular water are distinguished by the specific change in the drying rate during an isothermal drying procedure. Over time, we have successfully applied the method in several studies where the changes in either intracellular water content or in cell membrane integrity, affecting the drying profile of the cells, took place. An example of the results obtained by the technique is shown in Fig. 11, where can be seen the expectedly higher specific content of intracellular water for PHA non-accumulating culture as well as obvious cell desiccation caused by a hyperosmotic environment.

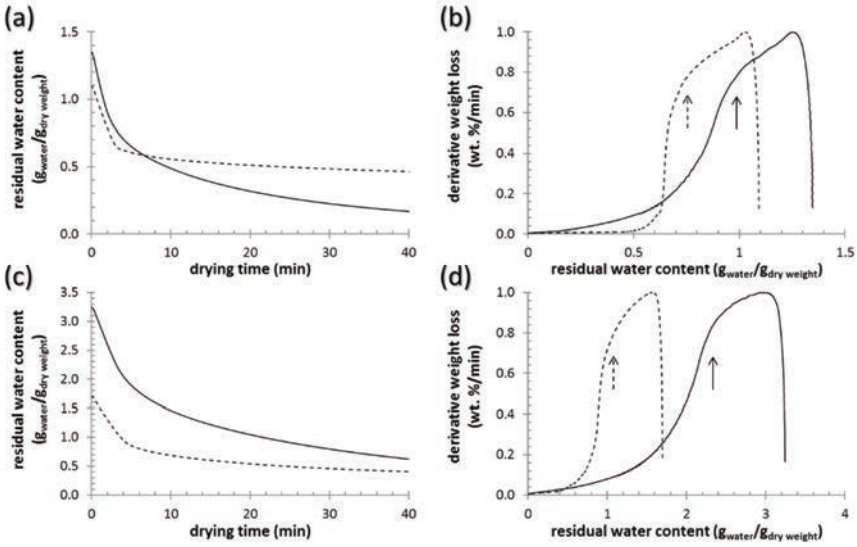

Fig. 11 Results of isothermal thermogravimetry determination of intracellular water content. Raw thermograms (*a, c*) *and drying rate profiles* (*b, d*) *of PHA containing cultures of C. necator* H16 (*a, b*) *and non-accumulating mutant C. necator* PHB^{-4} *in control isotonic buffer* (*solid line*) *and in 200 g/L NaCl* (*dashed line*).

2.2 Feeling stronger: role of PHA in bacterial stress-response

Although bacteria are among the most abundant organisms in nature, nature is not always very kind to them. Without a moment of rest, bacteria are challenged by the environment, being exposed to rapidly fluctuating physical conditions such as temperature, pH or salinity, and intensity of light, or to chemical restraints like limited availability of nutrients. To cope with the inhospitality of their natural habitats, bacteria developed numerous strategies which help them to face various stress conditions. It was likely just their astonishing variability in stress-response scenarios that made bacteria so successful in the process of natural selection in various highly competitive environments. [44, 45]

As it was already mentioned, PHA, since their discovery almost a century ago, were long considered simply carbon storage materials. Hence, their stress-coping role was seen just in providing energy during periods of starvation and their involvement in other protective mechanisms were overlooked, until the first study indicating their more complex biological role was published by Tal and Okon [46]. The authors reported that bacterial cells of *Azospirilum brasilense* accumulating PHA up to about 40% of CDM were

[XIX] Obruca, S., Sedlacek, P., Krzyzanek, V., Mravec, F., Hrubanova, K., Samek, O., Kucera, D., Benesova, P., and Marova, I. Accumulation of Poly(3-hydroxybutyrate) Helps Bacterial Cells to Survive Freezing. *PLOS ONE*. 2016, 11, e0157778.

significantly more resistant to UV irradiation, desiccation and osmotic pressure than cells containing a very low amount of PHA (about 5% of CDM).

We have followed up on these first works with an extensive research study, where we exposed the famous representative of PHA accumulating bacteria, *C. necator* H16, to various adverse conditions, and compared not only its overall stress-survival but also various specific aspects of its stress-response behavior, to those of the PHA non-accumulating culture (most often the PHA synthase deletion mutant *C. necator* PHB^{-4}). First, we paid attention to the resistance of the *C. necator* cultures against low temperature and freezing[XIX]. As far as approximately 80% of our planet's biosphere is permanently cold with average temperatures below 5°C and even in the remaining regions the temperature fluctuates wildly, low or cryo temperatures represent one of the most common types of adverse conditions that bacteria have to cope with. The mechanism of cell damage for this case is well-described and depends on whether or not the temperature decreases below the water freezing point. Above, the bacterial cells are usually capable of active defense against, usually involving synthesis and action of cold shock proteins or other specific cold-fighting metabolites. On the other hand, when the temperature reaches the value at which water starts freezing, most prokaryotes lose the ability to respond actively, which usually leads to the death of cells. As the extracellular ice crystals grow, osmotic pressure in the medium increases since the excluded solutes concentrate in a decreasing volume of water. This effect leads to "freeze dehydration", and has harmful consequences for challenged cells similar to that of cell exposure to an environment with high salinity. Other lethal effects are caused by the formation of intracellular ice crystals that bring damage to membranes and organelles and cause also the formation of intracellular gas bubbles. Last but not least, cells can also be damaged by reactive oxygen species formed in cells during freezing, and by the mechanical injury induced by the decreasing volume of the bacteria-inhabited channels of unfrozen liquid surrounded by growing ice crystals [47].

In our study[XIX], we have confirmed that the bacterial strain capable of PHA accumulation revealed a significantly higher ability to endure freezing than its PHA non-producing mutant strain. The difference between the viabilities of the respective bacterial strains increased with decreasing freezing temperature, the protective effect of PHA was hence the most obvious at -20°C where 84.1% of *C. necator* H16 cells retained viability, while only 34.5% of *C. necator* PHB^{-4} cells were identified as viable. We have also evidenced that the protective effect is not connected with direct metabolic utilization of PHA (polymer content in the cells did not change during the experiment).

A lot of effort was put into the paper to the detailed discussion of the mechanism of this cryoprotection provided by PHA. Taking together the specific effect of PHA on the activity of intracellular water, specific viscoelastic properties of PHA granules and the higher cellular content of cryoprotective 3-hydroxybutyrate molecule in PHA producing bacteria, we have proposed a concept of the simultaneous interplay of all these contributions in the complex protective effect of PHA. Specific physico-chemical properties of PHA-containing cells seem of crucial importance in this fine-tuned mechanism. The flexible scaffold of PHA granules with the liquid-like properties provides physical protection for cells against the formation of ice crystals and shearing stress associated with the freezing of extracellular water. Furthermore, the affected activity of intracellular water influences the rate of cell dehydration during freezing. It is well known that a compromise between a too high degree of cell dehydration and a total suppression of it is optimal in order to minimize the resulting cell mortality. It is therefore very likely that the effect of PHA presence on the rate of transmembrane transport of water may represent an important contribution to the overall cryoprotective strategy of PHA-producing bacteria.

Next, we turned our attention to the resistance of PHA-producing bacteria against UV irradiation. Again, UV light belongs among the most frequent environmental stress factors. It brings various harmful impacts on living organisms, for instance, UV light absorption induces oxidative pressure by stimulating the production of reactive oxygen species (ROS). Furthermore, it causes damage to the molecular structure of essential biomolecules, mainly nucleic acids, but also lipids or proteins [48]. Several reports dealing with the stress robustness of PHA producing bacteria indicated that also the presence of PHA granules in microbial

cells provides protection against UV radiation [49-52]; however, none of these studies focused on revealing the particular UV-protective mechanism of the granules.

Therefore, in our study[XVII] we exposed *C. necator* H16 and PHB^{-4} cultures to UVA radiation and measured cell viability at different time intervals. We confirmed that PHA accumulating strain showed significantly higher survival than the non-accumulating one. We complemented this study with a comprehensive investigation of light absorption and scattering properties of the cultures. We have shown that, as expected, PHA granules do not considerably absorb UV radiation but they are effective light scatterers. Apart from that, we revealed a significantly lower absorption of UV light by DNA molecules, and, moreover, a considerably reduced level of ROS in the PHA-accumulating cells. It is well known that PHA granules are not randomly distributed in bacterial cells, but they are specifically attached to DNA, the most UV-sensitive molecule which enhances their shielding effect [53].

Another environmental stress factor that, naturally, must not be missed in our study, is osmotic stress. Changes in external osmolarity are experienced by prokaryotes on a daily basis. Soil bacteria, for instance, are exposed to quick fluctuations in external salinity depending upon the weather. When bacterial cells are exposed to hypertonic conditions caused by a high extracellular concentration of solutes, water goes out of the cells causing quick dehydration of the cytoplasm. Moreover, as the volume of cytoplasm decreases, the cytoplasm membrane shrinks and separates from the outer layers of the cell envelope in the process known as plasmolysis. Conversely, when bacterial cells are exposed to hypotonic conditions, water influx tends to increase the cell volume and put significant mechanical forces on the membrane. Because the cytoplasm membrane is weak in tensile properties and cannot cope with significant volume changes (it was reported that is not capable of shrinking more than 2–5% [54]), both processes often result in a loss in integrity or even collapse of the cytoplasmic membrane (in the case of osmotic down down-shock referred to as hypotonic lysis).

Again, protective effects of PHA were reported before also for osmotically challenged bacterial cells [40, 50, 52]. We supplemented these works with a study[XX] where we confirmed that the PHA-accumulating wild-type strain (*C. necator* H16) survived osmotic up-shock much better than the PHA non-accumulating mutant (*C. necator* PHB^{-4}). Besides, we proved that the protective mechanism is not connected to the direct metabolic utilization of PHA, since we found that the osmotic up-shock did not induce any intracellular PHA degradation. Our multidisciplinary perspective, involving morphological and biophysical analyses of the intact and stress-exposed cultures, led us to the understanding that the PHA granules provide a scaffold-like effect to the cells and prevent them against massive plasmolysis which was manifested in PHA non-accumulating cells. An interesting contribution to this understanding was provided by thermogravimetric assay for the determination of intracellular water. As expected, the critical water content (that is considered equivalent to the content of water in the cells) decreased for osmotically challenged cells compared to the intact ones, however, we discovered that for PHA accumulating cells, this osmotically induced decrease in intracellular water content was significantly less pronounced compared to the cells unable of PHA accumulation. Another important observation was provided by cell morphology imaging by TEM (and complemented with cryoSEM). When the plasmolysis occurred in the very close vicinity of PHA granules, it was possible to observe that PHA partially stabilized membranes by "plugging" small gaps. Therefore, it is likely that the osmoprotective effect of PHA granules is at least partially enabled by the unique mechanical properties of amorphous intracellular PHA granules[XIX].

We later conducted a follow-up study[XXI] focused on the fate of the same bacterial cultures exposed to subsequent osmotic up- and down-shock. Once again, the PHA accumulating wild-type strain *Cupriavidus necator* H16 survived this challenging sequence much better than the PHA negative mutant. It was

[XX] Obruca, S., Sedlacek, P., Mravec, F., Krzyzanek, V., Nebesarova, J., Samek, O., Kucera, D., Benesova, P., Hrubanova, K., Milerova, M., and Marova, I. The presence of PHB granules in cytoplasm protects non-halophilic bacterial cells against the harmful impact of hypertonic environments. *New Biotechnology.* 2017, 39, 68–80.

[XXI] Sedlacek, P., Slaninova, E., Koller, M., Nebesarova, J., Marova, I., Krzyzanek, V., and Obruca, S. PHA granules help bacterial cells to preserve cell integrity when exposed to sudden osmotic imbalances. *New Biotechnology.* 2019, 49, 129–136.

evidenced both by the morphological and thermogravimetry assay that the PHA non-accumulating cells underwent massive hypertonic lysis, while the PHA-accumulating culture was capable of maintaining the cell integrity when suddenly transferred from hypertonic solution to distilled water. Isothermal TGA analysis proved to be a powerful technique for the detection of membrane integrity loss which was also confirmed by TEM analysis. To untie the down-shock behavior from the specific effects of previous hyperosmotic challenges, we included in this study also an investigation of the hypoosmotic challenge of PHA-accumulating halophilic bacterium *Halomonas halophila* which is adapted to high salinity and, therefore, did not experience any osmotic up-shock during the experiment. Also in this microorganism, exposure of the cells to hypotonic conditions (distilled water in particular) resulted in massive lysis of PHA-poor cells and considerable capability of keeping the cell integrity and viability in PHA-rich cells.

Beyond the scope of the above-mentioned studies, by the way of our research, we have met several additional manifestations of the increased cell robustness of PHA accumulating bacterial cells. For example, when we tested the usability of an analytical centrifuge in the analysis of PHA producing, we have found that boiled cultures of cells rich in PHA were much less prone to cell lysis than those with low PHA content. We have summarized the state of the art in understanding PHAs' role in stress-robustness in one review article[XXII] and one book chapter[XXIII]. Anyway, all the published reports and our original findings on the involvement of PHA in the stress-resistance of bacteria have led us to the conclusion that bacteria use PHA like we use a Swiss army knife (Fig. 12). Like a better tool can be found for each of its purposes, there are mechanisms more sophisticated and powerful, that some bacteria can use when coping with an individual stressor. Nevertheless, like in the Swiss knife, the main advantage stands in the versatility of the PHA effects. For both tools, a wide range of benefits is obtained for a reasonable price.

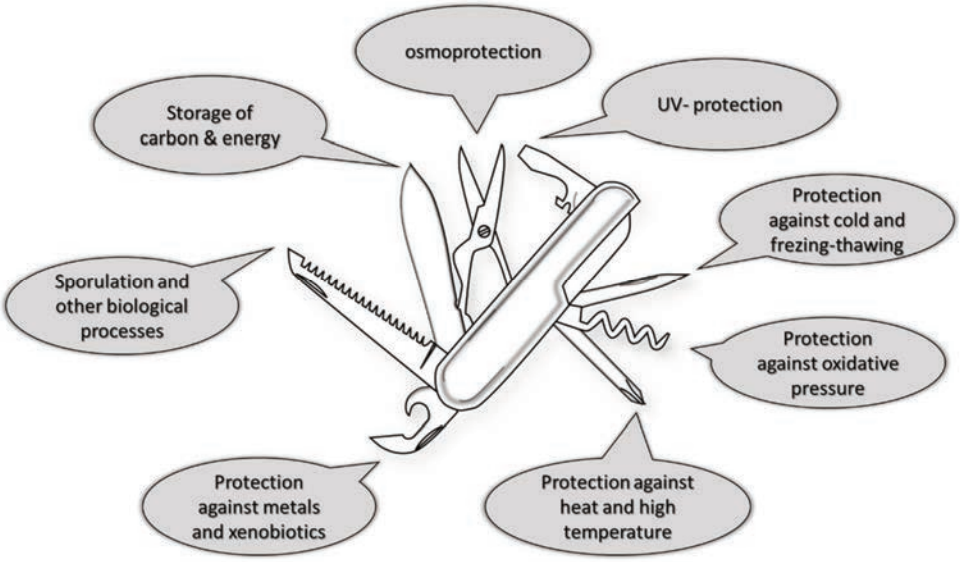

Fig. 12 Bacterial PHA granules are like a Swiss army knife: they offer versatile performance for a reasonable price

[XXII] Obruca, S., Sedlacek, P., Koller, M., Kucera, D., and Pernicova, I. Involvement of polyhydroxyalkanoates in stress resistance of microbial cells: Biotechnological consequences and applications. *Biotechnology Advances.* 2018, 36, 856–870.

[XXIII] Obruca, S.; Sedlacek, P.; Pernicova, I.; Kovalcik, A.; Novackova, I.; Slaninova, E.; and Marova, I. Interconnection between PHA and Stress Robustness of Bacteria. *In The Handbook of Polyhydroxyalkanoates*; CRC Press, 2020; pp. 107–132.

2.3 Another brick in the wall: monomer comes to the scene

Physical work is widely accepted as a great stress and anger relief. Nevertheless, imagine how relaxed and un-irritated would you feel, if you, for instance, were building a wall and, at the same time, someone next to you was demolishing the wall back to the bricks with a hammer? You may be asking why would he do that? Well, maybe just because he needed the bricks for another purpose.

Actually, nature provides numerous examples of how functional this apparently nonsense combination of constructive and destructive processes can be. One of those can be found also in the metabolism of polyhydroxyalkanoates in PHA-producing bacteria. It is well known that the PHA metabolism has a cyclic nature. It means that at a given moment, although the rate of PHA synthesis or hydrolysis prevails according to the current physiological state, both metabolic pathways are still active in the cell. The PHA metabolism is therefore referred to as the so-called "PHA cycle" [42]. Consequently, PHA-producing cells always contain a substantial amount of PHA monomers. So why do these cells aside from the energy investment into building the wall (PHA synthesis) maintain also the energy input into the wall demolishing (monomer production)? For what do they need a constant supply of the loose bricks?

At the time when we came to the topic of PHAs´ role in cell robustness, there was to our best knowledge single report indicating that 3-hydroxybutyrate (3HB) *per se* could provide a protective effect to the bacterial cells (or, more specifically, to the cellular proteins) [55]. Aside from the study focusing on the protective role of polymer PHA and its intracellular granules, we hence performed the experiments where we confirmed the interesting chaperonic effects of the monomer unit, and also the follow-up physico-chemical study where the light was shed on some of these effects from the viewpoint of the phase behavior of 3HB in aqueous solutions.

2.3.1 Protective effects of 3-hydroxybutyrate

In order to evaluate the protective effects of the monomer unit most frequently contained in the polymer structure of PHA, i.e. 3- hydroxybutyric acid (or 3-hydroxybutyrate as its dissociated form), we first investigated[XXIV] its capability of protecting model enzymes (lipase, lysozyme) from heat-induced or chemical (oxidative) denaturation. For this purpose, we used not only standard biochemical assays (enzyme activity measurement), but in parallel also physicochemical methods, namely DLS (monitoring the increase in the hydrodynamic volume of the proteins during the denaturation) and DSC (investigating the denaturation by its heat signature). Surprisingly for us at the moment, we have revealed that when compared at the same molar concentration, 3HB showed a greater protective effect than the well-known chemical chaperones, such as trehalose or hydroxyectoine. Furthermore, in the study[XIX] dealing with the role of PHA in the survival of microbes under freezing, we, to our best knowledge for the first time, manifested the structure-stabilizing effect of 3HB on an enzyme also during cyclic freezing/thawing. With the increasing addition of 3HB, the residual activity of the analyzed enzyme (lipase) increased significantly during the repetitive (up to 7 consecutive cycles) freezing and thawing. This protective effect was, again, comparable to that of trehalose, a well-recognized cryoprotectant.

2.3.2 On the hydration of 3-hydroxybutyrate

The protective effect of 3HB attracted our attention not only from the purely biological point of view. Bearing this essential role of water in mind, we decided to perform a comprehensive study[XXV] on the thermodynamics of the aqueous solutions of sodium salt of 3HB (Na3HB) as an inevitable first step to a better understanding of stabilizing effects provided by 3HB. Two distinct experimental strategies were used for this purpose: first, hydration of 3HB was studied using a combination of three different methods of

[XXIV] Obruca, S., Sedlacek, P., Mravec, F., Samek, O., and Marova, I. Evaluation of 3-hydroxybutyrate as an enzyme-protective agent against heating and oxidative damage and its potential role in stress response of poly(3-hydroxybutyrate) accumulating cells. *Applied Microbiology and Biotechnology.* 2016, 100, 1365–1376.

[XXV] Slaninova, E., Obruca, S., Kocherbitov, V., and Sedlacek, P. On the bioprotective effects of sodium 3-hydroxybutyrate: thermodynamic study of binary Na3HB-water systems. *Submitted for publication*.

sorption analysis, second, phase transitions in aqueous solutions of Na3HB were studied via DSC under equilibrium and non-equilibrium conditions, respectively.

Our experiments proved an outstandingly hydrophilic nature of 3HB which is at least comparable to, but in some perspectives even better than some well recognized compatible solutes such as trehalose. This represents a crucial finding not only with respect to understanding the natural protective role of 3HB in PHA-accumulating organisms but also from the view of its potential application in the technological fields in which stabilization of biological molecules is required. Apart from this, the study also revealed that sodium salt of 3HB can form, depending on the conditions (temperature, relative humidity), at least two different crystalline forms – anhydrous crystal and crystalline dihydrates.

The study also confirmed the high cryoprotective potential of 3HB in many aspects of the equilibrium (the corresponding phase diagram is shown in Fig. 13b) and non-equilibrium (Fig. 13c) phase behavior of the Na3HB/water mixtures. For instance, the effect of 3HB presence on the water freezing curve in the equilibrium phase diagram and the position of the eutectic point that represent the least achievable water freezing temperature (-28.1°C for Na3HB content of 40.5 wt.%) is, again, comparable to the effects found for compatible solutes and routinely used cryoprotectants such as trehalose, glycerol or sucrose. Similarly, the cryoprotective effects of 3HB can also be illustrated by its non-equilibrium behavior in aqueous systems. Water freezing was reduced partially (below Na3HB content of 40-50 wt.%) or completely (above this Na3HB content). Furthermore, in the region where the water freezes a significant freezing temperature depression was found again. Last but not least, from the total enthalpies of the ice melting endotherms (determined by DSC) we determined the amount of nonfrozen water to be approximately 1.35 g water per g of Na3HB (or 0.57 g water per g of water/Na3HB mixture). Once again, this illustrates the outstanding position of Na3HB among the recognized compatible solutes. For instance, published values of non-freezing water for sugars range from 0.21 g water per g of fructose, through 0.26 g water per g sucrose, to 0.31 g water per g trehalose [56].

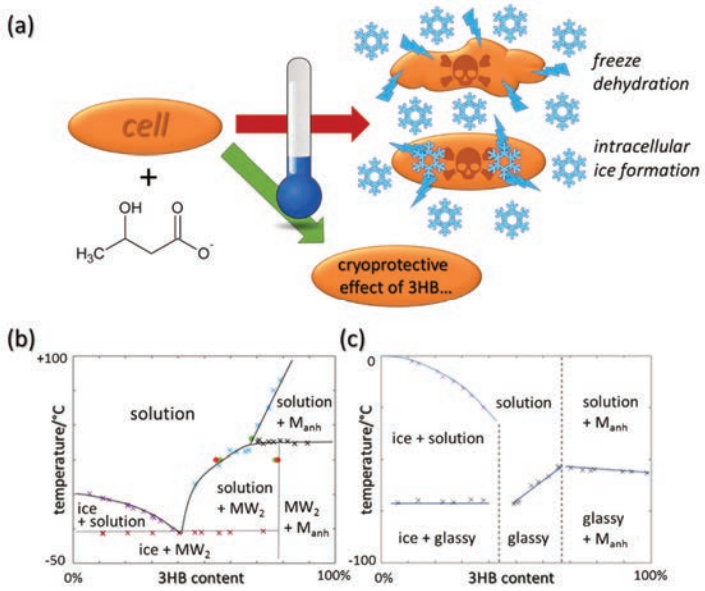

Fig. 13 *Cryoprotective role of 3HB as understood from the phase behavior of 3HB-water mixtures.* (*a*) *Schematic representation of the two main mechanisms of the lethal effect of freezing to cells.* (*b*) *The phase diagram in the equilibrium of 3HB-water complex using DSC* (×)*; sorption calorimetry* (●) *and DVS* (●). *All lines are drawn as a guide for the eye to follow the respective phase boundaries.* (*c*) *The phase diagram in non-equilibrium of Na3HB-water complex using DSC data from second scans represented by stars* (×). *All lines are drawn as a guide for the eye to follow the respective phase boundaries.*

Chapter 3:
Hydrogels: experimental model of biological systems, soft-matter material for future applications

"What do we mean by soft matter? Americans prefer to call it 'complex fluids.' This is a rather ugly name, which tends to discourage the young students."
(*Pierre-Gilles de Gennes*)

"Just because you are soft doesn't mean you are not a force."
(*Victoria Erickson*)

When something is soft, it is not necessarily weak as well. Soft-matter is in fact a family of exceptionally powerful materials. Among them, hydrogels represent yet special category. Hydrogels are three-dimensional networks of polymer chains (or associated colloidal particles in the case of particulate gels) that are able to retain a large amount of water in their swollen state. Since the term "hydrogel" first appeared in the scientific literature in 1894, these materials have continuously attracted substantial attention in research and development. Currently available hydrogels are materials of versatile composition, preparation, and properties [57]. They are utilized in numerous applications like biomedical, environmental, or in the fields of personal care and bioseparations [58]. In general, the essence of their technical functionality is rooted in their structure, i.e. network architecture, mesh or pore size, pore distribution etc., and in their binding ability based on a combination of chemical and physical structural features.

We have put a great effort to gain insight into this causal relationship, whose comprehension is crucial not only for understanding the specific material properties of commonly used existing hydrogels but also for the engineering of novel gel materials "tailor-made" for required properties. For this purpose, we have gradually - in the course of our hydrogel-related research - developed a unique methodology, that combines methods of macroscopic and microscopic analyses focusing on the three crucial aspects of the studied hydrogels: their morphological and ultrastructural architecture, barrier and release properties, and viscoelasticity (Fig. 14).

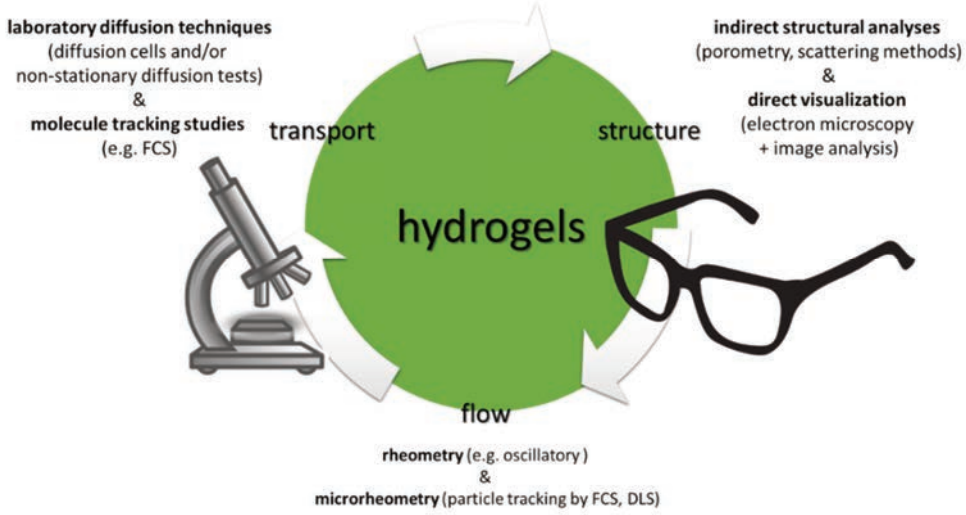

Fig. 14 Schematic representation of the developed methodology for multiscale experimental study on the relationships between internal structure, mechanical (flow) properties, and transport or barrier properties of hydrogels under investigation.

The story of a development of this unique methodology, that aims at providing a comprehensive understanding of composition–structure–performance relationships of hydrogels, is described in details in the full-length version of the thesis. Here, I will focus more on the applied research in which this multi-scale methodology was successfully utilized.

3.1 Semi-Interpenetrating Polymer Networks: Controlling structure and interactions independently

Hydrogels are especially attractive also from the "bio" perspective. They attract particular interest both in modeling real biological environments and in the field of biomedical applications. These were the first biomaterials designed intentionally for use in the human body. In biomedical uses, hydrogels benefit mainly from the outstanding biocompatibility that results from a combination of high water content and physicochemical similarity to the native extracellular matrix [59]. Their unique mechanical and transport properties make them material of choice mainly for controlled release systems, or in the field of tissue engineering. Therefore, there is still a need for new ways how to manipulate the internal architecture of gels in order to tailor their properties and dynamics, not only for creating novel biomaterials, but also to find appropriate experimental models on which behavior of complex and hardly explorable living systems could be better understood [60, 61].

We have recently put forward an original strategy, which aims to address the two critical issues of the hydrogel drug carriers – i.e. its mechanical and transport properties - independently of each other. The concept found its inspiration in our previous works on the solute-binding properties of humic substances, where we immobilized the dissolved humics in the supporting agarose gels. We have realized in this study, how specifically the relative contents of the inert gel-forming component (agarose) and the reactive substance (humic acids), contribute to the various aspects of the gel behavior.

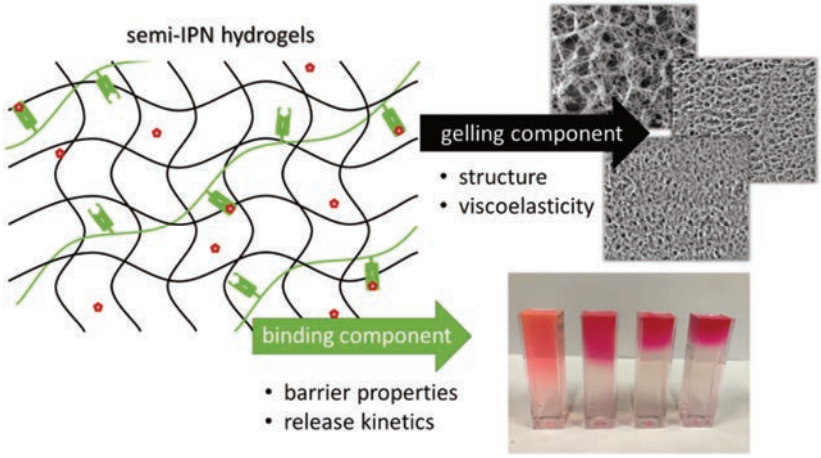

Fig. 15 Proposed strategy of tailoring performance and properties of semi-IPN hydrogels. An inert gel-forming polymer network is interpenetrated by a linear polymer component with the properly selected binding functionality. Mechanical and transport performance of the material is adjusted independently via manipulating the relative content of the two structural components.

The concept that we have proposed (it is represented schematically in Fig. 15) is based on semi-interpenetrating polymer networks (semi-IPNs) which, together with the interpenetrating polymer networks (IPNs) represent a novel and especially promising class of polymer blends. IPNs are defined by the International Union of Pure and Applied Chemistry (IUPAC) as "A polymer comprising two or more networks which are at least partially interlaced on a molecular scale but not covalently bonded to each other and cannot be separated unless chemical bonds are broken." [62]. Semi-IPNs differ from IPNs in the fact that the chains of the second polymer are dispersed in the network formed by the first polymer without forming

a separate network. We have suggested that semi-IPN hydrogels may be easily designed to provide independent control and adjustment of gel ultrastructure and all the associated properties (e.g. viscoelasticity) on the one side, and the binding ability and consequent barrier/release performance on the other. For this purpose, the semi-IPN hydrogels comprise in its dual network a "structure–ruling" gel-forming component which is interpenetrated by a "binding" polymer chains. The gel-forming component is physico-chemically inert – i.e. possesses a negligible binding affinity to the active substance. Its main task is to ensure a reproducible and controllable gelation process, providing gel architecture with adjustable parameters such as cross-linking density and average pore size, without interfering significantly with the kinetics of absorption and/or release of the active substance. On the other hand, the "binding" component, because of its significantly lower relative content, does not affect the internal morphology of the gel, but significantly improves its binding properties. Such a system hence offers an independent dual-tuning of mechanical and transport performance via manipulating the relative content of the two structural components.

We have performed a systematic case study of the proposed strategy on agarose-based semi-IPN gels. We have tested the incorporation of various interpenetrating components (poly-(styrenesulfonate) (PSS), alginate (ALG), hyaluronic acid, chitosan, quaternized dextran, etc.) in the supporting agarose matrix, and investigated the transport properties of diverse model solutes (cationic or anionic organic molecules) in the resulting semi-IPN hydrogels. The most important results were summarized in the comprehensive publication[XXVI]. The conclusions of this study strongly supported the validity of the proposed concept. In particular, it was found that the viscoelastic behavior of the gels (represented e.g. by the value of complex modulus) can be adjusted in a wide range by the gelling component (agarose) with the negligible effect of the interpenetrating component (results shown for PSS and ALG). On the other hand, the content of PSS as low as 0.01 wt.% of the gel (it means about 100× lower compared to agarose) resulted in a more than the 10-fold decrease of diffusivity in model-charged organic solute (Rhodamine 6G).

The pilot study summarized in the publication[XXVI], confirmed the great application potential of the proposed concept in the development of controlled-release hydrogel systems. Furthermore, it also demonstrated that the original analytical approach designed by us and applied in this study can be used as a valuable methodological framework providing complex insights into the composition–structure–performance relationships in hydrogel materials. Another indisputable advantage of the proposed concept is that it imposes no special requirements on the gelation procedure – various common gelling polymers can apparently be used as a gel-forming component. To make sure of this, we have recently performed some follow-up studies, where we confirmed the applicability of the concept on the poly(vinylalcohol) (PVA) gels, and most recently also on the poly(hydroxyethyl methacrylate) (PHEMA) gels.

3.2 Hydrogels: Multipurpose materials with versatile applications

As emphasized repeatedly throughout this text, in all my research interests I have always tried to combine the quest for fundamental knowledge with the rational transfer of the gained knowledge as close to real applications as possible. This is doubly true for the hydrogels, that intertwine throughout my scientific career, across actually all the research projects I have been involved in. In the previous section, I have demonstrated how we utilized the experience gained during the development of the diffusion-in-gel methodology for the reactivity mapping studies on natural compounds in the design of novel hydrogel materials for drug delivery. In this chapter, I will follow with a brief introduction of other types of hydrogel materials, that we designed and investigated according to the specific requirements of various applications.

3.2.1 Novel multi-purpose hydrosorbents for agricultural uses

In 1.1.3, I have already described some types of hydrogel forms of humic acids (HA) that we have proposed for use in agriculture and health-care. I have intentionally left apart from one specific type of gels

[XXVI] Trudicova, M., Smilek, J., Kalina, M., Smilkova, M., Adamkova, K., Hrubanova, K., Krzyzanek, V., and Sedlacek, P. Multiscale Experimental Evaluation of Agarose-Based Semi-Interpenetrating Polymer Network Hydrogels as Materials with Tunable Rheological and Transport Performance. *Polymers.* 2020, 12, 2561.

that we have paid special attention to – HA-containing superabsorbent composite hydrogels. These are novel multifunctional materials that we have suggested for agricultural and environmental applications. These hydrogels are based on conventional superabsorbent polymers (SAPs) – polyelectrolyte networks formed of chemically cross-linked polyacrylate or poly(acrylate-*co*-acrylamide) – that are, because of their exceptional water absorption and retention capacity, currently utilized in sanitary and hygiene supplies, but increasingly also in agriculture, where they are used primarily to enhance water retention capacity of low-quality soils. We have adapted the conventional polymerization procedure to incorporate into the structure of these SAPs also the humic component (commercial lignohumate) and inorganic nutrient component (NPK inorganic fertilizer). The final materials hence provide not only the great water swelling capacity (see Fig. 16), but also the capability of controlled release of the plant growth-promoting humic component as well as the inorganic nutrients.

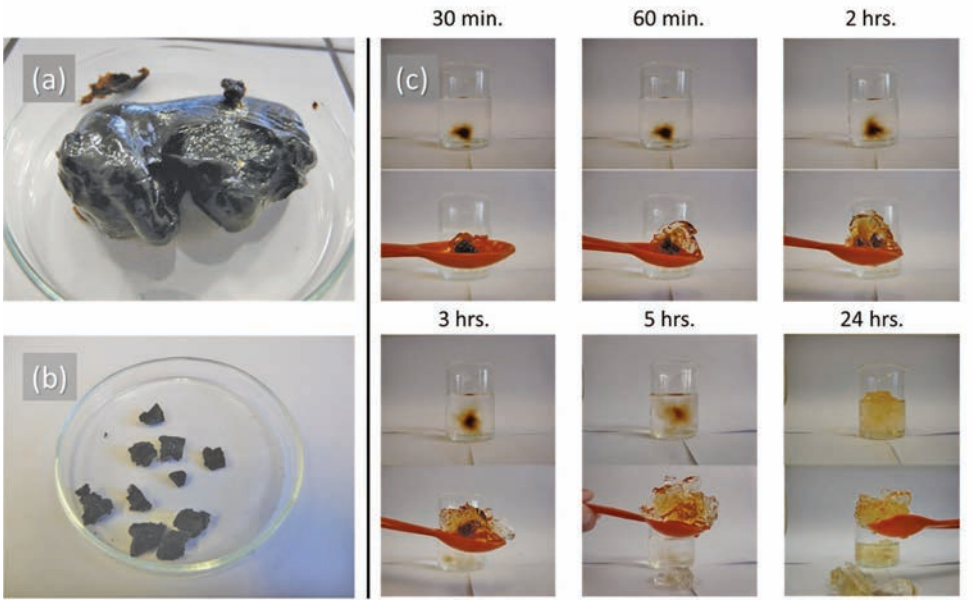

Fig. 16 Superabsorbent polymer (*SAP*) *material based on polyacrylate chains grafted on humic substances. Polymerized SAP as-prepared* (*a*) *and in the dried form* (*b*). *Monitoring the swelling of the SAP in deionized water* (*c*).

We have tested various combinations of SAP composition (with/without acrylamide), and contents of Lignohumate and NPK, respectively. Potassium peroxydisulfate was used as the initiator and N,N–methylenebisacrylamide as the crossing agent during the SAP preparation. We have performed a comprehensive study on how the composition of the material affects its structure in the dry form, swelling behavior in various aqueous environments, and mechanical properties in the swollen form (i.e. viscoelasticity of the formed hydrogel). Furthermore, to support their suggested application as novel controlled-release fertilizers, we have tested also the release of the active components as well as the biological activity of the gels (influence of the addition of SAPs on corn growth was studied in pot experiments). Among others, we have confirmed experimentally that the swelling of the SAPs was fast (completed always in 24 hours) and the final water absorbency of all samples was in the range 100 – 300 g/g, whereby the presence of Lignohumate had a positive effect on the swelling, contrarily to NPK that reduced the water absorbency. As expected the swelling ratio of the gel was conversely proportional to the strength and rigidity of the swollen gels. Similarly, the swelling also influenced the release of nutrients from superabsorbents. The release of mineral nutrients was ruled primarily by their contents in different samples.

Although the presence of Lignohumate had a negligible influence on the release of P and N, it increased the amount of released K. On the other hand, the release of the humic component was partially suppressed by the higher content of NPK. We have also evidence that the application of composite SAPs supported water retention of soils and the growth of corn. Better water management and a gradual supply of nutrients enhanced both height of plants and the length of roots. The most important results of this study were published[XXVII].

Obviously, the developed composite hydrogel materials represent the state-of-the-art controlled release systems for modern sustainable agriculture. It combines multiple functions – water management in soils, the release of the bio-stimulating humic component as well as inorganic nutrient elements, whose kinetics can be tailored by the composition of the material. We suggest the use of these materials mainly in problematic areas with dry soils and low levels of organic matter and nutrient elements.

3.2.2 Encapsulation of microbes in hydrogels

As it is quite obvious from the previous text, the wide range of topics to which I found the concept of biophysical chemistry useful and contributive, took me to various, apparently distinct, areas of research interest. Nevertheless, recently I found one special topic where the distinct routes of my previous scientific career began to reconnect. It is the topic of the research and development of bio-inoculants – the carriers of microbial cells intended for agricultural and environmental uses.

As already emphasized, improving the quality of arable soils represents nowadays one of the most urgent needs among all research fields covered by life-sciences. In this text, I have already paid great attention to the quality of soils regarding their chemistry. Nevertheless, the same emphasis should be put also on soil biology, or more specifically, microbiology. Currently, the development of biological agents based on plant growth-promoting (rhizo)bacteria (PGPR) for the restoration of soil fertility represents a hot topic in modern agricultural technologies. In general, PGPR affects plants or crops directly (biofertilization, rhizoremediation, stimulation of root growth, and plant stress control) or indirectly by reducing the impact of diseases. The mechanisms of plant growth-promoting effects of PGBR are summarized in Fig. 17.

Generally, the primary role of the formulation of an inoculant is to form a stable micro-environment that provides the microbial strain(s) with physical and/or chemical protection over a prolonged period, in order to avoid a rapid decrease of the cells' viability during storage and after being introduced into the soil [63]. The bioinoculant formulation design is hence aimed at providing a reliable source of living cells available to interact with plants and soil microbiome. Especially the liquid formulations often fail in this requirement because of their short shelf-life (2-3 months) and insufficient protection of the microbial cells after introduction to the soils. A longer shelf-life is provided by solid bio-inoculants. Nevertheless, the state-of-the-art formulations in the bioinoculants' production with respect to the effectiveness of cell entrapment and protection and the reproducibility of the preparation process are represented by the formulations based on the hydrogel carriers. Encapsulation of PGPR cells in hydrogels formed from cross-linked polysaccharides such as alginate and carrageenan has been proposed a long time ago as a technique to ensure the controlled release of plant beneficial microorganisms into the soil [64]. In general, the gel matrix assures mainly physical protection of the cells against various environmental stress factors, nevertheless, there are also strong shreds of evidence that polysaccharides play an important specific role in the mechanisms of abiotic stress protection of microorganisms [65]. However, as far as the agro-industrial technologies have the principal requirement of low cost, the task to find the appropriate technologically feasible and economically competitive PGPR encapsulation technique is still challenging [66].

Currently, the vast majority of hydrogel inoculant formulations use alginate as the gel-forming polymer. Alginate represents the family of linear polyanionic polysaccharides made up of L-glucuronic acid (G) and D-mannuronic acid (M). Alginate possesses numerous properties that make it the candidate-of-choice for the production of various gel carriers and controlled release systems: it is non-toxic, biocompatible, non-

[XXVII] Kratochvilova, R., Sedlacek, P., Porizka, J., and Klucakova, M. Composite materials for controlled release of mineral nutrients and humic substances for agricultural application. *Soil Use and Management.* 2021, 37, 460–467.

immunogenic, biodegradable, mucoadhesive, readily available, and has a relatively low cost. Ionic gelation is the most common method used to obtain alginate-based gels. In this process, alginate is cross-linked in aqueous solutions via chelating the Ca^{2+} (or other multivalent cation) by pendant carboxylic acid moieties of G units, generating 3D hydrogel networks that allow entrapment of other components dispersed in the aqueous solution (such as cells, dissolved bioactive compounds etc.). Currently, alginate is usually extracted from brown seaweeds, however, since only a few of the many species of brown algae are suitable and are limited in abundance and location for commercial alginate production, there is at present interest in the bacterial production of alginate-like polymers. Bacterial producers also provide alginate of defined monomer composition to gain determined properties instead of alginate isolated from seaweed, which in general suffers from heterogeneity in composition and quality [67].

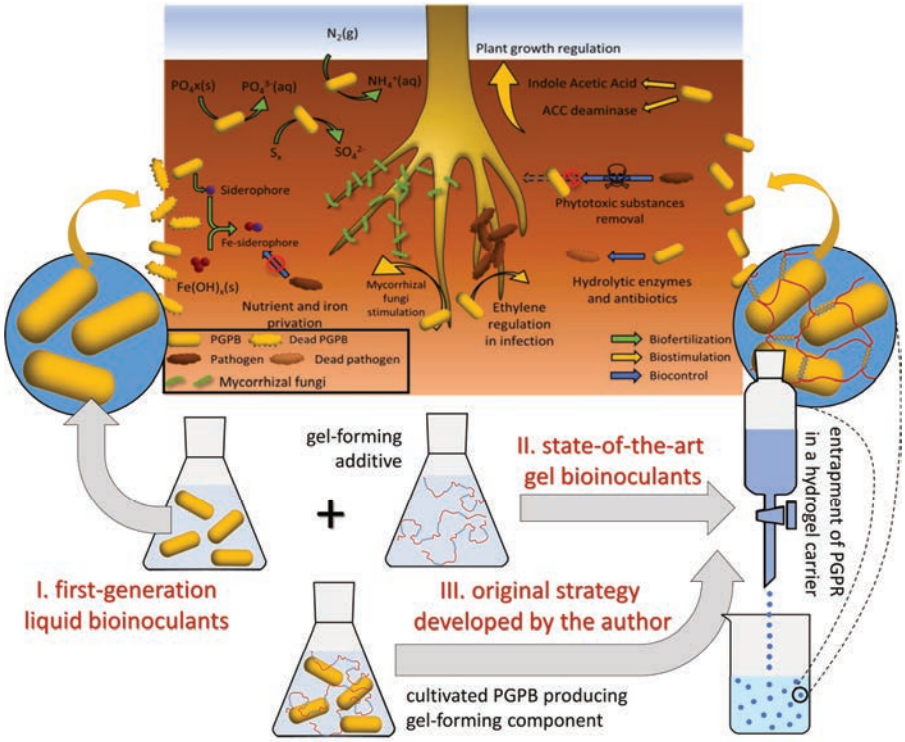

Fig. 17 Schematic representation of the recent trends in the development of bioinoculants (*from the 1st gen. liquid formulations to the state-of-the-art hydrogel carriers*) *together with the original strategy proposed and tested in the project* (*overview of the positive effects of PGPB on plants was adopted from Ferreira et al.* [68].

We have recently proposed a novel strategy for the preparation of PGPR-based biofertilizers. It is based on the entrapment of the bacterial cells in the hydrogel formed from alginate, which is produced directly by the PGPR bacteria. For this purpose, we have employed PGPR which belongs to the genus *Azotobacter*. The members of this genus are gram-negative nitrogen-fixing non-pathogenic bacteria. Probably the best-studied representative, *Azotobacter vinelandii*, is known to produce numerous low-molecular compounds providing stimulating effects on plants, such as indolacetic acid, gibberellins and cytokinins. Even more, interestingly, *A. vinelandii* has been recognized as an efficient producer both of extracellular alginate and intracellular PHA. It was exactly this combination of alginate and PHA production capability that attracted our attention. While the alginate biosynthesis induced a possibility to overcome one of the crucial technological and economic demands of the conventional gel inoculant production – i.e. the necessity to add an external gel-forming component, PHA accumulation carried a promise of enhanced stress robustness

of the bacterial cells, and, consequently, improved survival of the cells when applied in form of a bio-inoculant to a soil. Surprisingly, the combination of plant-growth-promoting effects and high production yields of alginate provided by the single bacterium has not attracted yet any attention in the design of bio-inoculants although it offers a significant simplification and streamlining of the process of their preparation. The fact that the gel-forming biopolymer is produced by plant-growth-promoting bacteria themselves is a crucial distinguishing sign and the most innovative feature of the proposed strategy that gives a great potential to simplify the preparation procedure and reduce the costs of the intended PGPR application.

In the preliminary experiments, we have already proved the validity of the proposed strategy. We are now capable of preparing PGPR cultures, that can be easily transferred to hydrogel form simply by the addition of calcium ions. We have also successfully induced in-situ ionotropic gelation in the cultivation medium via spontaneous evolvement of the calcium ions from an originally insoluble form. Currently, we are performing an in-depth optimization study on all the essential steps involved in the proposed technological process. The optimization of the cultivation properties has already provided a functional balance between the yield and quality of alginate and accumulated PHA content. Now we are focusing on optimization of the subsequent steps of the bio-inoculant production process – gelation and drying as well as a pilot evaluation of the biological activity of prepared bio-inoculants. Hereby, we wish to collect essential fundamental knowledge on the causal relationship between preparation procedure, structure and crucial properties of the hydrogel-entrapped bio-inoculants based on this original strategy. Furthermore, based on our experience with the bio-stimulating effects of humic substances, we also intend to incorporate these compounds as an additional component of the developed hydrogel bio-inoculant preparations.

Afterword

The main aim of this habilitation thesis was to introduce biophysical chemistry as the modern interdisciplinary scientific discipline that can offer fresh, unconventional conceptual perspectives on – but also powerful methodological apparatus for – answering miscellaneous research questions from the field of life sciences. Apart from the brief summarization of some historical moments that, in my opinion, helped to form the current state of the discipline, and besides emphasizing some crucial challenges that it nowadays faces, I tried to illustrate how a wide range of research interests can be covered under the wings of this discipline in a single scientific curriculum. I leave it up to the reader to assess how well the goal has been achieved by this text.

A friend of mine told me that, when working on his habilitation, he was feeling like writing memoirs. I was experiencing a kind of similar feeling when writing this thesis. Nevertheless, together with all the memories, even a stronger feeling came to my mind - gratitude. Gratitude to all who have helped me throughout my previous scientific career. My scientific supervisors, bosses, colleagues, and – last but not least – students. They all played an irreplaceable role in providing me with their experiences, knowledge, open-mindedness, inspiration, diligence, and enthusiasm. And, of course, my family, who showed boundless tolerance when scarifying the time spend with me for letting me do what I love. In spite of my name on the cover, this thesis is a collective work of all of these people.

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Abstract

The habilitation thesis presents the author's personal view of biophysical chemistry as a modern, distinctive chemical discipline. In the introductory part of the full-length thesis, the author presents his own perspective on the current state of the field, in the context of historical moments, which in his opinion have contributed to the formation of biophysical chemistry as an independent scientific discipline. Furthermore, the author presents a subjective view of the main challenges that this discipline currently faces, as well as the opportunities in which the author sees the main merit of biophysical chemistry in the near future. The second part of the work then summarizes the author's own contribution to the development of the state of knowledge in the field. This part is thematically divided into three sections, which gradually summarize the author's scientific contribution in the field of physical chemistry of biopolymers, the application of physical chemistry in the field of microbiology and biotechnology, and in research and development of hydrogel materials. Emphasis is placed on acquainting the reader with the unifying elements that these three seemingly different research topics combine in the author's scientific work - a general physico-chemical view, focused on the connection between thermodynamic state, supramolecular architecture and resulting system properties, and unique methodology, based on unconventional biophysical applications of standard physicochemical and spectroscopic methods.

Abstrakt

Habilitační práce prezentuje osobní pohled autora na biofyzikální chemii coby moderní svébytnou chemickou disciplínu. V úvodní části kompletní verze habilitační práce autor předkládá vlastní perspektivu současného stavu oboru, a to v kontextu historických okamžiků, které se dle jeho názoru klíčovou měrou přispěly k formování biofyzikální chemie jako samostatné vědní disciplíny. Dále autor prezentuje subjektivní pohled na hlavní výzvy, kterým tato disciplína v současnosti čelí, a také příležitostí, v nichž autor spatřuje hlavní přínos biofyzikální chemie v blízké budoucnosti. Druhá část práce poté sumarizuje vlastní příspěvek autora k rozvoji stavu poznání oboru. Tato část je tematicky rozdělena do tří oddílů, které postupně sumarizují autorův vědecký přínos v oblasti fyzikální chemie biopolymerů, aplikace fyzikální chemie v oblasti mikrobiologie a biotechnologie, a při výzkumu a vývoji hydrogelových materiálů. Důraz je kladen na seznámení čtenáře se sjednocujícími prvky, který tyto tři zdánlivě odlišná výzkumná témata v autorově vědecké práci spojují – na obecný fyzikálně-chemický náhled, zaměřený na spojitost mezi termodynamickým stavem, supramolekulární architekturou a výslednými užitnými vlastnostmi systému, a na unikátní metodologii, založenou na nekonvenčních biofyzikálních aplikacích standardních fyzikálně-chemických a spektroskopických metod.