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# **Poly(3-hydroxybutyrate): characterization and application as drug carrier matrix**

**Summary of Ph.D. Thesis**

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## 1. Introduction

One does not necessarily has to browse through the entire literature of biopolymers to conclude that the importance and significance of microbial polyesters is on the rise. The last few decades saw them evolving from a scientific peculiarity to an intensively studied and thoroughly characterized family of polymers bearing actual industrial potential. Their most important representative, poly(3-hydroxybutyrate) (PHB) can now be produced even on a large scale, as beside its application, its fermentation has also became an intensively studied area<sup>1,2</sup>.

A scaled-up production, however, has further benefits in terms of accessibility and affordability: with the appearance of novel, more effective fermentation techniques the price of PHB is continuously decreasing, the cost efficiency of a PHB product is competing now with that of PLA. Another important question is the relationship between the price of the polymer and the willingness of the researchers to use this particular material for their purposes: the more cost effective and accessible PHB becomes, the stronger it influences the trends in the field of biopolymers<sup>3</sup>.

One of the most important among these trends relates to medical and pharmaceutical applications. In the past few years, a vast number of biomedical applications have appeared (e.g. stents, sutures, or even implants), each of which is manufactured by using partially or exclusively PHB, not to mention the field of drug delivery, or the tissue engineering science, where PHB counts now as one of the most often used raw materials<sup>4</sup>.

While the popularity of PHB is clearly highlighted by the number of scientific areas already using this material, a profound investigation of the corresponding literature

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<sup>1</sup> Sharma, V., Misra, S., Srivastava, A., K., *Biocatal Agric Biotechnol.* **10**, 122-129, (2017)

<sup>2</sup> Wu, W., Lai, S. Y., Jang, M. F., Chou, Y. S., *J. Process Control* **23**, 1159-1168, (2013)

<sup>3</sup> Graupner, N., Müssig, J., *Compos Part A Appl Sci Manuf.* **42**, 2010-2019, (2011)

<sup>4</sup> Zhang, J., Shishatskaya, E. I., Volova, T. G., da Silva, L. F., Chen, G. Q., *Mater. Sci. Eng. C.* **86**, 144-150, (2018)

must also point out the reasons that result in the rapid expansion and success of the research projects focusing on the pure characterization, or on the practical application of this polymer. The most important among these is the biocompatibility of PHB: the metabolism of this microbial polyester yields 3-hydroxybutyric acid, which is a component of blood, thus an *in vivo* presence of this material does not expose the human body to any hazardous byproduct<sup>5</sup>.

Even though the metabolism of PHB has been proven to yield exclusively non-toxic products, the *in vivo* application of this polyester cannot be claimed to be safe without the knowledge of the kinetics of its degradation. While the degradation has already been investigated by a number of research groups aiming at the characterization of either the hydrolytically<sup>6</sup> or the enzymatically<sup>7</sup> initiated and maintained fragmentation of PHB, none of these publications proposed a kinetic description that is based on the mechanism of the process.

While the questions discussed above lack the otherwise crucial knowledge of the mechanism and kinetics of the metabolism of PHB could certainly highlight the incompleteness of this scientific field, a thorough review of the PHB related articles also reveals that the literature on the application of PHB as a drug carrier matrix is also lacking. Although a number of articles are devoted to a description of carrier matrices manufactured by using partially or exclusively PHB<sup>8,9</sup>, none of these attempts to determine kinetic parameters bearing actual physical significance, e.g. the diffusion coefficient. While some researchers use mathematical representations for the approximation of dissolution from PHB that are at least partially based on the principles

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<sup>5</sup> Pouton, C. W., Akhtar, S., *Adv Drug Deliv Rev.* **18**, 133-162, (1996)

<sup>6</sup> Holland, S. J., Jolly, A. M., Yasin, M., Tighe, B. J., *Biomaterials* **8**, 289-295, (1987)

<sup>7</sup> Tanio, T., Fukui, T., Shirakura, Y., Saito, T., Tomita, K., Kaiho, T., Masamune, S., *Eur J Biochem.* **24**, 71-77, (1982)

<sup>8</sup> Barouti, G., Jaffredo, C. G., Guillaume, S. M., *Prog Polym Sci.* **73**, 1-31, (2017)

<sup>9</sup> Sokolsky-Papkov, M., Agashi, K., Olaye, A., Shakesheff, K., Domb, A. J., *Adv Drug Deliv Rev.* **59**, 187-206, (2007)

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of physical chemistry<sup>10,11</sup>, a model that does not incorporate any semi-empirical factor and is still able to describe the release of an arbitrary drug has not been published yet.

The primary aim of this thesis is therefore to extend, improve and complete the field of PHB-related sciences by introducing novel ways that could be applied to the kinetic characterization of both the hydrolytically and the enzymatically catalyzed degradation of PHB. Besides the analysis and quantitative description of the behavior of this polymer under conditions modeling the parameters of the human body, we also intend to focus on the development of carrier matrices that could be applied to deliver and release drugs in a controlled manner. In the closing chapters of this thesis, we address the incompleteness of the drug release science by introducing fast and easy to implement techniques that can be applied for the determination of one of the most important kinetic parameter of dissolution (the diffusion coefficient) and to propose novel ways to monitor the process.

## 2. Materials and methods

Poly(3-hydroxybutyrate) granules were obtained from Metabolix Ltd. (Mirel M2100) with an approximate crystallinity of 60 %. The degradation studies were carried out on PHB films prepared by solvent casting and compression molding (Chapter 2 and 3), as well as on aqueous PHB suspensions (Chapter 4). The Mirel type PHB already mentioned above was used for the production of fibrous PHB scaffolds (Chapter 5). In this case, however, the technique that was applied for the production of the PHB fibers was wet spinning. Solvent cast PHB films were used in the studies discussed in the remaining chapters with the difference that in these cases the films were loaded with the molecules of drugs, i.e. quercetin (Chapter 6) and fuchsine (Chapter 7).

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<sup>10</sup> Mitran, R. A., Matei, C., Berger, D., Băjenaru, L., Moisescu, M. G., *Eur J Pharm Biopharm.* **127**, 318-325, (2018)

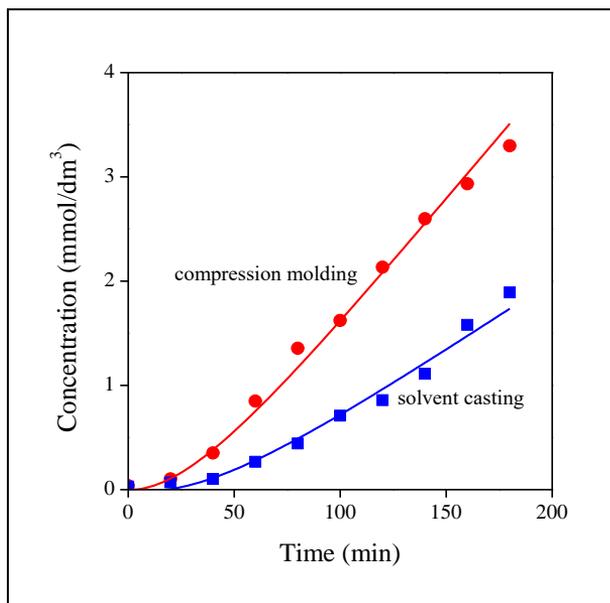
<sup>11</sup> Xu, X., Al-Ghabeish, M., Krishnaiah, Y. S. R., Rahman, Z., Khan, M. A., *Int J Pharm.* **494**, 31-39, (2015)

The degradation studies were carried out in alkali media (hydrolytic degradation) and in a dilute solution of the enzyme natively produced by the strain *Bacillus Megaterium* (enzymatic degradation). UV-Vis spectroscopy, HPLC, and LC-MS techniques were used for the monitoring, quantitative and qualitative analysis of degradation. Ethanol and PBS buffer were used in the dissolution studies presented in the second half of the thesis. Ethanol was the dissolution medium in the case of carrier matrices loaded with quercetin, while in case of fuchsin this role was assigned to a PBS buffer of pH and ionic strength corresponding to that found in the human body. The dissolution was monitored by UV-Vis spectroscopy and in Chapter 7 a camera, pictures of which have been processed in the MATLAB development environment.

### 3. Results

As discussed in the introduction, very little is known about the degradation of microbial polyesters, e.g. PHB. To fill this gap, we intensively studied both the hydrolytic, and the enzymatic degradation of PHB, and concluded that the depolymerization of this material can be facilitated both ways, although the former would require environmental conditions (strong alkali medium) that is not present in the human body. Therefore, in the case of products for *in vivo* applications, the enzymatic route is preferred.

The study of hydrolytically and enzymatically catalyzed PHB decomposition showed that in both cases the process can be quantitatively characterized by using kinetic models based on the chemical background of the reactions occurring during depolymerization. As in the case of hydrolytic degradation no kinetic model is presented in the literature that could be used for such quantitative description, we have decided to develop our own, which was subsequently applied for the successful and accurate approximation of the experimental results.



**Fig. 1** Fitting of the model to the kinetics of enzymatic degradation of PHB films. Detection was recursive UV-VIS spectroscopy.

In the case of enzymatic degradation, however, the situation is somewhat different, as the research on enzyme kinetics began as early as the beginning of the past century, thus there are already a number of mathematical approaches published in the literature, e.g. that of Michaelis and Menten. This model, however, is developed for reactions that are taking place in a homogeneous, aqueous medium, which,

due to its insolubility in water, PHB can never form. Therefore, we concluded that the modification of the original Michaelis-Menten approach is required that is able to take into consideration the inevitably heterogeneous nature of the reaction. We have subsequently applied our model for the description of the experimental data (**Fig. 1**) and have proven that the modified Michaelis-Menten approach can be used for the quantitative characterization, or even prediction of enzyme-catalyzed depolymerization.

This is the main, but not the only reason why we found our enzymatically catalyzed PHB depolymerization-related studies especially informative. We have also suspected, that the overall rate-determining step of the process is the adsorption of enzyme molecules onto the surface of PHB, but we could not investigate this phase of the reaction separately, as an active enzyme molecule – if successfully adsorbed on the surface of the polymer – will eventually catalyze the fission of the ester bond. This step, however, triggers the decomposition of the active complex and its desorption from the PHB phase, thus the separate analysis of the rate determining step is not possible.

Consequently, we have decided to carry out the in-depth analysis of depolymerization and searched for new methodologies that could be applied to overcome the difficulty described above. The solution was delivered by one of the achievements of gene technology, namely PCR (polymerase chain reaction) mutagenesis, which allows the creation of new gene sequences, and therefore, enzyme molecules, in which one or even multiple amino acids are exchanged to a different one. By implementing this technique, we created depolymerase molecules, which had their binding site left intact, but the active domain was neutralized. By using these molecules, we were able to analyze the adsorption step separately from (in this scenario impossible) catalysis, thus making possible the thorough characterization of the kinetics of enzyme adsorption. Furthermore, we concluded that by using these intentionally inactive enzyme molecules, the determination of the surface need of one molecule becomes also possible.

After completing the studies aiming at the characterization of the decomposition of PHB, we have turned our attention towards more application-oriented areas and investigated the possible use of PHB as a scaffold. A new spinning method has been developed exclusively for this purpose, which made possible the production of PHB fibers of an especially narrow distribution of diameters – a result that would have been otherwise especially difficult to achieve if we insisted on the application of the more or less conventional method of electrospinning.

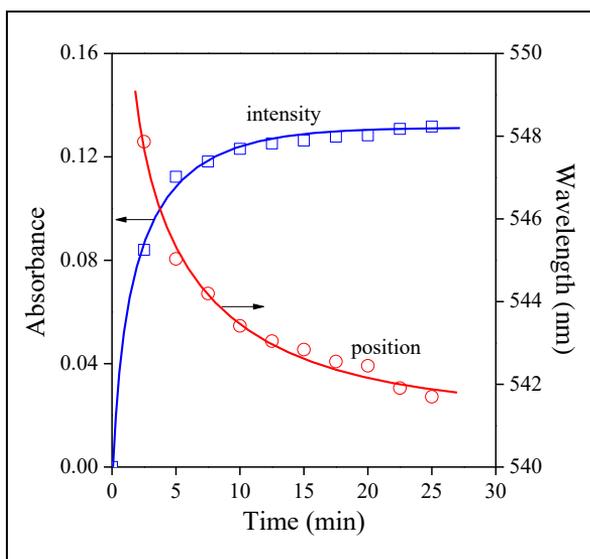
We have also studied the possibility of using these fibrous scaffolds (**Fig. 2**) as carrier matrix, and concluded that the presence of entrapped drug molecules does not modify the spinning procedure, thus the fine-tuning of the diameter of the fibers is still possible. In the closing



**Fig. 2** Digital optical micrograph of PHB fibers produced by wet spinning at 0.08 ml/min spinning rate.

part of this study, we have also shown that dissolution can be described and predicted by solving Fick's second law numerically for the case of cylindrically symmetric diffusion.

Besides the application of PHB as carrier matrix, we have also invested a considerable amount of energy into the development of novel techniques, which could be used for the monitoring of the dissolution process. As a part of this effort, we developed an entirely new approach for the fast and cost effective determination of diffusion coefficient – all this without the need of the rather obsolete instrumentation generally used for the acquisition of such data. We also showed that the monitoring of concentration gradients in amorphous PHB films yields results that correlate closely with those obtained by other methods – a further factor that underlines the reliability of our approach.



**Fig. 3** Changes in the maximum and intensity of visible absorption during a release experiment. Symbols: (□) intensity (○) position of the absorption peak.

hypsochromic shift (**Fig. 3**) which can be used for the quantitative characterization of the dissolution of the drug into the surrounding medium.

The other technique we developed provides a fast and cost effective way to monitor the entire dissolution process. As a part of this research, we found that the recording chromatic shifts observed in the UV-VIS spectrum used for enzyme assays can be utilized also for dissolution studies. We showed that dissolution inevitably alters the absorption characteristics of the drug in some extent, resulting in a

## 4. New scientific results

1. With the thorough study of the hydrolytic degradation of PHB films, we proved that degradation takes place mainly in the bulk of the samples and not on their surface. We also showed that degradation does not occur randomly, but with larger frequency at the end of the chains [1].
2. By assuming that the hydrolysis of PHB is a  $S_N2$  type bimolecular nucleophile substitution reaction, we developed a kinetic model, which describes the formation of various degradation products. We considered also the diffusion of the degradation products and thus the concentration of the monomer could be predicted also in the aqueous solution. Such a model did not exist before [2].
3. During the study of the enzymatic degradation of PHB, we showed that degradation proceeds in two stages, an accelerating stage during which the enzyme adsorbs on the surface of the film, and a steady state with constant rate. We modified the Michaelis-Menten model to describe the kinetics of degradation quantitatively, which takes into account the heterogeneous nature of the degradation reaction [2].
4. We prepared a deactivated enzyme by point mutagenesis to separate the adsorption of the enzyme on the surface of the polymer from catalysis. By using the model developed earlier, we could describe the kinetics of adsorption and degradation separately. We determined the rate constants of the various processes for the first time and the surface need of an enzyme molecule as well [3].
5. We prepared a fibrous scaffold with fibers of uniform diameter from PHB by wet spinning and determined the kinetics of drug release from the scaffold. We proposed a novel approach based on Fick's laws, which allows the quantitative description of release kinetics very accurately without any simplification or the introduction of empirical constants. The model allowed also the estimation of the diffusion coefficient [5].

6. We developed a completely new method for the determination of the diffusion coefficient of certain, colored drugs in amorphous PHB, which is a reliable alternative of the conventional permeation measurements. The method is based on the generation of a concentration gradient in the polymer film and the determination of its dependence on time. The fitting of the numerical solution of Fick's second law onto the measured values provides directly the targeted diffusion coefficient [4].
7. We developed another new, alternative method for the study of drug release and the determination of the diffusion coefficient of the drug in the polymer. The method is based on the hypsochromic shift of the absorbance of active molecules resulting in changes in the UV-Vis spectra as an effect of changing environment. The solution of Fick's second law under the initial and boundary conditions of the experimental setup and the numerical solution of the equations allow the quantitative analysis of the experimental results and the prediction of release kinetics [6].

## 5. List of publications

### Papers used for the preparation of this thesis

1. Polyák, P., Szemerszki, D., Vörös, Gy., Pukánszky, B.: Mechanism and kinetics of the hydrolytic degradation of amorphous poly(3-hydroxybutyrate), *Polymer Degradation and Stability* **140**, 1-8 (2017)  
DOI:10.1016/j.polymdegradstab.2017.03.021; IF:3.193; I:3(3); [80%]
2. Polyák, P., Dohovits, E., Nagy, G.N., Vértessy, B.G., Vörös, G., Pukánszky, B.: Enzymatic degradation of poly-[(R)-3-hydroxybutyrate]: mechanism, kinetics, consequences, *International Journal of Biological Macromolecules* **112**, 156-162 (2018)  
DOI:10.1016/j.ijbiomac.2018.01.104; IF:3.909; I:2(2); [80%]

3. Polyák, P., Urbán, E., Nagy, G.N., Vértessy, B.G., Pukánszky, B.: The role of enzyme adsorption in the enzymatic degradation of an aliphatic polyester, *Enzyme and Microbial Technology* **120**, 110-116 (2019)  
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4. Polyák, P., Szemerszki, D., Benke, H.C., Pukánszky, B.: A novel method for the determination of diffusion coefficients in amorphous poly(3-hydroxybutyrate), *Polymer Testing* **63**, 342-348 (2017)  
DOI:10.1016/j.polymertesting.2017.08.037 IF:2.247 I:2(2) [80%]

## Manuscripts

5. Polyák, P., Bartha, K., Benke, H.C., Pukánszky, B.: Quantitative determination of release kinetics from fibrous poly(3-hydroxybutyrate) scaffolds, *in preparation*
6. Polyák, P., Tilinger, D.M., Pukánszky, B.: A simple spectroscopic method for the determination of the release kinetics of drugs from PHB, *in preparation*

## Other publications

1. Polyák, P., Hári, J., Pukánszky, B.: A halloysit mint ásványi töltőanyag és polipropilén kompozitjainak tulajdonságai, *Műanyag és Gumi* **50**(4), 156-159 (2013)
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3. Kirschweng, B., Polyák, P., Pukánszky, B., Vörös, Gy.: A poli(3-hidroxibutirát) hidrolitikus degradációja, *Polimerek* **1**(5), 136-140 (2015)
4. Hári, J., Polyák, P., Mester, D., Mičušík, M., Omastová, M., Kállay, M., Pukánszky, B.: Adsorption of an active molecule on the surface of halloysite for controlled release application: interaction, orientation, consequences, *Applied Clay Science* **132-133**, 167-174 (2016)
5. Polyák, P., Rácz, P., Rózsa, P., Nagy, G.N., Vértessy, B.G., Pukánszky, B.: The novel technique of vapor pressure analysis to monitor the enzymatic degradation of PHB by HPLC chromatography, *Analytical Biochemistry* **521**, 20-27 (2017)

6. Polyák, P., Farkas, Á.E., Somogyi, B., Pukánszky, B.: Poli(3-hidroxiibutirát) mikroszemcsék előállítása és alkalmazása hatóanyag-hordozó mátrixként, *Polimerek* 4(1), 26-32 (2018)
7. Hegyesi, N., Hodosi, E., Polyák, P., Balogh-Weiser, D., Pukánszky, B.: Controlled degradation of poly- $\epsilon$ -caprolatone for resorbable scaffolds, *in preparation*
8. Hegyesi, N., Zhang, Y.C., Kohári, A., Polyák, P., Sui, X.F., Pukánszky, B.: Enzymatic degradation of PLA/cellulose nanocrystal composites, *submitted to Biomacromolecules*

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1. Polyák, P., Horváth, Zs., Hári, J., Pukánszky, B.: PLA/halloysite nanocomposites: structure and properties, Bipoco 2012, May 27-31, 2012, Siófok, Hungary
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5. Polyák, P., Hári, J., Omastová, M., Mičušík, M., Pukánszky, B.: Halloysite nanotube as functional filler in polymer nanocomposites, BYPOS 2014, 16-20 June, 2014, Zázrivá, Slovakia
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8. Polyák, P., Szemerszki, D., Pukánszky, B.: Determination of diffusion coefficients in amorphous poly(3-hydroxybutyrate) matrices, ABIQG Meeting, 2016. december 8, Budapest, Hungary
9. Polyák, P., Dohovits, E., Pukánszky, B., A PHB enzimátikus degradációja, Oláh György Doktori Iskola Konferencia, 2016. február 11, Budapest
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12. Faludi, G., Polyák, P., Kirschweng, B., Pukánszky, B.: Hydrolytic degradation of poly(3-hydroxybutyrate) films: mechanism and kinetic description of the reaction, 33rd PDDG Conference, September 1-5, 2017, Taormina, Italy
13. Hegyesi, N., Hodosi, E., Polyák, P., Balogh-Weiser, D., Pukánszky, B.: Controlled degradation of poly- $\epsilon$ -caprolactone for resorbable scaffolds, Bipoco 2018, September, 2-6, 2018, Balatonfüred, Hungary
14. Polyák, P., Farkas, Á.E., Somogyi, B., Pukánszky, B.: Preparation and application of poly(3-hydroxybutyrate) microparticles as drug carrier matrices, Bipoco 2018, September, 2-6, 2018, Balatonfüred, Hungary
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