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

Ph. D. Thesis

by

Péter Polyák

Supervisor: Béla Pukánszky

Laboratory of Plastics and Rubber Technology
Department of Physical Chemistry and Materials Science
Budapest University of Technology and Economics

Polymer Physics Research Group
Institute of Materials and Environmental Chemistry
Research Centre for Natural Sciences
Hungarian Academy of Sciences

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Introduction

Since the appearance of the first synthetic polymer on the market, they generally have been classified as raw materials that are applied for the production of industrial goods in especially large volumes. While this characteristic of plastics has undoubtedly helped polymer technology to leave behind industries that historically produced goods from other materials, e.g. metals, wood, glass, paper or even concrete, recently polymer industry has changed considerably and it focuses now on products with a significant intrinsic value as well.

The most important of these products are manufactured for the medical and pharmaceutical industry [1-4]. Decades ago, such applications definitely did not exist, as the right polymers were not available for the purpose. Naturally, recursively attempts were made to utilize the achievements of polymer related research in the medical industry [5,6], yet these ideas were either immature, or just simply not sufficiently efficient to present any industrial potential. This incapability of the polymer industry to contribute to medical sciences certainly did not result from the inferior quality of the research projects, but scientists simply did not have the appropriate materials for the purpose.

However, the tide of contemporary science undoubtedly has turned and scientists have an astonishing number of novel materials now that can be applied also in the field of medical and pharmaceutical industry without presenting any harm to the human body [7-10]. The change was brought about by the appearance of biopolymers on the market. Although the definition of “biopolymer” is still the subject of debate among scientists, it does not affect their very fast development; the number of research projects that are devoted to the investigation of these materials increases continuously. The rapid development of biopolymers resulted in an increase in their in vivo application as well [11-15].

Figure 1.1 Chemical structure of poly(3-hydroxybutyrate), abbreviated both in the literature and in this thesis as PHB
The Laboratory of Plastics and Rubber Technology of the Department of Physical Chemistry and Materials Science at the Budapest University of Technology and Economics, together with the Institute of Materials and Environmental Chemistry at the Hungarian Academy of Sciences have invested considerable amount of time and energy into the study of biopolymers over the past decade. However, polymers with intended use in the pharmaceutical and medical industry have not been thoroughly investigated yet. Consequently, the goal of this thesis is to open up this new area and the results presented in it focus on this question. Our primary aim is to introduce and characterize one of the polymers most frequently used in the field of pharmaceutical and medical science, poly(3-hydroxybutyrate) or PHB. We will show that it can be used as drug carrier matrix and complement characterization with the kinetic study of drug release, which is inevitably required to produce a carrier phase that can be applied safely even under in vivo conditions.

1.1 Microbial polyesters in drug delivery

PHB belongs to the family of microbial polyesters, which, as suggested by their name, are synthesized by microorganisms [16-24]. The vast majority of these microorganisms are bacteria, for which the polyester acts as intracellular carbon and energy storage [25-29].

![PHB accumulates as intracellular inclusions in the cytoplasm, as seen on the TEM micrographs recorded by Obruca [29]. Scale bar = 5 µm (A) and 2 µm (B)](image)

This phenomenon was discovered as early as 1926 by the French scientist, Lemoigne [30], yet his finding has remained a mere curiosity for several decades. At this point, one could assume that the event that brought Lemoigne’s result to sunlight was the
rapid advance of industries that require biocompatible polymers (e.g. the medical and pharmaceutical fields) yet oddly, things have happened quite differently.

The event that triggered the re-discovery of microbial polyesters was the continuous accumulation of polymeric waste [31-37], which around the end of the past century was feared to grow beyond control [31-37]. Therefore, scientists started to look for polymers that – unlike commodity polymers like polyolefins – can be degraded by microorganisms being present in the soil, thus the eventual, and often inevitable, polymer contamination of the environment would not grow out of proportion [38-41].

Although such polymers are relatively easy to find (e.g. starch or plasticized starch [42-46]), the producer has to cope with the difficulty that the mechanical properties of these materials are often significantly inferior to those of polyolefins that severely limits their application [47-49]. Moreover, a polymer natively synthesized by microorganisms that can be degraded by enzymes is often prone to unwanted degradation prior to the end of its life cycle, which, especially if the degradation begins during the application of the product, can be embarrassing for the manufacturer and unpleasant for the consumer. However, such difficulties can be avoided, if the mechanical properties of the biopolymer used are close to those of polyethylene (PE) or polypropylene (PP), and simultaneously has a life cycle that is not limited by premature degradation.

Poly(lactic acid) (PLA) is the most important polymer that meets all these requirements, thus it gained importance in several fields of life, mostly in packaging [50-54]. Although the practical importance of PLA is undoubted, its application has several drawbacks as well. The most important is its stepwise production, which consists of the initial fermentation of the monomer followed by subsequent polymerization. Although several microorganisms produce lactic acid, none is able to synthesize the polymer as well [55].

Microbial polyesters appeared at this point. As mentioned previously, they were discovered and partially characterized almost a century ago, yet their importance became evident when the scientific community started to look for polymers that can be produced by bacteria in a single fermentation step [56-59]. Consequently, these polyesters are often compared to PLA, as in the article of Graupner [60] or Hamad [61]. One must not ignore the fact, though, that their real potential lies primarily in their application in the field of medical sciences and not as a substituent to polyolefins, even if this actually triggered the re-discovery of microbial polyesters.

We must also emphasize here the fact that the complete substitution of polyolefins with PHB cannot be achieved at all, as this biopolymer has somewhat worse mechanical characteristics as PE or PP. According to the paper of Chanprateep [62], copolymerization may improve the properties of PHB, but even in the best case, a hydroxybutyrate-hydroxyvalerate copolymer (PHB-HV) might compete with PLA [62] and not with polyolefins. Other researchers, like Peterson [63] or Tanase [64], have followed a different approach and prepared reinforced PHB composites, but the incorporation the reinforcement requires an additional step, which brings us back to the initial problem of producing biodegradable polymers in a single step.
Research projects aiming at the substitution of polyolefins with microbial polyesters are of limited importance, scientists generally focus on their application in other areas, e.g. in medical and pharmaceutical applications, which usually need polymers that can be applied safely under in vivo conditions [65-69]. PHB is a perfect choice from this point of view, as its monomer, 3-hydroxybutyric acid, is the normal component of human blood, thus the in vivo application of the polymer does not produce any toxic metabolite [70].

Accordingly, PHB could be utilized as drug carrier matrix, as its in vivo presence does not expose the human body to any harmful effect [70]. The same applies to short-time implants [71-73], but recent studies also proved that due to the biocompatibility of PHB, it can be used even in long-time applications, e.g. for the coating of certain implants [74-76]. Since PHB is compatible with living tissues, it can also facilitate controlled release even over long times, e.g. in the case when the concentration of a certain drug must be maintained between the limits of the therapeutic window. Up to this point, we treated carrier matrices and implants separately, but practical applications often require both functions: an implant is often doped with at least one disinfectant or anti-inflammatory drug, thus the application of a microbial polyester as drug carrier matrix and as implant practically cannot be distinguished [77,78].

When we speak about the multi-functionality of microbial polyesters, we must also take into consideration one of the most rapidly advancing fields of medical sciences, tissue engineering [79-83]. Microbial polyesters are the perfect choice for the preparation of tissue frameworks, i.e. scaffolds [79-83]. Moreover, these materials can be processed using several techniques that are currently applied for the preparation of scaffolds, e.g. electrospinning [84-87], sol-gel techniques [87] and 3D printing [87-89].

![Microscopic images of scaffolds fabricated by electrospinning (A), sol-gel method (B) and 3D printing (C) [87]]

The articles devoted to the preparation of scaffolds obviously focus on the technique used, but researchers interested in the application of the device often pay more
attention to the doping of the polymer with various drugs to facilitate the growth of the cell culture, for example [90]. Accordingly, even if microbial polyesters are applied in the field of tissue engineering, they act as carrier matrices as well [90].

We have seen now that biopolymers became an essential part of medical science, but their safe application still requires the thorough analysis of their behavior and life cycle under various conditions, including in vivo. One of the most crucial questions is related to the degradation of the polymer, as it modifies both the release kinetics of the entrapped drug and the residence time of the matrix in the human body. Accordingly, several research teams studied the degradation of microbial polyesters; the most relevant results are compiled in the next section.

1.2 Degradation of microbial polyesters

1.2.1 Hydrolytic degradation

Although the degradation of polyesters can be initiated and maintained in several ways, a number of these would require environmental conditions, which are not present in the human body (e.g. high temperature, high pressure), thus these methods are generally not covered by articles published in the field of medical science. However, degradation techniques, which assume an aqueous environment, either acidic or alkaline, are intensively studied, as the human body meets this condition.

A considerable number of publications was dedicated to the investigation of the hydrolytic degradation of PHB and its copolymers [91-93]. Although these studies are rather diverse, they agree that the degradation of PHB in aqueous media is a base catalyzed hydrolytic reaction, in which the rate of the reaction is primarily determined by the concentration of the hydroxide ions [91-93]. Unfortunately, the results of the various studies are quite difficult to compare mainly because of the different methods applied for the preparation of PHB films and pellets (film casting [91,92], microencapsulation followed by cold pressing [91], injection molding [94]), even when two research teams used the same polymer or copolymer.

Moreover, none of the papers cited above attempted to describe the mechanism and kinetics of the hydrolytic degradation of the biopolymer studied. The theoretical background of polyester hydrolysis has been described already by Flory [95], who found that it is a reversible reaction, which eventually reaches equilibrium. However, the formation and the hydrolysis of polyester macromolecules differ from each other. Based on the analysis of his experimental results, Flory [95] concluded that the hydrolytic fragmentation of polyesters occurs with larger probability at ester groups located close to the end of the polymer chain with the consequence that metabolites with smaller molecular mass form with larger probability.

Accordingly, the most probable product of chain fragmentation is the monomer, and the amount of other oligomers decreases with increasing molecular weight. Position dependent hydrolysis rates are needed to describe the inhomogeneous distribution of
metabolites quantitatively [95]. Even though none of the polymers mentioned and studied by Flory [95] was a microbial polyester, we might assume that also the hydrolysis of PHB follows the characteristics described above.

The theoretical treatment of Flory [95] was based on the assumption of homogeneous reaction; hydrolytic degradation was carried out in solution. However, under practical conditions, the degradation of biopolymers often occurs heterogeneously both in composting and in vivo, like in the case of implants. The other factor that makes the application of hydroxide ions as catalyzers cumbersome under in vivo conditions is that measurable rate of degradation occurs exclusively if these ions are dissolved in the aqueous medium at a rather high concentration. A large concentration of hydroxide ions (strongly alkaline medium), however, cannot be tolerated by the human body, thus degradation catalyzed only by hydroxide ions is of limited practical significance. In order to overcome this difficulty, other type of catalysts, e.g. enzyme molecules must be used.

1.2.2 Enzymatic degradation

Enzymatic reactions are very specific and well defined thus the use of a PHB hydrolase enzyme natively synthesized by prokaryotes (bacteria) or eukaryotes (fungi) [96] results in the efficient degradation of the polymer. Several papers reported the production and characterization of extracellular PHB hydrolase enzymes produced by various bacterial strains, such as Alcaligenes faecalis [97, 98], Comamonas testosteroni [99], Pseudomonas lemoignei [100], Pseudomonas pickettii [101], while others used enzymes natively produced by fungi Fusarium solani [102], Paecilomyces lilacinus [103], Penicillium funiculosum [104].

As extracellular enzymes are secreted by the organism, cell disruption is not required as an intermediate step during the production of the protein. Besides the simplicity of their synthesis, extracellular enzymes have the advantage that they are not very sensitive to environmental factors like high temperature, acidic or basic pH, or the presence of oxidizing or reducing agents. Besides the monomer, these extracellular enzymes usually produce longer metabolites [105], while one of them natively produced by the strain Paucimonas lemoignei yields predominantly oligomers [105]. On the other hand, as shown by Chen et al. [105], the novel intracellular enzyme natively produced by Bacillus megaterium yields solely 3-hydroxybutyric acid in the controlled degradation of PHB.

Besides the amount, type and composition of the degradation products, the kinetics of degradation is at least as important for most practical purposes. Composting technology has well-defined cycle times, while the in vivo degradation of medical devices requires the exact knowledge of degradation rate. Although a number of papers published on enzyme catalyzed heterogeneous reactions describe the dependence of the rate of degradation on enzyme concentration, like those of Mukai [106], Scandola [107], or Timmins [108], very little is known about the time dependence of these reactions. The kinetics of enzyme-catalyzed hydrolysis is often described with the model proposed by Michaelis and Menten in 1913 [109]. However, the model was developed and is valid
only for homogeneous reactions, but in practice, either in composting or in medical applications, degradation takes place in a heterogeneous medium, thus the original model must be modified accordingly.

The most important modification concerns the first step of any heterogeneous enzyme catalysis, namely the adsorption of enzyme molecules on the surface of the polymer. This is exactly what Kumar [110] did, who introduced two consecutive steps into the Michaelis-Menten model mentioned above, the adsorption of the enzyme and the actual catalysis. His model takes into account also the second or higher order kinetics of adsorption in the presence of two or multiple enzymes [110].

Besides Kumar, Kari [111] has also investigated the question of enzyme adsorption. He has found that the coverage of the surface by the enzyme is an important parameter, which influences the kinetics of the reaction, thus he introduced an additional parameter into the equations describing the quasi steady state of the reaction [111]. The modified model described the experimental data satisfactorily and the approach proved that the appropriate modification of the original Michaelis-Menten model could be applied even if the substrate is not soluble in water [111].

Although the modification of existing models might predict the kinetics of a particular reaction reasonably well, the authors themselves admit that their approach have severe limitations in the accuracy of the predicted data [110]. Consequently, a number of groups working in the field of heterogeneous enzyme kinetics developed semi-empirical or empirical models for the quantitative description of a specific heterogeneous substrate-enzyme reaction [112-114]. Diao [112], for example, studied the enzymatically catalyzed hydrolysis of a number of cellulose derivatives and estimated the time dependence of the average molecular weight of the degrading polymer by a particular solution of the differential equation describing first order kinetics. While Diao [112] ignored the formation and presence of different products, Kadam [115] developed a model based on the time dependent concentration of the most frequent metabolites forming during the enzymatically catalyzed degradation of lignocellulose [115]. His approach is entirely empirical, but rather successful, since it can predict values surprisingly close to those obtained by measurements [115].

Based on the success of the above presented approaches, we can conclude that by using an appropriate model, which describes the time dependence of in vivo degradation of aliphatic polyesters, makes the application of PHB matrices completely safe, as the entire lifespan of the product can be predicted reasonably accurately. PHB can be used for the preparation of devices with a variety of forms and applications, a brief description is given about these devices in the next section.

1.3 Devices applied as a carrier matrix

Fields extensively using microbial polyesters also include medical applications, in which products consisting one or more of these materials fulfil various purposes. One of the most prominent of these fields is the application of biopolymers as scaffolds in
tissue engineering [116-121]. A closer study of the literature reveals that microbial polyesters are used for the replacement of tissues of astonishing diversity, including heart valve [117], vascular [118], bone [119], cartilage [120], as well as nerve conduit tissues [121]. As shown by the number of areas in which these biopolymers are used routinely, the family of microbial polyesters is an accepted class of material in biomedical applications. The use of PHB scaffolds increased even further, when Peng [122] have found that it does not cause carcinogenesis even if the polymer remains under in vivo conditions for a long time [122].

The advantageous characteristics of microbial polyesters listed above clearly makes them very important raw materials in the field of tissue engineering, which drastically increased the demand for high quality polyhydroxy-alkanoate scaffolds. The increased interest in the preparation of scaffolds obviously resulted in the increase of papers published in this area; a vast number of articles are devoted to the description of techniques used for this purpose [123-128]. Among many others, the most important methods are electrospinning [124-128], salt leaching [129], or solution casting [130].

![SEM images of neat PHB scaffolds (A), and scaffolds with cells grown on them (B) [126]](image)

Although these techniques have been available for decades now, new methods are also developed, which could revolutionize the field. The most important is 3D printing that makes possible the generation of scaffold geometries of almost any arbitrary shape, which, especially in the case of overly sensitive cell cultures, could be crucial for success. One must keep in mind that in the case of fiber spinning, the conformation and spatial arrangement of the fibers is inevitably random [124].

While in the case of scaffolds, 3D printing is only an option, standard geometries must be used in other fields, e.g. for orthopedic pins, stents, cardiovascular patches, articular cartilage repair devices, tendon repair devices, or nerve guides [116]. In these cases, injection molding would be either especially cumbersome, or even impossible to use. Moreover, some of the devices mentioned above must have dimensions of a rather
narrow tolerance, e.g. a stent used for the treatment of aortic stenosis must fit the arteria perfectly. This kind of tight tolerance may be achieved by injection molding, but current manufacturing techniques rely almost exclusively on 3D printing, for which microbial polyesters are the perfect choice of materials as claimed by Saska [131], Pereira [132] or Ausejo [133].

We have seen now how versatile microbial polyesters are in the field of biotechnology and medical sciences. Their real potential, however, does not lie in the diversity of the geometries in which they can be produced, but in their capability of carrying various drugs or pharmaceuticals [134]. Research projects of a considerable scientific significance rarely use any of these biopolymers as a simple product having a pre-defined shape, but exploit them as carrier matrices as well [134, 135]. When an implant or a simple surgical device like a suture is prepared, one inevitably must be certain that the drug initially entrapped in the polymer is released in a predictable manner; otherwise, its concentration may not be located within the therapeutic window. Concentrations outside the window must be avoided, since an insufficiently small concentration of the drug may not fulfil its purpose, while the opposite could be even more dangerous, i.e. an overdose might cause a number of side effects.

Consequently, the description and prediction of the release kinetics of a drug used in therapy has always been an especially important topic in the field of drug delivery science. Although the models developed and applied for this purpose are rather diverse from the aspect of both the mathematical apparatus used and the targeted area of application, we categorize and present them in the next section.

1.4 Quantitative characterization of release kinetics

1.4.1 Mathematically exact methods

The first attempts to describe the kinetics of drug release relied heavily on the canonical laws of physical chemistry and used Fick’s laws of diffusion as starting point. One of the most important and most frequently referenced work is the study of Fujita [136] published as early as 1952, which demonstrated that the numerical solution of Fick’s second law can be expressed as an infinite sum of exponential functions [136]. While Fujita was undoubtedly the first to come up with this idea, the importance of his article was somewhat pushed into the background by the frequently cited book of Crank [137], which was published four years later, in 1956. Crank expressed the time dependence of the \( m_t / m_{\infty} \) ratio, where \( m_t \) is the mass of the drug already dissolved, \( m_{\infty} \) is the mass of the drug dissolved at thermodynamic equilibrium in the following way

\[
\frac{m_t}{m_{\infty}} = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} e^{-\frac{D(2n+1)^2\pi^2}{l^2}t} 
\]

where \( t \) is time, \( D \) the diffusion coefficient of the small molecular weight component in the matrix and \( l \) the thickness of the layer in which the diffusion takes place. Note that
this is a mathematically exact solution of Fick's second law, which does not include any approximation or empirical factor. One may wonder, therefore, why nobody is actually using the equation introduced above. Well, the first and most prominent reason is the nature of the right hand side of Eq. 1 consisting of an infinite sum of functions, which cannot be fitted to experimental data neither with simple linearization, nor by using more complex iterative regression techniques like the Levenberg-Marquardt algorithm. Researchers, who still prefer to fit formulae derived from the laws of diffusion and possibly retain the accuracy of the mathematical apparatus, inevitably have to introduce some kind of simplification and/or empirical factors.

1.4.2 Semi-empirical approaches

The simplest solution for this computational difficulty is offered by the article of Sanchez-Garcia [138], who simply kept the first six terms of the sum shown in Eq. 1 and omitted the rest. Despite its simplicity, the practice of keeping a pre-defined number of exponential terms and disregarding the rest is a frequently used approach, which was followed by Miguel [139] or Iordanskii [140] as well. The latter considered only the first term of the exact solution presented above and fitted Eq. 2

\[
\frac{m_t}{m_\infty} = 1 - \frac{8}{\pi^2} e^{-\frac{D}{\ell^2}t}
\]  

onto his empirical data. The most important advantage of using only one exponential term is the simplicity of its linearization, which is generally followed by the calculation of linear regression providing the slope of the straight line closely related to the diffusion coefficient required [139, 140]. While such approaches are undoubtedly easy to use, even the authors admit that they describe and approximate the measured values accurately only at long times, towards the end of the experiment, when \( t \to \infty \) [140].

In order to overcome this problem, but still insisting on using a formula derived from the mathematically exact solution of Fujita and Crank, researchers like Schwarzl [141] have modified Iordanskii’s model and developed a mathematical apparatus, which, although complex and somewhat difficult to comprehend, at least contains parameters with actual physical meaning [141]. Although some authors prefer models with actual physical background, others are not that fastidious, and they are ready to accept modifications, which do not have any relationship to the more or less conventional starting point of these derivations, i.e. Fick’s laws of diffusion. At least some of these solutions provide good correlation with the experimental data.
1.4.3 Empirical models

Mitran [142], for example, raised time to the power of $n$ and claimed that this modification is essential for the creation of a model with increased accuracy

$$\frac{m_t}{m_\infty} = 1 - e^{-kt^n}$$

(3)

Even though the comparison of similar models used in different fields of polymer science is not the aim of this thesis, it might be worth to mention that the Avrami model [143] used 24/7 for the analysis of polymer crystallization has exactly same form.

$$X = 1 - e^{-kt^n}$$

(4)

Although this similarity is a mere coincidence, it emphasizes that in many cases theoretical accuracy and physical significance is clearly subordinated to simplicity and easy application of the models used routinely. When the goal is the development of a model for routine calculations, the theoretical background of molecular diffusion and the principles of physical chemistry do not hinder the selection of the formula, thus practically any function can be used, which roughly describes the tendency outlined by the experimental data recorded during a dissolution study.

One may wonder, therefore, which functions approximate the best a saturation curve. We can select, for example, the logarithmic function. Browsing the scientific databases for a couple of minutes and one hits the article of Xu [144], who claims that the function

$$Q_{aq} = K \ln(t) + Q'$$

(5)

can provide a fairly acceptable approximation of the results collected experimentally. In the article of Xu [144], $Q_{aq}$ indicates the amount of drug already dissolved into the aqueous medium. A similar strategy would lead to the articles of Takeru Higuchi, who, unlike the Xu mentioned previously, earned wide recognition and appreciation with his work published in 1960 and onwards [145-147]. Although the original Higuchi model, as well as the rather numerous solutions derived from it, containing time with the exponent of 0.5 provides false data at $t \to \infty$, because the square root function is divergent, it is widely accepted and constantly used. The model is usually used in its original form containing only three parameters, $D$, the diffusion coefficient, $C_s$, the amount of dissolved drug, and $A$, the initial amount of drug contained by the carrier

$$m_t = \sqrt{DC_s(2A - C_s)} \cdot t$$

(6)

but researchers often attempt to modify it for two main reasons. The first is to extend the time range the model, since at $t \to \infty$, the model predicts the dissolution of infinite amount of drug. The second reason is to provide better correlation between the measured and calculated data at the beginning of the dissolution study. One of the most frequently cited
articles using this kind of modification was written Paul, who proposed the following modification to the Higuchi model [148]:

\[ m_t = \sqrt{2Dc_s(A - \frac{1}{2}C_s)(\sqrt{t} - \sqrt{t_0})} \]  \hspace{1cm} (7)

Besides Paul, Dukhin [149] and Petropoulos[150] also modified the model, but one can find entire review articles containing a summary and comparison of Higuchi-related models published in the literature. Such a paper was published by Siepmann [151], for example.

While the work of Siepmann is thorough and profound, it does not compile all models derived from the original Higuchi equation, as there are authors who did not know, or just simply did not mention the origin of the square root function used for the kinetic characterization of dissolution experiments. These articles, therefore, are more difficult to find, however, we still present a couple of examples here. Obayemi [152], for example, successfully applied the square root function for the approximation of release data obtained on PLGA microparticles, or Yoon [153], who used the following differential quotient

\[ D = \left( \frac{\pi}{16} \right) \left[ \frac{d}{d(\sqrt{t})} \right]^2 \left( \frac{M_t}{M_{\infty}} \right)^2 \]  \hspace{1cm} (8)

for the quantitative characterization of release kinetics and for the estimation of the diffusion coefficient. As the denominator of this quotient contains the square root of time, it can be classified as a derivative of the Higuchi model, even if Yoon [153] did not mention this fact.

We have seen now the large number and diversity of models used for the description of release kinetics. The reader may also notice that each mathematical formula used currently is an approximation which describes experimental data either towards the end of the dissolution experiment (exponential and logarithmic functions) or at the beginning of the process (square root functions), but somewhere inevitably fails to provide accurate results. In this thesis, besides the identification of degradation mechanisms, we also propose a computational technique, which is reliable over the entire time interval of the experiment and is especially suitable for the description and prediction of dissolution studies in the case of fibrous matrices, e.g. scaffolds.

1.5 Conclusions

A thorough survey of the literature clearly pointed out the fact that microbial polyesters became one of the most important polymers in the field of medical and
pharmaceutical industry. Their behavior and degradation under *in vivo* conditions was studied and characterized thoroughly, on the one hand, and several devices were developed which are used in various fields of health care, on the other. An astonishing number of methods emerged as the result, which may be diverse, but a large number of them are based on the same polymer family. A further conclusion drawn is the multifunctionality of these devices, only a few of them are designed to fulfill a single purpose, e.g. to provide a static framework for living tissues in the way scaffolds do. The majority of them has multiple additional tasks, amongst which the most important is their ability to function as a biocompatible carrier matrix. Such a matrix having actual industrial potential, however, releases its content according to well-characterized kinetics, which renders the kinetic characterization of dissolution processes an especially important and intensively studied field. Several studies were devoted to the introduction of various mathematical models for the description of the time dependence of drug release, some of which are based on the principles of physical chemistry, while others are completely empirical. A rather broad spectrum of semi-empirical approaches exist as well, which are claimed to provide a reliable approximation of experimentally collected data. The final conclusion of this survey, however, must be the observation that the knowledge on the *in vivo* application of microbial polyesters is still insufficient in many aspects and needs further study and development.

1.6 Scope

Although the hydrolytic degradation of the most intensively studied microbial polyester, PHB, was reported in many papers, none of the publications includes a model, which describes the kinetics of hydrolytically catalyzed degradation. Most of the papers present the mass loss and/or the amount of metabolites formed during degradation, but none of them introduces a model that predicts the outcome of the degradation. In Chapter 2, we present our approach based on the mechanism of $S_N2$ type nucleophile substitution, which describes and predicts adequately the time dependence of the hydrolytically catalyzed degradation of PHB.

Just like in the case of hydrolytic degradation, the results published in the literature on the enzymatic degradation of PHB up to now cannot not be considered complete either. The most important and still missing point is the characterization and application of intracellular enzymes. The main reason for using extracellular proteins instead of intracellular ones is that the former are generally easier and cheaper to synthesize and often more resistant against environmental factors like temperature, pH, the oxidative/reductive nature of the aqueous media or its ionic strength. However, also the application of an intracellular enzyme can be advantageous. In Chapter 3, we demonstrate that the application of the intracellular enzyme synthesized natively by the strain *Bacillus megaterium* results in much larger degradation activity than in the case of the more or less conventional extracellular enzymes. Moreover, as the intracellular enzyme used is an exokinase, only the monomer of PHB is produced during degradation, which considerably facilitates both the monitoring and the modeling of the degradation process. This advantage makes possible the introduction of a kinetic model based on the
Michaelis-Menten approach. The model was modified to take into account the heterogeneous nature of the reaction and describes the time dependence of enzymatically catalyzed degradation quite well.

The model introduced in Chapter 3 describes the kinetics of enzymatic degradation well and could be used even for intracellular enzymes. However, usual experiments with active enzymes do not allow the separation of the individual steps of the degradation. The reason lies in the inseparable nature of the two steps of enzyme catalysis. As soon as the protein absorbs on the surface of the polymer, the active complex forms and this leads immediately to the next step, which is either its decomposition or the actual catalysis. Accordingly, adsorption and catalysis cannot be analyzed separately by using native enzymes, even if the former step is crucial for the overall reaction rate. Gene technology helps to solve this problem and the approach is described in Chapter 4. The modification of the gene sequence determining the primer structure of the enzyme by exchanging the serine or cysteine block to alanine in the active centrum renders the protein inactive. The modification of the active centrum does not alter the kinetics of adsorption, thus point-mutated molecules allow the detailed analysis of the adsorption step without the biasing effect of the subsequent step, catalysis.

The degradation studies created the proper basis for our further work, for the preparation of products and devices from PHB, which might be used in the pharmaceutical and medical industry. As described in Chapter 5, scaffolds are often used as carrier matrices for active compounds, usually drugs. Dozens of articles are available in the literature focusing on such application of scaffolds, but very few of them describe the kinetics of drug release quantitatively. In the chapter, we show that one does not necessarily has to compromise between mathematical accuracy and convenience in the selection of a model used for the quantitative description of the results of dissolution experiments. A novel approach is introduced, which is based on Fick's laws of molecular diffusion, but at the same time is able to describe and predict accurately the outcome of a release study.

The closing chapters of this thesis are devoted to a third topic, to the characterization of the kinetics of drug release. An easy to implement technique is introduced in Chapter 6, which can be applied routinely for the measurement of the most important kinetic parameter, the diffusion coefficient of the drug initially entrapped in the polymer. The proposed method requires only a camera, which is widely available these days and a drug that have an absorption band anywhere in the wavelength range of visible light. The generation and observation of concentration gradients inside the PHB film allows the direct calculation of the diffusion coefficient, which would be especially difficult, if one used any of the conventional techniques extensively applied today like permeation studies.

In the last chapter, in Chapter 7, a further measurement technique is introduced and discussed, which can be applied routinely without requiring any expensive instrumentation or cutting edge technology. We prove that the phenomenon of hypsochromic shift can be applied for the monitoring of the dissolution of a drug from a carrier matrix, and in this case practically any drug, as this phenomenon inevitably occurs
Introduction

every time when a molecule leaves the carrier phase and enters the dissolution medium. With the introduction of these easy to use, cost effective, very practical and informative techniques, we truly hope to help fellow scientists who are working with microbial polyesters and are eager to find new methodologies that could help them along the way.

1.7 References

**Chapter 2**

**Mechanism and kinetics of the hydrolytic degradation of amorphous poly(3-hydroxybutyrate)**

### 2.1. Introduction

The continuously escalating industrial interest towards microbial polyesters can be traced back to several reasons [1-11], amongst which the most important is originating from their biocompatibility [11]. Therefore, although these materials, or in some cases their copolymers can be applied as commercial products as well [12-26], their real potential lies in their *in vivo* applications [27-31]. An *in vivo* application inevitably requires knowledge on the degradation of the polymer, otherwise the length of the time interval in which the polymer is still present in the human body cannot be estimated. Therefore, several research groups have studied the hydrolytic degradation of microbial polyesters, often focusing on their most important representative, poly(3-hydroxybutyrate), PHB [32-35].

These studies, however, did not propose any kinetic approach that could be used for the prediction of the purely hydrolytic decomposition of an arbitrary PHB product. While an appropriate mathematical model is not available in the literature yet, the theoretical background of polyester hydrolysis has been already described, and is found in the book of Flory [36]. Based on the principles of polyester hydrolysis discussed in the above referenced book, we propose a novel kinetic model, which simultaneously considers the heterogeneous nature of degradation and accounts for the diffusion of the main metabolites. A further goal of this study is to introduce and determine the rate constants of hydrolytic degradation, which have not been published before and are not available otherwise.

### 2.2. Experimental

#### 2.2.1. Materials

Poly(3-hydroxybutyrate) granules were obtained from Metabolix Ltd. (Mirel M2100, ≥99.5 % purity) with an approximate crystallinity of 60 %. The aqueous media used to degrade the PHB films consisted of technical grade NaOH (Molar Chemicals Ltd.) and distilled water, while the HPLC eluent was a $\mathrm{H_3PO_4/KH_2PO_4}$ phosphate buffer consisting of components purchased from Molar Chemicals Ltd. ($\mathrm{H_3PO_4}$) and Fluka GmbH ($\mathrm{KH_2PO_4}$), respectively. Technical grade chloroform stabilized with 1 % EtOH (Molar Chemicals Ltd.) and laboratory grade acetonitrile (Promochem Ltd.) were used.

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for the extraction of metabolites from degraded polymer films. The 3-hydroxybutyric acid with a purity of ~95% used for the calibration of the HPLC detector and the UV-VIS spectrophotometer was supplied by Sigma-Aldrich.

2.2.2. Preparation of PHB films

Amorphous poly(3-hydroxybutyrate) films were prepared by compression molding and solvent casting. Films of approximately 0.97 g mass were compression molded using a Fontijne SRA 100 machine at 120 kN, 3 min, 220 °C and at a cooling rate of about 30 °C/min. Films of approximately 97 mg mass were cast onto a glass surface from a chloroform solution of 2 m/m% of the polymer and subsequently kept at constant temperature (25 °C) and relative humidity (50%). Compression molding produced amorphous films with an approximate thickness of 100 μm and a surface area of 192 cm². The surface area of the solvent cast films was the same, but their average thickness was much smaller (~10 μm).

2.2.3. Characterization, measurements

The hydrolytic degradation of PHB was monitored quantitatively by four independent techniques. Two of them, gravimetric analysis and metabolite extraction, were applied in order to characterize the polymer phase during hydrolysis, while the other two, UV-VIS spectrophotometry and high performance liquid chromatography (HPLC), were employed to provide information on the quality and quantity of metabolites present in the aqueous phase.

For gravimetric analysis amorphous PHB films were prepared, weighed, and subsequently placed into a 100 ml flask containing aqueous media with the pH value of 13.0, 12.5, 12.0 and 7.0, respectively. The flasks were sealed and then opened after a given degradation time (7, 14, 21 and 28 days). The degraded films were washed, dried and kept in a room of constant temperature (25 °C) and relative humidity (50%).

To determine the metabolites present in the polymer, degraded PHB films were dissolved in 5 ml chloroform, the solution was later extracted with 20 ml of acetonitrile. The acetonitrile phase was centrifuged at 13000 rpm for 10 minutes in order to remove the precipitated PHB from the extracting solvent and then subsequently filtered with a PTFE syringe frit of 45 μm average pore size. The centrifuged and filtered extracting solvent was analyzed with a reverse phase liquid chromatograph (Merck-Hitachi LaChrom Elite) equipped with a LiChroChart 250-4 column. The column contained LiChrospher 100 RP-18 type end-capped silica with an average particle diameter of 5 μm and pore size of 100 nm. Laboratory grade acetonitrile was used as eluent with the isocratic and constant flow of 1 ml/min. The reverse phase LC system was equipped with a diode array (DAD) detector. The detector was set to record the wavelength range of 190-300 nm with a sampling time of 400 ms.

The composition of the aqueous phase was analyzed by UV-Vis spectroscopy using a Thermo Scientific Unicam UV-500 instrument in the 190-400 nm wavelength
Hydrolytic degradation of amorphous PHB

range. Spectra were recorded in a quartz cuvette of 1 cm thickness on samples degraded for various length of times. The qualitative analysis of the aqueous media was performed with the HPLC system already mentioned, but using a H$_3$PO$_4$/KH$_2$PO$_4$ phosphate buffer of pH 3.0 at the concentration of 10 mmol/dm$^3$ as eluent. The pH of the buffer must be set to a low value to keep the metabolites in their protonated form which has significantly longer retention time than ions. The ionized metabolites of PHB degradation are practically inseparable when they are present in an aqueous media of high pH, i.e. in the degradation solution. For the sake of unbiased UV-detection, the phosphate acid and salt was applied at a relatively small concentration (10 mmol/dm$^3$) and the problem of low puffer capacity was overcome by adjusting the pH of the solutions to pH 7.0 just prior injection.

2.3. Results

The results of the experiments are reported in several sections. The time dependence of hydrolytic degradation followed by various techniques is presented in the first. The composition of the degradation products is discussed in the next, followed by the presentation of the kinetic model used for the quantitative analysis of the results. Diffusion coefficients and rate constants are discussed in the next section including consequences for practice.

2.3.1. Degradation kinetics

The time dependence of hydrolytic degradation can be followed by various methods. One of the simplest and most often used one is gravimetric analysis, the determination of the decrease of weight as a function of time. Weight loss measured in alkali solutions of various pH values are plotted against time in Fig 2.1 for films prepared by solvent casting.

The figure highlights the most important features of the degradation: the rate of mass loss depends on the pH of the medium and the time dependence is distinctly nonlinear. The slightly accelerating rate indicates that the hydrolytic reaction occurs also inside the polymer film and not only on the surface, but this assumptions needs further verification. The most important difference between surface and bulk erosion is that in the former case reaction rate depends on the surface area of the sample and it is independent of its volume. In the case of bulk degradation, however, chain fragmentation occurs also inside the polymer and thus reaction rate depends on sample volume. Accordingly, one needs films with the same surface, but different thickness, i.e. volume, in order to determine reliably the character of degradation, to decide if it is mainly a surface or a bulk process. Contradictory statements have been published in the literature claiming either exclusive surface [32-34] or bulk degradation [37].
Figure 2.1  Weight loss of solvent cast PHB films degraded in aqueous media of pH 13.0 (□), pH 12.5 (○), pH 12.0 (△), pH 7.0 (▽) plotted as a function of time.

Figure 2.2  Effect of thickness on the weight loss of compression molded (□) and solvent cast PHB films (○) with an average thickness of 100 and 10 μm, respectively. The samples were degraded in aqueous media of pH 13.0.
The degradation of films prepared by compression molding and solvent casting is compared to each other in Fig. 2.2. The samples had the same surface area, but different thicknesses, 10 vs. 100 μm for solvent cast and compression molded samples, respectively. The comparison of the two functions clearly shows that degradation rate depends strongly on sample volume. The positive correlation together with the nonlinear time dependence of weight loss confirms that the hydrolytic degradation of PHB takes place mainly in the bulk of the sample and not on its surface.

The measurement of the mass loss of the samples gives direct information about the kinetics of degradation. However, degradation kinetics can be followed also by the analysis of the degradation medium. Degradation products have a definite absorbance in the UV-Vis spectrum in the range of 190 and 250 nm, thus changing intensity offers information about the amount of soluble components forming during degradation. UV-Vis spectra recorded on the degradation media are presented in Fig. 2.3.

![UV-Vis spectra of aqueous degradation solutions recorded with 4 hour intervals. Compression molded film degraded at pH 13.0.](image)

A definite absorption peak appears on the spectra indeed, indicating the formation of one or more degradation products and confirming that degradation kinetics can be followed in this way as well. However, the products of the degradation (monomer, dimer, etc.) are of a similar chemical characteristics, thus their absorption bands could overlap. Accordingly, the peaks presented in Fig. 2.3 might be the sum of absorptions
resulting from the presence of more than one metabolite. To determine the quality and quantity of PHB chain fragments solved into the aqueous phase, the components must be separated first which can be done by liquid chromatography.

The chromatograms obtained are very simple. A small peak appears at around zero retention time, which does not change with the time of degradation and obviously belongs to the ions present in the solutions. The intensity of the second peak, on the other hand, increases with degradation time as shown in Fig. 2.4.

![HPLC chromatograms recorded on degradation solutions as a function of degradation time. Sampling frequency: 4 hour.](image)

The time dependence of this second peak is very similar to that of the UV-VIS absorption peaks shown in Fig. 2.3. The lack of any other peak on the chromatogram of the degradation media obtained after various times of degradation indicates that only one degradation product, possibly the monomer, forms during degradation. The lack of other components may have several reasons.

The first one is closely related to the diffusion of possible metabolites. The diffusion coefficients of the 3-hydroxybutyric acid (monomer) and the 3-(3-hydroxybutanoyloxy) butanoate (dimer) in the PHB phase are unknown, but the dimer is expected to have a smaller diffusion coefficient, because of its larger size. It might not diffuse sufficiently fast to be detected in the aqueous phase. The other reason might be
the high basicity of the aqueous phase, in which the catalyst (hydroxide ions) is present in a relatively large concentration. Even if the dimer diffuses into the aqueous medium, it may hydrolyze immediately due to the large concentration of hydroxide ions.

The two methods, i.e. UV-Vis spectrophotometry and HPLC chromatography may supply different information about the products dissolved in the degradation solution. The intensity of the peaks detected by the two techniques is plotted against each other in Fig. 2.5. The correlation is very close with a small deviation at longer degradation times, probably because the concentration of the metabolite is too large for accurate detection. We may conclude that the two methods offer similar information about the degradation of PHB in aqueous medium under the effect of basic catalysis and can be used for the determination of degradation kinetics.

![Graph](image)

**Figure 2.5** Correlation between the intensity of UV-VIS absorption (peak height) and chromatographic peak area recorded on aqueous degradation solutions after various degradation times.

The area under the chromatographic peak recorded on the degradation solution after various length of times on compression molded and solvent cast films are plotted against the time of degradation in Fig. 2.6. The correlations correspond exactly to those determined by gravimetric analysis proving that both the measurement of the weight of the solid films or the analysis of the degradation solution reflects the same degradation kinetics.
Figure 2.6  Effect of thickness on the kinetics of hydrolytic degradation of PHB films. Integrated peak area related to the monomer is plotted against time. Symbols: (□) compression molded, (○) solvent cast films. The samples were degraded in aqueous media at pH 13.0.

The acceleration of degradation and the difference in degradation rate for the two kinds of samples confirm that degradation occurs rather in the bulk of the sample than on its surface. Although the kinetics of degradation is clearly shown by these measurements, we can only guess the composition of the degradation products, which we assume to be mainly the monomer, but further experiments are needed to prove this assumption.

2.3.2. Composition of the degrading solid

The measurement of the weight loss of samples during degradation and the analysis of the degradation solution revealed the mechanism and kinetics of the hydrolytic degradation of PHB. The first approach does not offer any information about the quality of the degradation products, while we can only speculate on them in the second case. To obtain further information about the composition of the degradation products and to determine the quality and quantity of the metabolites present inside the polymer, PHB films were dissolved in chloroform, then extracted by acetonitrile and subsequently the solution was analyzed by HPLC chromatography.
Hydrolytic degradation of amorphous PHB

Chromatograms obtained on the extracting solution after various degradation times are shown in Fig. 2.7. Three peaks appear in the chromatograms with different intensities and at different retention times. The peak at intermediate retention time belongs to chloroform mixed in acetonitrile and its intensity is independent of the time of degradation. The first and third peaks, however, change intensity with degradation time thus they must belong to metabolites formed during degradation.

![Chromatogram](image)

**Figure 2.7** Identification and quantitative analysis of degradation products extracted from degrading PHB films. Chromatograms recorded on acetonitrile solutions used for the extraction of metabolites from PHB films dissolved in chloroform.

The compound eluting at the shortest time was identified as the monomer, 3-hydroxybutyric acid by injecting the acetonitrile solution of the monomer purchased commercially onto the column. The resulting chromatogram exhibited a single peak located at exactly same retention time (1.2 minutes) as the first peak in Fig. 2.7. The identification of the second compound eluted at longer time is more difficult. It is safe to assume that it is the dimer, but this compound [3-(3-hydroxybutanoyloxy)butanoate] is not available commercially and could not be applied as an internal standard.

The hydrolytic fragmentation of a polyester molecule is expected to occur with higher probability at ester groups located close to the end of the polymer chain [36]. Accordingly, metabolites with smaller molecular mass must form with higher probability
and thus the most probable product of chain fragmentation is the monomer, while the second is the dimer. As the dimer has a smaller dipole moment than the monomer, it should elute from the column at longer times. There was some indication of a third peak at even longer retention times which might belong to the trimer, but the intensity of the peak was hardly larger than the stochastic noise of the measurement thus the identification of this compound was impossible.

Accordingly, only the first two metabolites (monomer and dimer) were used for the quantitative characterization of the time dependence of hydrolytic degradation. The chromatographic peaks recorded after various degradation times are presented in Fig. 2.8. Unlike in gravimetric analysis (see Fig. 2.1), the amount of monomer extracted from the polymer phase appears to follow a saturation-like characteristic. A similar tendency can be observed for dimer molecules, which indicates that the concentration of metabolites in the polymer films does not increase exponentially as determined by the measurement of weight or the analysis of the degradation solution.

![Figure 2.8](image)

**Figure 2.8** Changing intensity of the chromatographic peak assigned to the monomer with degradation time. Chromatograms recorded on acetonitrile solutions.

The area under the peaks belonging to the two degradation products was converted to concentration and this latter is plotted against time in Fig. 2.9. The amount of the two metabolites approaches a saturation value indeed. The concentration of the monomer and the dimer seems to reach its corresponding maximum value with different
time constants; monomer concentration appears to converge faster to its plateau. We must consider here that the hydrolytic fragmentation of a polyester molecule occurs with higher probability at ester groups located closer to the end of the polymer chain [36], i.e. monomers form faster than dimers.

Moreover, dimers may decompose to monomers with time yielding further monomer molecules. However, different time constants do not explain the saturation tendency observed. Here, one must consider also time dependent reaction rates and the diffusion of the metabolites into the degradation solution. Decomposition increases, while diffusion decreases their concentration and obviously an equilibrium is reached in the rate of the two processes as degradation proceeds. Since diffusion strongly influences the composition of the solid and the degradation solution, it must be taken into account in the development of a reliable kinetic model for the description of the hydrolytic degradation of PHB.

Figure 2.9 Effect of degradation time on the concentration of metabolites in degrading PHB films. The films were prepared by compression molding and degraded at pH 13. Symbols: (□) monomer, (○) dimer, the solid regression curves have been calculated by using our kinetic model discussed in detail in the next chapter.
2.3.3. The kinetic model

In order to describe the kinetics of degradation, we must know the mechanism of the reaction. Hydrolysis can be classified generally as bimolecular nucleophile substitution ($S_N2$), which begins with the attack of a nucleophile agent on the ester group. The attack results in the formation of an activated complex anion, which, in the presence of water, hydrolyzes immediately. The rate determining step is the formation of the activated complex thus the overall rate of ester hydrolysis depends only on the concentration of the hydroxide ions.

In our case, however, simply the fragmentation of the macromolecular chain would not result in the direct mass reduction of the PHB film, since the metabolites must leave the polymer first. Accordingly, the overall rate of bulk degradation is determined by two parallel processes: the diffusion of the catalyst ions into the polymer and the diffusion of the metabolites into the solution. The diffusion coefficients of the catalyst ions and that of the metabolite molecules in the PHB phase must be known in order to identify the rate determining process. Unfortunately, these coefficients have not been published yet. Since the rate of diffusion depends on the size and dipole moment of the diffusing species, only the competition between the catalyst ions and the monomer must be considered. All other metabolites are much larger, consequently they diffuse much slower. All the above considerations result in the assumption that the diffusion of the hydroxide ion is considerably faster than that of the monomer thus the overall rate of weight loss is determined by the diffusion of the metabolites into the aqueous phase.

Since chain fragmentation is catalyzed by hydroxide ions, the rate of the degradation reaction is primarily determined by their concentration. Because their diffusion rate is considerably faster than that of any metabolite, their concentration is assumed to be constant from the very beginning of the degradation. Accordingly, the initial rate of hydrolysis is defined as

$$\frac{d[m](t)}{dt} = k_i [OH^-]$$

where $[m]$ is the concentration of any arbitrary metabolite, $k_i$ is its initial rate coefficient, while $[OH^-]$ is the nominal concentration of catalyst ions. Since the latter is assumed to be independent of time, it can be merged with the rate constant $k_i$ to obtain $k_i^*$.\[2.1]

Polyester hydrolysis eventually reaches its equilibrium implying that the overall rate of the hydrolysis decreases with increasing amount of the reaction product, i.e.

$$\frac{d[m](t)}{dt} = k_i^* - k_m [m](t)$$

where $k_m$ is the rate constant related to the time required to reach equilibrium. The results presented in Section 3.1 indicated that two metabolites form in the polymer during degradation; $\text{Eq. 2.2}$ must be specified for both of them.
Changes in the concentration of the monomer can be described by Eq. 2.3

$$\frac{d[M](t)}{dt} = k_{i,M} - k_M [M](t)$$ (2.3)

while the equation for the dimer takes the same form, but index M for the monomer changes to D.

As Eq. 2.3 is a simple inhomogeneous, first order, linear differential equation, it can be solved analytically. The final solution gives the time dependence of monomer and dimer concentration in the following form

$$[M](t) = C_1 e^{-k_MT} + \frac{k_{i,M}}{k_M}$$ (2.4)

$$[D](t) = C_2 e^{-k_DT} + \frac{k_{i,D}}{k_D}$$ (2.5)

The analytical solutions presented above allow us to compare the prediction of the model to the measured values. After calibration the fitting of the model to the experimental results yielded the correlations shown by solid lines in Fig. 2.9. The agreement between the prediction and the measurements is excellent confirming the validity of our approach. It proves that degradation proceeds to equilibrium and that the rate of dimer formation is much slower than that of the monomer. Although the calculated concentration values are valid for the solid phase and concentrations used for comparison were measured in the extract, their time dependence is certainly representative and can be applied for the calculation of time constants. The fitting procedure yielded the values of 0.32 1/day and 0.13 1/day for $k_M$ and $k_D$, respectively. The values of the time constants indicate that the formation of monomer molecules reaches its equilibrium significantly faster than that of the dimer, indeed. The dependence of the rate of metabolite formation on the size of the molecule also confirms that the rate of hydrolysis depends on position along the chain [36].

Although the hydrolytic degradation of PHB is described by the model presented above, the decreasing mass of PHB films and the increasing concentration of the monomer in the aqueous phase has not been described kinetically yet. As mentioned above both is related to the diffusion of metabolites. Only monomers were detected in the aqueous phase, thus only the diffusion of the monomer will be considered in the kinetic treatment. Diffusion through a plane, through the surface of the PHB film in our case, can be described by Fick’s first law

$$J = -D \frac{\partial c(x, t)}{\partial x}$$ (2.6)

where $J$ is the diffusion flux, $D$ the average diffusion coefficient, $c$ concentration, while $x$ is the spatial coordinate (position).

The analytical form of $c(t)$ was given above (see Eqs. 2.4 and 2.5). However, the metabolite concentration of the aqueous phase is also required in order to calculate the
infinitesimal concentration change through the surface of the polymer film, \( [\hat{\partial}c(x, t)] \). Since the concentration of 3-hydroxybutyric acid remains rather small throughout the reaction, the driving force of diffusion is expected to be constant. Therefore, the numeric value of the differential term in Eq. 2.8 is assumed to be a linear function of the metabolite concentration inside the polymer given by Eqs. 2.4 and 2.5.

\[
J = -D^* \left[ C_M e^{-k_M t} + \frac{k_{i,M}^*}{k_M} \right] 
\]

Eq. 2.7 defines the molar flux of monomer molecules through the surface of the polymer film with a known size \( (A_s = 192 \text{ cm}^2) \). Since the volume of the aqueous media is also known \( (V = 100 \text{ ml}) \), the molar amount of metabolites can be converted immediately into concentration

\[
J = \frac{1}{A_s} \frac{d n_M(t)}{dt} = \frac{1}{A_s} V \frac{d C_M(t)}{dt} \quad (2.8)
\]

The substitution of Eq. 2.7 into Eq. 2.8 and rearrangement leads to the indefinite integral

\[
c_M(t) = -D^* A_s \frac{1}{V} \int \left[ C_M e^{-k_M t} + \frac{k_{i,M}^*}{k_M} \right] dt 
\]

which after integration gives the concentration of the metabolite in the degradation medium as a function of time

\[
c_M(t) = -D^* A_s \frac{1}{V} \left[ C_M e^{-k_M t} + \frac{k_{i,M}^*}{k_M} t \right] + C \quad (2.10)
\]

To fit Eq. 2.10 to the experimental data, detector signals must be converted into concentration which was done by calibration. Eq. 2.10 was then fitted to the experimental data using the Levenberg-Marquardt algorithm. The prediction of the model and the experimental data converted into concentration are compared to each other in Fig. 2.10. The agreement is excellent both for compression molded and solvent cast films showing that the kinetic model proposed describes properly the concentration of the monomer in the degradation solution and the kinetics of hydrolytic degradation generally. The fitting procedure allows now the determination of rate constants otherwise not available.
Figure 2.10  Kinetics of the hydrolytic degradation of PHB films. Symbols: (□) compression molded, (○) solvent cast. The samples were degraded in aqueous media at pH 13.0. The solid lines represent correlations fitted according to the proposed model taking into account also the diffusion of the monomer into the degrading solution (see Eq. 2.10).

2.3.4. Parameters, consequences

The model contains altogether seven parameters. The first two are the surface area of the PHB films and the volume of the aqueous medium, and they are defined by the experimental conditions. The value of the kinetic coefficients (diffusion coefficient, rate constants) are determined primarily by the pH of the degradation medium and by the preparation method of the film used for the study. Parameter $C$ is an integration constant, its value is defined by the initial condition of the process, by the concentration of the metabolite at the beginning of the degradation. If the aqueous media does not contain any degradation product at $t = 0$, then $C$ equals to

$$C = D^* A_s \frac{1}{V} \frac{C_M}{-k_M}$$

(2.11)

The actual values of the parameters determined by the fitting procedure described above are compiled in Table 2.1. While the applicability of the model is completely independent from the dimensions one wishes to use during the collection of the empirical data, for the sake of convenience we decided to measure time in days, and
concentration in mmol/dm$^3$. Therefore, the parameters listed below are submitted in the following dimensions: mmol/dm$^2$ (pre-exponential factor), 1/days (time constant), while the linear coefficient is dimensionless.

Table 2.1  

<table>
<thead>
<tr>
<th>pH value</th>
<th>Preparation$^a$</th>
<th>Pre-exponential factor ($D^<em>C_M^</em>-k_M^*$)</th>
<th>Time constant ($k_M$)</th>
<th>Linear coefficient ($k_{i,M}/k_M$)</th>
</tr>
</thead>
<tbody>
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<td>13.0</td>
<td>CM</td>
<td>3.01E+05</td>
<td>0.0512</td>
<td>19200</td>
</tr>
<tr>
<td></td>
<td>SC</td>
<td>2.32E+05</td>
<td>0.0434</td>
<td>9230</td>
</tr>
<tr>
<td>12.5</td>
<td>CM</td>
<td>2.16E+05</td>
<td>0.0418</td>
<td>11300</td>
</tr>
<tr>
<td></td>
<td>SC</td>
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<td>0.0225</td>
<td>6530</td>
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<tr>
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<td>0.0457</td>
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<tr>
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<tr>
<td></td>
<td>SC</td>
<td>4.70E+04</td>
<td>0.0162</td>
<td>2810</td>
</tr>
</tbody>
</table>

$^a$ Method of film preparation; CM: compression molding, SC: solution casting

The first quantity, the pre-exponential factor contains several parameters, the diffusion coefficient of the monomer, the integration constant, $C_M$, and the time constant, $k_M$. The quantity gives the concentration of metabolites produced by the initial, accelerating phase of the hydrolysis. Unfortunately diffusion rate cannot be determined separately from the model, we need independent measurements to obtain it. The time constant offers information about the deviation of kinetics from linearity during the accelerating phase of hydrolytic degradation, i.e. the curvature of the concentration vs. time function. Finally, the third quantity, the linear coefficient, gives the rate of the reaction at infinite time.

The table clearly shows that all values related to the rate of the reaction increase with increasing pH of the aqueous medium indicating that the main factor is the concentration of the hydroxyl ions acting as catalyst. Although the diffusion coefficient cannot be determined separately, its dependence on the factors studied (pH, method of film preparation) can be estimated, since neither $C_M$ nor $k_M$ depend on them. The data of
the table indicate that the rate of monomer diffusion increases with increasing pH, probably because of changes in local morphology as degradation proceeds. The importance of the structure of the films is shown also by the difference in the parameters determined for compression molded and solvent cast films. The two preparation methods result in films with different free volumes leading to dissimilar rates of diffusion. Obviously the molecules in films prepared by solvent casting are closer to equilibrium, have smaller free volume which leads to slower diffusion. With the help of the parameters determined, the time of degradation can be predicted reliably, if degradation conditions are known. With all probability the model can be used also for the description and prediction of the hydrolytic degradation of other aliphatic polyesters like PLA, but also of various copolymers.

2.4. Conclusions

The study of the hydrolytic degradation of PHB films prepared by compression molding and solvent casting, respectively, proved that degradation takes place mainly in the bulk of the samples and not on their surface. The overall rate of degradation depends strongly on pH, it increases with increasing pH values. The bulk-like nature of the degradation was also confirmed by the analysis of degradation products within the degrading polymer. Metabolite extraction and chromatography proved that degradation does not occur randomly, but with larger frequency at the end of the chains; basically only the monomer and the dimer was found in the degrading polymer. By assuming that the hydrolysis of PHB is a $S_N2$ type bimolecular nucleophile substitution reaction, a kinetic model was proposed which describes the formation of various degradation products. Weight loss and the concentration of the metabolites in the aqueous phase depends also on the diffusion rate of the components, the diffusion of the monomer proved to be the rate determining step. Diffusion was accommodated into the model and thus the concentration of the monomer could be predicted also in the aqueous solution. The correlation between prediction and experimental result is excellent. The model can be extended for the description of the degradation of other aliphatic polyesters as well.

2.5. References

of Biological Macromolecules 59, 170-177 (2013)
Chapter 3

Enzymatic degradation of poly-[(R)-3-hydroxybutyrate]: mechanism, kinetics, consequences

3.1. Introduction

Although the trend that the polymer industry is searching for materials that could be produced from renewable sources [1-4] is not new at all, novel techniques aiming at the production, application and decomposition of the most important polyhydroxyalkanoates, e.g. PLA [4-10] or microbial polyesters [11-24] are still regarded as the most intensively studied topic in the field of biopolymers. This phenomenon can primarily be traced back to their biodegradable nature. A biodegradation could be implemented in several ways, the most straightforward (hence the most prevailing) is enzymatic degradation, which can be executed even if hydrolytic decomposition [25-29] cannot be applied, e.g. under in vivo conditions.

In this study, we investigated the enzymatic degradation of PHB, and quantitatively characterized the process by using a kinetic model, which is based on the original Michaelis-Menten approach [30], yet describes and predicts the outcome of heterogeneous enzyme reactions.

3.2. Experimental

3.2.1. Materials

Poly(3-hydroxybutyrate) granules were obtained from Metabolix Ltd. (Mirel M2100, ≥99.5% purity) with an approximate crystallinity of 60%. HIS-tagged poly(3-hydroxybutyrate) depolymerase enzyme molecules were produced by recombinant Escherichia Coli bacteria [strain: Origami DE3 (Novagen), plasmid: pGS1865 bearing the depolymerase gene of the bacteria Bacillus Megaterium] purified by affinity chromatography on a Ni-nitrilotriacetic acid (NTA) agarose column.

3.2.2. Sample preparation

Amorphous poly(3-hydroxybutyrate) films were prepared by compression molding and solvent casting techniques, both of which have already been described in chapter 2.2.2. The method that was used to a production of the solvent cast films,

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however, differed from that of described previously at one point: solvent cast films were tempered at 25 °C during an extended period of time (one week) in order to minimize the amount of chloroform still present in the film, that could denature the enzyme molecules.

3.2.3. Methods

The enzymatic degradation of amorphous poly(3-hydroxybutyrate) films was carried out in Erlenmeyer flasks, at 37 °C with continuous stirring at 200 rpm. The aqueous media consisted of 100 mmol/dm$^3$ NaCl and 20 mmol/dm$^3$ Tris/HCl buffer [tris(hydroxymethyl)-aminomethane hydrochloric acid salt] adjusted to pH 8.0. The amorphous polymer films and the enzyme solution were simultaneously added to the Erlenmeyer flasks, the latter in a quantity to provide 7 μg/ml enzyme concentration. The experimental conditions used (37 °C temperature, pH 8.0 basicity, 7 μg/ml enzyme concentration) were selected from a previously published paper reporting the dependence of maximum enzyme activity on these factors [31].

Enzymatic degradation was followed with UV-VIS spectrophotometry (UNICAM UV-500, wavelength range: 200-300 nm) and HPLC chromatography (Merck-Hitachi LaChrom Elite, equipped with a DAD detector set for the same 200-300 nm wavelength range). The HPLC column (LiChroChart 250-4) contained LiChrospher 100 RP-18 type end-capped silica (5 μm average pore size), the eluent was a phosphate buffer of 10 mmol/dm$^3$ at pH 3.0. Both the UV-VIS spectra and the HPLC chromatograms were recorded with a time interval of 20 min over 3 hours. Besides repetitive sampling, UV-VIS measurements were carried out also with an online UV-VIS spectrophotometer (PharmaTest PTWS 600) in order to measure the time dependent spectrum of the degrading medium not biased by intermittent sampling. The composition of the samples degraded for various times were analyzed also by mass spectrometry (TA Instruments SDT 2960 MS).

3.3. Results and discussion

The results of the study are reported in several sections. Data obtained on the kinetics of enzymatic degradation are reported in the first. The model developed for the quantitative analysis of degradation kinetics is presented in the next one, followed by the estimation of parameters and the discussion of the results including their practical relevance.

3.3.1. Degradation kinetics

UV-VIS spectra recorded on the degradation media after various times are presented in Fig. 3.1. The absorption peak appearing at around 215 nm in the UV-Vis spectra can be assigned to the specific absorption of the monomer 3-hydroxybutyric acid. Peak maxima were found to shift slightly towards smaller wavelengths; maximum absorbance was always determined at the corresponding wavelength of the maximum and not at 215 nm.
Figure 3.1  UV-VIS spectra recorded on aqueous media containing compression molded films degrading for various length of times.

Figure 3.2  Degradation kinetics of poly(3-hydroxybutyrate) films determined after intermittent sampling by UV-VIS spectroscopy. Symbols: (○) compression molding, (□) solvent casting.
The shift in the position of the absorbance peak might be caused by a number of effects, one of them is the formation of metabolites with a specific UV absorbance close to that of the monomer (~215 nm). The UV spectra of different metabolites which might form during degradation is expected to be very similar to that of the monomer that makes quantitative analysis quite difficult. Maximum absorbance measured both on compression molded and solvent cast films is plotted against the time of degradation in Fig. 3.2.

In order to separate the components of the solution obtained after various times of degradation, samples were injected onto a reverse phase liquid chromatograph. The chromatograms recorded on various samples are presented in Fig. 3.3.

![Chromatograms recorded on aqueous media after various times of degradation of compression molded PHB films.](image)

The first peak appearing at 100 s belongs to the Tris/HCl buffer cation; its concentration remained constant (10 mmol/dm$^3$) in the entire time span of the degradation experiment. The height of the second peak, however, depends on time proving the formation of increasing amounts of a product, possibly the monomer. The lack of additional peaks indicates that longer metabolites do not form or the HPLC column cannot separate them from the monomer.

To exclude the latter possibility, MS spectra were recorded at the end of the degradation period, after 3 hours (Fig. 3.4).
The only peak of relevance appears at 103.15 g/mol. The molar mass of 3-hydroxybutyric acid is 104.11 g/mol, but at pH 8.0 practically all molecules of a weak acid with the pKₐ value of 4.8 are present in ionized form. Deprotonation is expected to decrease the molar mass of the dimer [3-(3-hydroxybutanoyl) oxybutanoic acid] as well, and thus its presence should have appeared as a peak at 189.22 g/mol. Because of the lack of any peaks in this range or above, one may safely state that the only product of the enzymatic degradation is the monomer.

The results presented above clearly show the general tendency of the kinetics of enzymatic degradation, but the plotted values (Figs. 3.2 and 3.4) are inevitably biased by systematic and stochastic errors caused by the nature of repetitive intermittent sampling. In order to minimize these errors, additional samples were prepared and their degradation monitored with a fully automated on-line UV-VIS measurement system. The results of the measurements are plotted in Fig. 3.5 and they confirm completely those obtained by recursive sampling. Compression molded films degrade faster than those prepared by solvent casting, and degradation proceeds through an initial accelerating stage to achieve constant rate later.
Automated sampling carried out by a software-controlled peristaltic pump has the undeniable advantage of providing quasi-continuous flow of the aqueous medium through the measurement cell, but certain issues may arise about the accuracy of UV detection also here. The online UV-VIS spectrophotometer requires an initially specified wavelength, at which absorbance is recorded continuously as a function of time during the measurement. This wavelength was set to 215 nm, resulting in systematic error, since the position of maximum absorption shifts towards smaller wavelengths with increasing degradation time (see Fig. 3.1).

3.3.2. The model

Fig. 3.2 shows that the rate of degradation initially increases and eventually, after 40-60 min, it becomes constant. The linearity of the correlations reveals that degradation carried out both on compression molded and solvent cast films proceeds similarly with a constant rate. An appropriate kinetic model may help to explain the initial accelerating stage.

Among many others, Michaelis and Menten [30] proposed a model to describe the kinetics of enzymatic reactions. In their original publication, they presented a two-step model, which included the formation of an enzyme-substrate complex first, and then its subsequent decomposition. This latter step may yield an unmodified substrate or a
product molecule. Neither of them modifies the enzyme, the protein remains intact in both cases. The model is usually expressed as

\[
E + S \rightleftharpoons k_1 ES \rightarrow k_{cat} E + P
\]  

(3.1)

where \(E\) is the enzyme, \(S\) the substrate, \(ES\) the activated complex, and \(P\) the product. Each of the reaction steps has its own rate constant, i.e. \(k_1\), \(k_{-1}\) and \(k_{cat}\). The determination of the rate constants requires the knowledge of the concentration of the components and the kinetic order of the reaction, which, according to the original article, corresponds to the number of reactants participating in the respective reaction step.

According to these assumptions, the concentration of the enzyme increases by the decomposition of the activated complex and decreases by the formation of the \(ES\) complex

\[
\frac{d[E](t)}{dt} = -k_1[E](t)[S](t) + k_{-1}[ES](t) + k_{cat}[ES](t)
\]  

(3.2)

The reaction is calculated similarly for the \(ES\) complex; its concentration increases by its formation and decreases by its decomposition, i.e.

\[
\frac{d[ES](t)}{dt} = +k_1[E](t)[S](t) - k_{-1}[ES](t) - k_{cat}[ES](t)
\]  

(3.3)

The concentration of the substrate changes only in two reactions, in the formation of the \(ES\) complex and during its decomposition

\[
\frac{d[S](t)}{dt} = -k_1[E](t)[S](t) + k_{-1}[ES](t)
\]  

(3.4)

Finally the rate of the formation of the product molecules is affected only by the concentration of the \(ES\) complex

\[
\frac{d[P](t)}{dt} = +k_{cat}[ES](t)
\]  

(3.5)

Although the Michaleis-Menten model described above is quite simple, preliminary calculations based on its differential equations (Eqs. 3.2-3.5) usually provide surprisingly exact results. In our case, however, the model has to be modified to take into account the heterogeneous character of enzyme catalyzed hydrolysis.

The first modification to be made is related to the formation of the enzyme-substrate complex. In the degradation of polyhydroxyalkanoates the substrates are ester groups and only those located at the surface of the polymer film are able to participate in the reaction and form an activated complex. The approximate diameter of a PHB depolymerase molecule is about \(8 \pm 3\) nm [31], which makes its diffusion inside the polymer phase practically impossible. As the surface of the PHB film placed into the aqueous media remains constant throughout the 3 hours of the measurement, thus the number of ester groups located on the surface can be assumed constant as well.
The adsorption kinetics of the enzyme molecules onto the surface of the film must be also considered and accommodated into the model. Since the formation of an activated complex requires a free enzyme molecule and a free ester group on the surface, only a monomolecular layer of the enzyme can be active and catalyze the degradation reaction \([31]\). Accordingly, the total amount of active enzyme molecules adsorbed on the surface of the polymer is rather small and thus enzyme concentration \([E]\) is regarded as constant in the model.

The application of the modifications described above leads to the following equations

\[
\begin{align*}
\frac{d[E](t)}{dt} &= 0; \quad E_0 = \text{const} \quad (3.6) \\
\frac{d[S](t)}{dt} &= 0; \quad S_0 = \text{const} \quad (3.7) \\
\frac{d[ES](t)}{dt} &= +k_1E_0S_0 - k_{-1}[ES](t) - k_{\text{cat}}[ES](t) \quad (3.8) \\
\frac{d[P](t)}{dt} &= +k_{\text{cat}}[ES](t) \quad (3.9)
\end{align*}
\]

where \(E_0\) and \(S_0\) are the constant number of enzyme molecules and ester groups located on the surface of the polymer film, respectively.

### 3.3.3. Application of the model, parameters

The differential equation system presented above (Eqs. 3.8 and 3.9) must be solved in order to obtain the unknown \([ES](t)\) and \([P](t)\) functions. The analytical solution obtained takes the following form

\[
\begin{align*}
[ES](t) &= Ce^{-(k_{-1}+k_{\text{cat}})t} + k_1E_0S_0 \frac{1}{k_{-1} + k_{\text{cat}}} \\
\quad + k_{\text{cat}} \frac{k_1E_0S_0}{k_{-1} + k_{\text{cat}}} t + C' \\
[P](t) &= C \left( k_{\text{cat}} \frac{k_1E_0S_0}{k_{-1} + k_{\text{cat}}} e^{-(k_{-1}+k_{\text{cat}})t} \right) \quad (3.10)
\end{align*}
\]

The graphical form of the functions expressed by Eqs. 3.10 and 3.11 are plotted in Fig. 3.6, which shows the concentration of the activated complex ([ES]) and that of the product ([P]) as a function of time as predicted by the modified Michaelis-Menten model.
Enzymatic degradation of PHB

According to the model derived above, the total number of enzyme molecules adsorbed on the polymer surface approaches a constant value. Doi and his colleagues [31] studied the adsorption kinetics of several PHB depolymerase enzymes, and although none of them was the strain *Bacillus megaterium*, the obtained enzyme-substrate complex vs. time plots were quite similar to that predicted by our modified Michaelis-Menten model (Fig. 3.6). According to the model, product concentration goes through an initial accelerating phase with increasing adsorption of enzyme molecules, but as the total number of ES complexes reaches its maximum, the formation rate of product molecules also converges to a constant value, i.e. the product concentration vs. time function becomes linear.

While the concentration of the ES complex was not measured in our recent study, product formation was monitored with several independent methods. In order to be able to compare measured data with the prediction of the model, absorbance values must be converted to concentrations, and the model equation (Eq. 3.11) fitted to the measured data. The conversion was done by calibration using commercial 3-hydroxybutiric acid, while the fitting was carried out with a nonlinear iterative method using the Levenberg-Marquardt algorithm.

In order to facilitate the fitting procedure and the determination of the constants of the model, Eqs. 3.10 and 3.11 are further simplified. The merging of the rate constants

---

**Figure 3.6** Changing concentration of the intermediate complex ([ES]) and that of the product ([P]) as a function of time as predicted by the Michaelis-Menten model modified for heterogeneous reaction (Eqs. 3.10 and 3.11).
$k$ and the integration constant $C$, as well as introducing parameter $\lambda$ in the form $\lambda = k_1 + k_c$, simplifies the exponential part of Eq. 3.11 and the linear term can be modified in a similar way. Taking into consideration the initial condition of the model, i.e. $[P](0) = 0$, shows that the $C$ constant equals the preexponent $(A)$ of the simplified equation. All these simplifications result in the final form of the equation which can be used for fitting and the estimation of the parameters, i.e.

$$[P](t) = A \cdot e^{\lambda t} + p \cdot t - A$$  \hspace{1cm} (3.12)

where $\lambda$ indicates the time necessary to reach the stationary state of the degradation reaction, $p$ is the formation rate of the product molecules (monomer) and thus the rate of degradation in the stationary stage, while $A$ is the nominal amount of monomer formed in the first, nonlinear stage.

The simplified formula was fitted to the experimental results obtained both by intermittent sampling and on-line UV-VIS detection. The results of the fitting procedure are shown in Figs. 3.7 and 3.8, respectively.

Figure 3.7  Fitting of the model to the kinetics of enzymatic degradation of PHB films. Detection was recursive UV-VIS spectroscopy.

The fit is excellent in all cases proving that the model is adequate for the description of the kinetics of the enzymatic degradation of PHB, but most probably also for that of other aliphatic polyesters.
Figure 3.8  Fitting of the kinetic model of enzymatic degradation onto the data obtained by online UV-VIS measurements.

The comparison of the measured and predicted data also shows, especially for films prepared by compression molding, that the rate of degradation decreases at longer times, deviates from the predicted line. A probable reason for the deviation is the denaturation of the enzyme, but this tentative explanation needs further study and proof. Another interesting phenomenon is the slower degradation of solvent cast films. Only tentative explanations can be given here too. Although both were amorphous, the morphology of the two films might be different, compression molding at high temperature might result in some degradation, and finally the solvent used for casting might not have been removed completely and could have led to the denaturation of the enzyme. Although a final and unambiguous explanation cannot be given for these phenomena, the new model may help considerably the identification and the quantitative determination of the effect of factors influencing the enzymatic degradation of aliphatic polyesters.

The fitting of the model to the experimental data yielded also numerical values for the parameters of Eq. 3.12 which are listed in Table 3.1.
### Table 3.1

<table>
<thead>
<tr>
<th>Preparation method</th>
<th>Parameter</th>
<th>Detection method</th>
<th>Recursive sampling</th>
<th>Online measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compression molding</td>
<td>$p$ (mmol/dm³/min)</td>
<td></td>
<td>0.0241</td>
<td>0.0244</td>
</tr>
<tr>
<td></td>
<td>$\lambda$ (1/min)</td>
<td></td>
<td>-0.0302</td>
<td>-0.0237</td>
</tr>
<tr>
<td></td>
<td>$A$ (mmol/dm³)</td>
<td></td>
<td>0.8311</td>
<td>0.9890</td>
</tr>
<tr>
<td>Solvent casting</td>
<td>$p$ (mmol/dm³/min)</td>
<td></td>
<td>0.0133</td>
<td>0.0168</td>
</tr>
<tr>
<td></td>
<td>$\lambda$ (1/min)</td>
<td></td>
<td>-0.0249</td>
<td>-0.0223</td>
</tr>
<tr>
<td></td>
<td>$A$ (mmol/dm³)</td>
<td></td>
<td>0.6648</td>
<td>0.8043</td>
</tr>
</tbody>
</table>

The parameters clearly show, as mentioned above, that the degradation of solvent cast films is generally slower than that of the compression molded ones; the rate of the second, stationary stage ($\lambda$) is significantly slower for solvent cast than for compression films and 3-hydroxybutyric acid concentration in the first, nonlinear phase ($A$) is also smaller. The attention must be called here to the fact that parameters determined by different detection methods, i.e. intermittent and online, show excellent agreement in spite of the inherent deficiencies of both techniques.

Beside reaction rates, parameter fitting provided valuable information about the adsorption of enzyme molecules as well. The larger absolute values of the time constant ($\lambda$) indicates that a shorter time is required to reach the equilibrium in adsorption in the case of compression molded than for solvent cast films. In the latter case the nonlinear phase seems to be longer implying that the adsorption kinetics of enzyme molecules depends on the method used to prepare the PHB films, probably on their morphology or surface quality.

### 3.4. Conclusions

Amorphous poly(3-hydroxybutyrate) films prepared by compression molding and solvent casting were degraded with the hydrolase enzyme natively synthesized by the strain *Bacillus megaterium*. The results showed that the enzyme catalyzes the degradation of PHB indeed. 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. Biodegradation produces the (R)-3-hydroxybutyric acid monomer in high purity, no
metabolites or side products were detected in the degradation product. The kinetics of degradation was described quantitatively by a modified form of Michaelis-Menten model. Modifications had to take into account the heterogeneous nature of the degradation reaction. The new model assumes constant substrate and enzyme concentration, which simplifies treatment considerably. The parameters determined by the fitting of the model to the experimental values were consistent and did not depend on the method of detection (recursive or on-line). On the other hand the rate of degradation depended significantly on the technique used for the preparation of the films indicating parameters not accounted for during the study. The degradation of PHB by the strain used offers a simple way for the economic production of 3-hydroxybutyric acid, a compound used in chemical synthesis or as a component of biomedical systems.

3.5. References


Chapter 4

The role of enzyme adsorption in the enzymatic degradation of an aliphatic polyester

4.1. Introduction

We truly hope that the previous chapter has successfully highlighted the importance of enzyme catalyzed polymer degradation and gave an insight to the kinetic background of these kind of reactions. Obviously, studies reported independently of us show the use of either the Micaelis-Menten [1-4] or the Briggs-Haldane [5-9] kinetics in their original form, yet we must point out again, that polymer degradation differs fundamentally from the reaction to which the above mentioned models were initially applied. The first difference is the heterogeneous nature of a polymer degradation, which, as proven by the articles referenced as [10-14], has already been addressed by a number of researchers, but the enzyme catalyzed decomposition of polymers has not been described completely yet.

This is mainly due to the fact that none of the research groups active in this field have attempted to assess the consecutive steps of enzyme catalyzed depolymerization separately, thus no information concerning the characteristics of the enzyme adsorption and the subsequent catalysis is available in the literature. By using the achievements of gene technology, such as PCR mutagenesis [15-23], however, we could create enzyme molecules, which adsorb on the surface, but do not catalyze the hydrolysis of the ester bond. Consequently, a separate analysis and kinetical characterization of the adsorption step becomes possible.

4.2. Experimental

4.2.1. PCR mutagenesis

Enzyme molecules carrying an inactive catalytic and an intact binding domain have been prepared by the site-directed mutation of the intracellular enzyme natively synthesized by the strain Bacillus megaterium (ATCC 11561). The gene of the enzyme have been isolated, characterized, and kindly supplied by Chen [24] in a pGS1941 plasmid. The plasmid has been extracted from the carrier E. coli Origami (DE3) strain (Novagen) by using a NucleoSpin Plasmid Miniprep (Macherey-Nagel GmbH). The original gene sequence of the catalytic domain is the following

in which the thymine-guanine-thymine codon encodes the cysteine located in the catalytic domain of the enzyme. In order to substitute cysteine by alanine, the codon must be changed to gct. The forward and reverse primers applied during the PCR have been synthesized by Sigma-Aldrich:

Forward primer: 5’ccggtctg gct gcaggaggaagt 3’
Reverse primer: 3’cacaaataacggccagac cga cgtcctc 5’

By using the primers of the base sequence indicated above, the PCR mutagenesis has been carried out in a Thermo Hybrid PCR Spin (OyaGen) device. The Phusion® Site-Directed Mutagenesis Kit (Thermo Fisher Scientific-Finnzymes) containing a Phusion® Hot Start II High-Fidelity polymerase enzyme was applied.

The product of the PCR has been purified with methylated DNA digestion catalyzed by DpnI nuclease, and was subsequently transformed into an E. coli RosettaBL21 (DE3) strain. Enzyme molecules carrying the inactivated catalytic domain were expressed by growing the transformed RosettaBL21 culture in LB medium at 37 °C, which was later treated with a 0.1 mmol/dm³ IPTG (isopropyl β-D-1-thiogalactopyranoside) solution. Cells were collected, disrupted by sonication, and subsequently centrifuged at 10000 rpm for 30 min. The remaining supernatant was used for the purification of HIS-tagged enzyme molecules by affinity chromatography on a Ni-nitrilotriacetic acid (NTA) agarose column. In order to compare the characteristics of the enzyme molecules having an inactive catalytic site to the original enzyme molecules, the native enzyme has also been expressed by using the method described above on an E. coli Origami (DE3) strain containing the original, pGS1941 plasmid.

4.2.2. Preparation of PHB suspensions

Poly(3-hydroxybutyrate) (PHB) granules were obtained from Metabolix Ltd. (Mirel M2100, ≥99.5% purity) with an approximate crystallinity of ~60%. An 5 m/m % solution of the polymer was prepared in chloroform (Molar Chemicals Ltd., ~98 %) and then it was precipitated in technical grade denatured ethanol (Molar Chemicals Ltd., ~96 %). The ethanol was intensively stirred during precipitation with an Ultra-Turrax (Type T-25, IKA-Werke GmbH) at the high rate of 24 000 rpm in order to obtain a suspension of large interface area.

The polymer particles were separated by filtration, then washed first with distilled water, and then with the buffer used for the enzymatic degradation. This buffer consisted of an aqueous solution of Tris/HCl (10 mmol/dm³) and NaCl (100 mmol/dm³), both components supplied by Fluka GmbH. The buffer also contained the co-factor of the enzyme (Ca²⁺, applied in a form of CaCl₂ salt, purchased from Sigma-Aldrich) in a concentration of 5 mmol/dm³, and it had a pH value of 8.0.

The washing steps were followed by the re-suspension of the polymer particles in the puffer of the enzymatically catalyzed degradation. The suspension was stirred with
an Ultra-Turrax at 6000 rpm in order to hinder the aggregation of the polymer particles and to achieve the largest water-polymer interface possible.

4.2.3. Degradation

The degradation of the polymer was carried out in the presence of the original enzyme and the one modified by site-directed mutagenesis, respectively. Both enzymes were added to the polymer suspensions (37 °C, 200 rpm) at the concentration of 7 μg/ml. Twelve samples were taken from the suspensions after 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120 minute reaction times. The polymer particles were separated from the suspension in a centrifuge at 10000 rpm to avoid biasing the measurement of enzyme concentrations.

The clear supernatant obtained was analyzed by high performance liquid chromatography (Merck-Hitachi LaChrome Elite, equipped with a DAD detector). Chromatograms were recorded in the 200-350 nm wavelength range at a scanning period of 400 ms. The HPLC column (LiChroChart 250-4) contained a LiChrospher 100 RP-18 type end-capped silica (5 μm average particle size), the eluent used was a 10 mmol/dm³ phosphate buffer with the pH of 3.0.

Besides the measurement of the concentration of the degradation products by HPLC, the size of the dispersed PHB particles was also determined in a suspension not containing any enzyme and in one subjected to degradation for 120 min catalyzed by either the native or the inactivated enzyme. The size distribution of PHB particles was determined by using a HORIBA Partica LA 950 A2 type apparatus.

4.3. Results and discussion

The results are presented in several sections. The effect and efficiency of point mutagenesis is analyzed in the first, followed by the presentation of the adsorption kinetics of the enzyme. A model describing kinetics quantitatively is introduced in the next section, while considerations related to the surface need of an enzyme molecule are presented in the final section of the paper.

4.3.1. Effect of mutagenesis

The efficiency of the site-directed mutagenesis was checked indirectly by the determination of degradation products in the aqueous media surrounding the polymer. Since the depolymerase enzyme Bacillus megaterium was shown to produce exclusively the monomer, 3-hydroxybutyric acid during degradation [24], the qualitative analysis of the metabolites is relatively simple. However, 3-hydroxybutyric acid must be separated from the other UV-absorbing components present in the buffer (tris and molecules of the depolymerase enzyme) that is achieved by the application of an eluent with the pH value of 3.0, since under acidic conditions the acid (pKₐ = 4.7) is in the protonated form, its retention time is relatively long. The ionic molecules of tris and the enzyme are expected to have a retention time corresponding to the t₀ value of the column.
Figure 4.1  
*HPLC chromatograms recorded on degradation suspensions at different reaction times. The amount of the single product of the hydrolysis, 3-hydroxybutyric acid, increases with time.*

Figure 4.2  
*Lack of any peak on the HPLC chromatogram of the degradation medium containing the inactive enzyme; mutagenesis was successful.*
The chromatograms of the degradation solutions containing the original, active enzyme are presented in Fig. 4.1. According to the considerations presented above, two peaks can be observed in the chromatograms shown, one at \( t_0 \) and one at the retention time of the degradation product. The appearance of the peak assigned to the elution of 3-hydroxybutyric acid prove that the active enzyme catalyzes the degradation of PHB indeed, and changes in the area under the peak can be used for the quantitative analysis of degradation kinetics. The chromatograms recorded on solutions containing the modified enzyme are shown in Fig. 4.2. The absence of the retention peak of 3-hydroxybutyric acid proves that mutagenesis was successful and the inactive enzyme does not catalyze the depolymerization of PHB.

Another way to check the activity of the enzymes used is the measurement of the size of the dispersed polymer particles. The size distribution of the original particles, and those obtained in the presence of the active and inactive enzyme, respectively, after 120 hours of reaction are presented in Fig. 4.3.

![Figure 4.3](image_url)

**Figure 4.3** Size distribution of PHB particles in the degradation medium. Symbols: (solid) initial suspension, (dashed) suspension containing the inactive enzyme, (short dot) suspension containing the active enzyme.

The figure clearly shows that the size distribution of the original particles and those extracted from the solution containing the inactive enzyme are identical. However, particle size is considerably smaller in the suspension containing the active enzyme.
indicating that degradation took place in the suspension in this case. The difference in particle size confirms again that mutagenesis was successful and further evaluation of the recorded chromatograms allows the quantitative analysis of adsorption kinetics.

4.3.2. Adsorption kinetics

The concentration of the enzyme in the solution must be measured in order to follow the adsorption kinetics of the enzyme on the surface of the PHB film. However, the enzyme is in the ionized form in the suspension, thus it cannot be separated by HPLC. On the other hand, it contains aromatic groups, e.g. tryptophan, which absorbs at around 260 nm, thus enzyme concentration can be determined by UV spectroscopy. The spectra recorded on suspensions after different adsorption times are presented in Fig. 4.4. The maximum of the absorbance is around 250 nm instead of the usual 260 nm. However, absorbance depends on the chemical environment of the molecule as well as on the structure of the enzyme itself, which may explain the shift. The quantitative analysis of enzyme adsorption was carried out at this wavelength to improve the accuracy of the measurement.

Figure 4.4  UV spectra recorded on the product appearing in the HPLC chromatogram at t = 2.1 min retention time. Changing enzyme concentration with increasing time.
Chromatograms recorded with detection at 250 nm are presented as a function of the time of adsorption in Fig. 4.5. The figure clearly shows that the concentration of the inactive enzyme decreases with increasing time, i.e. enzyme adsorption can be followed by the approach indeed.

![Chromatogram](image)

**Figure 4.5** Changing intensity of the chromatographic peak of the enzyme with increasing adsorption time.

The amount of both the active and the inactive enzyme was determined in the suspension by the integration of chromatographic peaks and it is presented in Fig. 4.6. The amount of both enzymes decreases initially and then approaches a value representing chemical equilibrium, which depends on the type of the enzyme.

Decreasing concentration of the modified enzyme indicates that it retained its capability of absorbing on the surface of the polymer, an important observation since enzymes are very sensitive to any change in their primary structure. The difference in the equilibrium concentrations of the two enzymes results from the catalytic activity of the original enzyme. When it catalyzes the degradation reaction, the active complex decomposes; the enzyme leaves the surface and transfers into the suspension. Accordingly, catalysis results in the desorption of the enzyme from the surface and increases its concentration in the suspension. The inactive molecule remains on the surface after adsorption, thus its concentration decreases continuously in the degradation medium.
Figure 4.6  Dependence of the amount of the enzymes (peak area) in the suspension on the time of adsorption. Symbols: (□) active enzyme, (○) inactive enzyme.

Figure 4.7  Difference in the rate of adsorption of the active and the inactive enzyme. The active enzyme apparently adsorbs in smaller amounts than the inactive during the same period.
Catalytic activity changes also the apparent rate of adsorption. The concentration of active and inactive enzymes is plotted against each other in Fig. 4.7. At the beginning of the experiment the concentration of the two enzymes are the same, but later the deviation depends on the rate of the reaction/adsorption processes. The apparent rate of adsorption is smaller for the active enzyme, since it desorbs from the surface after each successful catalysis and its concentration is larger in the suspension than that of the inactive enzyme.

Since in the case of inactive enzyme molecules catalysis is not possible, they either can be adsorbed on the surface forming an enzyme-substrate complex (abbreviated as ES) or located in the aqueous media. Consequently, the number of ES complexes can be calculated by simply subtracting the measured amount of enzymes still present in the aqueous media (abbreviated as E) from their initial concentration (7 µg/ml). The two quantities, i.e. enzyme concentration in the suspension and the calculated amount of adsorbed molecules, are plotted against time in Fig. 4.8. The determination of the time dependence of the [ES] and [E] concentrations allows us the characterization of the kinetics of enzyme adsorption.

**Figure 4.8** Dependence of the concentration of the components on adsorption time. Symbols: (□) enzyme, (○) enzyme-substrate complex. The solid lines are functions predicted by the kinetic model proposed.
4.3.3. The kinetic model

Our approach is based on the Michaelis and Menten model, which has already been described in detail in chapter 3.3.2. Although this mathematical representation is quite simple, calculations based on its differential equations (Eqs. 3.2-3.5) often provide surprisingly exact results. In our case, however, the model inevitably must be modified in order to account for the heterogeneous conditions and the inactivity of the modified enzyme.

The substrates are ester groups in these experiments located at the surface of the PHB particles present in the suspension. Their number remains the same throughout the experiment, since the enzyme adsorbed on the surface cannot catalyze their hydrolysis. Consequently, the geometry of the suspended polymer particles does not change over time either. Since the modified enzyme cannot catalyze the hydrolysis of PHB, the rate of product formation is zero and also the concentration of the product is zero throughout the entire measurement. Taking into account these two modifications and considering that the concentration of two of the components, the substrate and the product, is independent of time, the equations introduced in chapter 3.3.2 become much simpler

\[
\frac{d[E](t)}{dt} = -k_f'[E](t) + k_r[ES](t) \\
\frac{d[ES](t)}{dt} = +k_f'[E](t) - k_r[ES](t)
\]

(4.1)

(4.2)

where the original rate constant for the formation of the ES complex \(k_f\) is merged with the constant concentration of the substrate \(S_0\) to give the modified constant, \(k_f'\).

Eqs. 4.1 and 4.2 form a linear, homogeneous, first order, autonomous differential equation system, which can be solved analytically. The solution takes the following form

\[
[E](t) = C_1 \frac{k_r}{k_f'} - C_2 e^{-(k_r+k_f')t} \\
[ES](t) = C_1 + C_2 e^{-(k_r+k_f')t}
\]

(4.3)

(4.4)

where \(C_1\) and \(C_2\) are integration constants the value of which can be determined by the fitting of the equations to the experimental data, while \(k_f'\) and \(k_r\) are constants expressing the rate of adsorption and desorption, respectively. Fitting was done by the nonlinear iterative Levenberg-Marquardt algorithm and the functions obtained are indicated by solid lines in Fig. 4.8. The agreement between measurements and calculations is excellent, thus the kinetics of adsorption and desorption of the enzyme on the substrate can be described with the model with acceptable accuracy.

The rate constant \(k_f'\) and \(k_r\) can be calculated from the integration constants, \(C_1\) and \(C_2\) and from the initial and boundary conditions. At \(t = 0\) the concentration of the enzyme was 7 µg/ml, while that of the ES complex zero. At \(t = \infty\) the system is in dynamic equilibrium and the values can be obtained by extrapolation from the experimental data. \([E](t_\infty) = 2.29\) and \([ES](t_\infty) = 4.71\) µg/ml, respectively, were obtained for their value. Considering that at \(t = \infty\), the exponential terms are infinitely small in Eqs. 4.3 and 4.4,
we obtain the values of 4.71 µg/ml and -4.71 µg/ml, respectively, for \( C_1 \) and \( C_2 \). The knowledge of the integration constants allows us the calculation of the rate constants \( k_f' \) and \( k_r \), for which 0.0519 l/min and 0.0253 l/min, respectively, were obtained. The relation between these values, the rate of the adsorption being more than twice as large as the rate of the desorption shows that the chemical equilibrium is shifted towards adsorption. Adsorption is obviously thermodynamically favorable, thus in the state of equilibrium the majority of the enzyme molecules are adsorbed on the surface of the polymer. Although the values of \( k_f' \) and \( k_r \) obtained apply exclusively to our conditions, enzyme and substrate, the approach can be generalized to other similar systems.

One must emphasize here that the rate of adsorption and desorption of an enzyme on a polyester substrate has never been determined before, thus we cannot compare the obtained values to published data. On the other hand, the rate of catalysis, i.e. \( k_c \) values have been measured and published before (see Cornish [25], Cook [26], or Bisswanger [27]). The analysis of published data shows that the enzymes with the fastest catalysis rates falling in the range of \( k_c = 10^1\text{-}10^6 \) l/min are the metabolizers, followed by the restriction enzymes with a rate not significantly smaller than \( k_c = 10^1 \) l/min. The slowest restriction enzymes are generally operating at a rate of \( 10^{-3} \) l/min. On this virtual scale, the slowest catalysts are the ligases attaching two molecules or groups to each other. Their rate is slower than the value of \( 10^{-3} \) l/min mentioned above, and covers the range of \( 10^{-3} - 10^0 \) l/min [25-27]. The knowledge of the rate constant of the various enzymes offers the possibility to compare our calculated values directly to those published in the literature. The rate of adsorption of the enzyme natively produced by the strain *Bacillus megaterium* is significantly slower than the catalysis of the metabolizers, generally operating at a rate of \( 10^1\text{-}10^6 \) l/min. Thus, we can say that the adsorption of our enzyme occurring at the calculated rate of 0.0519 l/min is certainly not instantaneous; in fact, it is the rate-determining step in the enzymatic degradation of PHB.

### 4.3.4. Surface need of an enzyme molecule

The amount of enzyme molecules located on the surface of the PHB particles is given by the difference in their concentration at equilibrium (see Fig. 4.8) and at the start of the experiment (7 µg/ml). The surface area available for adsorption can be calculated from the number and the geometry of PHB particles present in the aqueous phase. As the latter would be difficult to obtain for individual particles, their shape is approximated with that of a sphere. The size distribution of the particles presented in Fig. 4.3 can be converted into surface as shown in Fig. 4.9.

The conversion of the size distribution into surface distribution and the integration of the function leads to the cumulated surface area of the particles present in the suspension. If the surface is completely covered by the enzyme, the surface need of one molecule can be calculated as the ratio of the area \((1.04\cdot10^{17} \text{ nm}^2)\) and the total number of molecules adsorbed \((7.95\cdot10^{15})\). Accordingly, the surface occupied by one enzyme molecule is 13.1 nm².
Figure 4.9  Differential and cumulative surface area distributions of PHB particles suspended in the adsorption medium. Symbols: (solid) differential curve, (dashed) integral curve.

The surface need of the PHB depolymerase enzyme natively synthesized by *Bacillus megaterium* has not been published yet, but information exists for other, similar enzymes. According to Kasuya [28], for example, the extracellular depolymerase enzyme of *Alcaligenes faecalis* occupies a surface of $17 \pm 8 \text{ nm}^2$, which is in the same range as the value obtained by us. The deviation might be explained by the fact that the catalytic domain of the enzyme applied by the Japanese researchers was not inactivated prior the measurement, thus their sample was far from complete coverage. The other factor, which must be taken into account, is the difference in the composition and structure of the enzymes. Even if prokaryotic polyhydroxyalkanoate depolymerase enzymes are often of a similar size, the radius of the globule determined by the secondary and tertiary structure of the polypeptide chain varies from enzyme to enzyme, and thus the areas occupied by them on the surface are also different.

4.4. Conclusions

The enzyme catalyzed degradation of poly(3-hydroxybutyrate) is a two-step process consisting of the adsorption of the enzyme on the surface of the PHB film and the cleavage of ester bonds. A deactivated enzyme was prepared by point mutagenesis to separate the two steps from each other in order and study enzyme adsorption independently of catalysis. Measurements carried out with active and inactive enzymes proved that mutagenesis was successful and the modified enzyme did not catalyze
Adsorption of enzyme molecules

degradation. Based on the Michaelis-Menten approach, a kinetic model was proposed which could describe the processes quantitatively; the agreement between prediction and the measured data was excellent. The separation of the two processes allowed the determination of the kinetics of the adsorption of the enzyme. The rate constants of the adsorption and desorption process were determined for the first time. The comparison of these constant to reaction rates showed that adsorption is not instantaneous and it is the rate-determining step in the enzymatic degradation of PHB. The results obtained allowed the determination of the area occupied by one enzyme molecule to be 13.1 nm². The separation of the two steps makes possible prediction and the control of the degradation process.

4.5. References

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Chapter 5

Quantitative determination of release kinetics from fibrous poly(3-hydroxybutyrate) scaffolds

5.1. Introduction

In the previous chapters, we have thoroughly characterized the decomposition of PHB, but the possibilities inherent to the biodegradability and biocompatibility of this material have not been covered yet. Due to these beneficial features, microbial polyesters are especially suitable for biomedical applications [1-7], among which scaffolds are gaining more and more significance [8-10]. A scaffold, however, is often used to entrap and deliver certain drugs, thus the profound characterization of such a carrier matrix inevitably requires the quantitative analysis and kinetic characterization of the release as well [11-26].

While each of the articles referenced here as [11-26] contain mathematical approaches for the description of the release of an arbitrary drug, they are either too complex to be applied for a routine determination of kinetic parameters, or too simplistic to be able to provide reliable prediction over a wider timescale. In this work, we introduce a novel approach which combines the advantageous features of the currently available models. The approach applies a computational technique that is simultaneously based on the principles of physical chemistry, and provides an accurate estimation over the entire time interval studied.

5.2. Experimental

5.2.1 Materials

The spinning of the fibrous matrix required PHB, chloroform and ethanol, amongst which the first two were obtained by a purchase of the same products that have already been described in chapter 2.2.1. Denatured ethanol was supplied by Molar Chemicals Ltd., with a purity of 96 %. The model drug used in the experiments was quercetin and it was purchased from Sigma-Aldrich as a product of ≥95 % purity.

5.2.2 Preparation of poly(3-hydroxybutyrate) fibers

The poly(3-hydroxybutyrate) fibers were prepared by wet spinning from chloroform solutions of 8 m/m%. The polymer and the solvent (chloroform) were placed

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4Polyák, P., Bartha, K., Benke, H.C., Pukánszky, B. *in preparation*
into a glass flask in an amount to form a 5 m/m% solution. The solvent was then refluxed at its boiling point (approximately 62 °C) for 8 hours to form an opalescent solution with oil-like consistency. In order to remove the contamination from the solution, it was cooled down and then let standing for 48 hours, during which inhomogeneities separated as supernatant onto the top of the chloroform phase. The supernatant containing the insoluble contaminations was then separated from the homogeneous solution. Subsequently, the solution was placed into a bath heated to 40 °C until the concentration of the solution reached the desired value of 8 m/m%. Evaporation took a relatively short time, about 2 hours.

The solution prepared was filled into a syringe of 10 milliliters volume and then put into a syringe pump. The rate of spinning was 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10 and 0.11 ml/min, respectively. The stream of polymer solution was led into ethanol of 96% purity to precipitate the fibers. The precipitated fibers were removed from the precipitating medium, washed, dried and conditioned in a room of constant temperature (25 °C) and relative humidity (50 %) for 24 hours.

The concentration of quercetin was 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/ml in the chloroform solution containing the polymer. Although chloroform is an excellent solvent of quercetin, its homogenization required 3 hours and continuous stirring at 300 rpm because of the rather large amount of polymer already present in the solution.

Since ethanol is also a relatively good solvent of quercetin, fibers could not be precipitated in it without losing some of the active component during spinning. In order to prevent the diffusion of quercetin into the precipitating media, the ethanol phase contained quercetin as well. The concentration of quercetin in the precipitating ethanol was selected to provide approximately equal chemical potential of quercetin in the polymer and the ethanol phases. The concentration to achieve this was determined by a preliminary experiment. A solvent-cast PHB film of 0.1 x 8 x 12 cm dimensions containing quercetin was placed into 100 ml ethanol. The thermodynamic system was let to reach equilibrium, which took 30 min. The initial concentration of quercetin in the film was 25 mg/g. The determination of quercetin in the film and the solution at equilibrium allowed the calculation of its relative solubility in the two phases. The ratio of concentrations was approximately 1:10, thus the quercetin concentration of the ethanol used for the precipitation of PHB fibers was always set ten times larger than that of the polymer.

5.2.3 Dissolution experiments

20 mg of polymer fibers were placed into a flask containing 100 ml of ethanol stirred continuously at 50 rpm. The amount of quercetin dissolved into the ethanol phase was determined by UV-Vis spectrophotometry (the device used was a Unicam UV-500 type spectrophotometer). Quercetin has a strong absorption at 375 nm, thus after calibration the monitoring of the intensity of this peak allowed the determination of concentration. In order to track the time dependence of dissolution as well, samples were recursively taken from the dissolution media after every 5 minutes.
5.3. Results and discussion

The results are discussed in several sections. The characteristics of the fibers important for the calculations are described first, and then release kinetics is presented subsequently. Quantitative analysis and modeling is followed by the comparison of the experimental results to prediction, while the determination of the diffusion coefficient is presented in the next section. The proposed and published models are discussed in the last part of the paper together with comments on practical relevance.

5.3.1. Fiber characteristics

As shown by Fig. 5.1, the spinning method described in the experimental section yields fibers with a rather narrow distribution of diameters.

Figure 5.1  Digital optical micrograph of PHB fibers produced by wet spinning at 0.08 ml/min spinning rate.

This feature of the technology is extremely advantageous for the modeling and evaluation of the results. A further advantage of the technique is the close correlation between the rate of spinning and the diameter of the fibers. The correlation is presented in Fig. 5.2 and shows that the diameter of the fibers depends linearly on spinning rate.
**Figure 5.2** Average diameter of wet spun fibers plotted against the rate of spinning. Symbols: (○) neat PHB, (□) PHB fibers containing entrapped quercetin molecules.

This relationship can be used for the control of fiber thickness, which, as we will see later, determines the kinetics of drug release. We must also note that electrospinning, the most widely used technique for the preparation of fibers and fibrous scaffolds, is difficult to apply for our system, since the dipole moment of chloroform is too small to form a Taylor cone. In our case, the dielectric characteristics of the solvent do not play any role.

### 5.3.2. Release kinetics

A series of UV-Vis spectra recorded during the release of the drug from the fibers is presented in **Fig. 5.3**. The model drug, quercetin, has only one prominent absorption peak in the visible wavelength range, which has a maximum at 375 nm. **Fig. 5.3** clearly proves the presence of quercetin in the dissolution medium at continuously increasing concentrations. It is much easier to follow the change of concentrations, if peak maximums are plotted against time as shown in **Fig. 5.4**.
**Figure 5.3** UV-vis spectra of the dissolution media containing a PHB scaffold of an average diameter of 91.5 µm. The spectra were recorded at increasing times. Initial quercetin content: 0.6 mg/ml.

**Figure 5.4** Time dependence of the intensity of the characteristic absorbance of quercetin in the dissolution medium. Effect of fiber diameter. Symbols: (△) 68.0, (○) 77.8, (□) 91.5 µm.
Only the results of three of the ten series of measurements are plotted in the figure in order to avoid confusion and facilitate interpretation. The figure clearly demonstrates the saturation-like tendency of dissolution and also the effect of fiber diameter on the rate of drug release, i.e. the thinner is the fiber, the faster is the diffusion of the active compound into the surrounding medium. The close correlation between the thickness of the fiber and release rate allows us to control and adjust the release characteristics of the fiber to the intended application.

Release rate can be characterized quantitatively in several ways. One could apply one of the models presented in the introductory part and use its time constant for characterization. Since one of the goals of our paper is to propose a new approach for the quantitative characterization of release kinetics, we simply introduce a characteristic time instead, the time, which is necessary for 95 % of the drug to diffuse into the surrounding medium. This specific time, $t_{95}$, is plotted against the average diameter of the fibers in Fig. 5.5.

![Graph](image)

**Figure 5.5** Effect of the average diameter of the fibers on the time required to reach 95 % of the amount of quercetin to dissolve into the surrounding medium ($t_{95}$).
The figure clearly proves that our spinning method can be used for the control of release rate. In the case of slow release, thick fibers must be prepared, while fibers with small diameter should be prepared when fast release is required. The correlation presented in Fig. 5.5 would allow the adjustment of release rate with an acceptable accuracy. The thickness of the fibers can be controlled by the modification of the rate of spinning as demonstrated in Fig. 5.2.

5.3.3. Quantitative analysis, modeling

As mentioned above, the quantitative analysis of the experimental results, the determination of a time constant and the diffusion coefficient, requires a model. This could be one of the simplified models introduced in the experimental part, but we propose a more exact approach. Our model is based on the partial differential equation of Fick’s second law that describes the case of cylindrically symmetric diffusion (Eq. 5.1), i.e.

\[
\frac{\partial}{\partial t}[c(r, t)] = D \left[ \frac{\partial^2}{\partial r^2}[c(r, t)] + \frac{1}{r} \frac{\partial}{\partial r}[c(r, t)] \right]
\] (5.1)

Functions that satisfy this partial differential equation contain one dependent and two independent variables. Since the shape of the fibers produced by the spinning technique used is cylindrical and their thickness is practically identical, the results obtained are very suitable for the analysis.

The first step of the calculations is the setting of the initial and boundary conditions of the experimental setup. Since the precipitating medium contains the necessary amount of quercetin, molecular diffusion is prevented through the surface of the fiber during its spinning. Accordingly, the concentration of quercetin has the same value everywhere inside the polymer phase, while it is zero outside the fiber, as the dissolution medium does not contain any quercetin molecule at the start of the experiment. The boundary condition is defined by the relative amount of the fiber and the dissolution medium. At the beginning of the release study, the concentration of the drug is obviously zero at the boundaries of the fiber, in the solvent. Later on, however, the concentration of quercetin increases, but because of the large volume of the ethanol and the small amount of fiber used, it can be regarded zero throughout the entire experiment.

Eq. 5.1 cannot be solved analytically, but only by numerical methods. A software was written in the development environment of Origin 9.0, which uses the forward Euler method for calculation. The solution obtained for the space and time dependence of concentration, i.e. the \(c(r,t)\) surface shown in Fig. 5.6. In order to facilitate interpretation even further, the dependence of the concentration of the drug on the independent variables, i.e. the surface function \(c(r,t)\) shown in Fig. 5.6, is presented in a two-dimensional plot in Fig. 5.7.
Figure 5.6  Three-dimensional representation of the c(r,t) function satisfying the differential equation of cylindrically symmetric diffusion (Eq. 5.1).

At the beginning of the experiment, the concentration is at its maximum along the entire diameter and equals zero in the dissolution medium. Consequently, a vertical line appears at the polymer-ethanol interface, at ±25 µm, as the calculation example considers a fiber of 50 µm thickness.

Further concentration profiles belonging to time coordinates t > 0 outline the expected tendency, the drug dissolves first from the outer regions of the fiber, then concentration starts to decrease even in the very center of the cross-section and the decrease proceeds until drug remains in the polymer. Theoretically, not every quercetin molecule is expected to leave the fiber, the concentration in equilibrium is determined by the ratio of the chemical potentials of quercetin in ethanol and PHB, respectively. However, the volume of the dissolution medium is so large compared to the total volume of the fiber that the amount of drug present in the polyester phase in thermodynamic equilibrium is negligible and the concentration profiles of Fig. 5.7 converge towards a horizontal line located at the abscissa.
5.3.4. Comparison to experiments

In order to compare the experimental results to those of the simulation, the amount of quercetin dissolved in the surrounding solution must be calculated from the profiles presented in Figs. 5.6 and 5.7. First, we calculate the total amount of quercetin that was present in the fiber at the beginning of the dissolution experiment, i.e. \( c_{t=0}(r) \), by the integration of the concentration profile at \( t = 0 \)

\[
A_{t=0} = \int_{-R}^{+R} c_{t=0}(r) \, dr \tag{5.2}
\]

The concentration of quercetin present in the fiber can be calculated in a similar way at any time \( t > 0 \). The amount of drug dissolved can be calculated by subtraction, i.e.

\[
A_{diff} = \int_{-R}^{+R} c_{t=0}(r) \, dr - \int_{-R}^{+R} c_{t>0}(r) \, dr \tag{5.3}
\]

The graphical representation of the calculation is shown in Fig. 5.8.
Figure 5.8  A graphical demonstration of the determination of the amount of dissolved drug through the calculation of integrals.

The figure shows the integration of concentration at a specific moment, but integrals must be calculated for the entire time interval of the experiment. This has been done and thus the amount of the model drug can be calculated now in both the film and the solution.

In order to calculate the amount of drug dissolved from the scaffold at any given time, we need the volume of the fibers, which is given as

\[ V = HA = H \pi r^2 \]  \hspace{1cm} (5.4)

where \( A \) is the cross-section, \( H \) the length and \( r \) the radius of the fiber. The mass of the drug is obtained by the multiplication of the mass of the polymer and the concentration of the drug, i.e.

\[ m_q = \rho_{PHB} H \pi r^2 c_q \]  \hspace{1cm} (5.5)

where \( \rho_{PHB} \) is the density of the polymer and \( c_q \) is the concentration of the drug. We must take into account the fact that \( c_q \) depends on the spatial coordinate \( (r) \), thus we have to rewrite Eq. 5.5 using the integrals of Eq. 5.3. Accordingly, we obtain
Application of PHB as a carrier matrix

\[ m_q = \rho_{PHB} H \pi \left[ \int_0^R c_{t=0}(r) \, dr^2 - \int_0^R c_{t>0}(r) \, dr^2 \right] \] (5.6)

which allows the calculation of the mass of the drug present in the dissolution medium at any \( t > 0 \) time.

The calculation of the integrals makes possible the comparison of the results of the measurements to prediction. The density of the polymer is 1.25 g/cm\(^3\). The length of the fiber \((H)\) was calculated from the mass of the sample and the diameter of the fibers obtained from optical micrographs (see Fig. 5.1). The comparison of the experimental results and prediction can be done only numerically, since no explicit solution exists for Fick's second law in our case. An algorithm was written in the development environment of Origin 9.0 to carry out the operation. Based on our results presented in Chapter 6, an initial value was selected for the diffusion coefficient. Then the algorithm compared the calculated data to the measured values and determined the difference in the sum of squares of the two sets. Iteration was stopped when the difference between two consecutive cycles was smaller than 0.05 percent of the absolute value of the sum of squares computed in the previous cycle. The results of the calculations is seen in Fig. 5.4, in which the solid lines were calculated by using the approach described above. The close correlation between the data obtained experimentally and by computation validates our method and proves that release kinetics can be quantitatively characterized without introducing any simplified or entirely empirical models.

5.3.5. Diffusion coefficient

The approach has the further benefit that it yields also the diffusion coefficient. The value of the diffusion coefficient obtained from the calculations was 8.6x10\(^{-8}\) cm\(^2\)/s, which compares well with the value of 3.5x10\(^{-8}\) cm\(^2\)/s obtained by a different method (see Chapter 6). The diffusion coefficient was determined for fibers of 50 \( \mu \)m thickness. Most probably, the difference of the two values was caused by a systematic error resulting from the large surface to volume ratio of the fibers used in this study. With decreasing fiber diameter, the bias would increase even further.

The bias is caused by the fact that in the thin fibers, quercetin molecules have to travel a relatively short way inside the polymer phase and they reach the surface of the fiber rather fast. The absolute concentration of quercetin in the liquid phase remains very small throughout the entire measurement. Accordingly, the chemical potential of quercetin is especially high in PHB and relatively low in ethanol and thus the driving force, which initiates and maintains a flux of quercetin through the boundary phase is significantly faster than the diffusion inside the polymer bulk. Since in the case of carriers with a large specific surface area the thickness of the bulk and that of the boundary phase are similar, the bias related to the thermodynamically driven phase transport is inevitably significant. Consequently, although the method presented in this work allows the determination of the diffusion coefficient, we recommend the application of a different, more reliable technique instead (see Chapter 6).
5.3.6. Comparison of models

The capability of our approach to provide accurate description of the release kinetics of a drug from a fibrous scaffold has been proven above and demonstrated amply in Fig. 5.4. We can further validate the approach if we compare it to models published in the literature. Such a comparison could point out the advantages and drawbacks of the various approaches and indicate the weakness of empirical or semi-empirical models. It also points out the time range, in which the various models reasonably describe experimental results or on the contrary, the range in which they commit the largest error.

First, we compare our exact solution to models, which describe time dependence with an exponent of 0.5 (see Chapter 1). The two approaches are compared in Fig. 5.9.

![Figure 5.9](image)

**Figure 5.9** Comparison of the mathematically exact solution (solid) to models based on the square root of time (dashed).

The square root function is obviously not a bad choice when one wants to describe only the beginning of the dissolution process. However, **Fig. 5.9** also points out the weakness of this function showing that the deviation becomes larger with increasing time. Accordingly, this approach first introduced by Higuchi [20-22] can be used only at short times with any hope for accuracy.

Unlike the above mentioned approach of Higuchi, simple exponential models are more reliable towards the end of the dissolution experiment, at very long times, and
they fail to provide accurate approximation around the origin. The solutions are compared in Fig. 5.10, which shows that both curves converge towards the same value.

![Graph comparing solutions](image)

**Figure 5.10** *Comparison of the mathematically exact solution (solid) to models based on exponential functions (dashed).*

The dashed curve is a plot of a simple exponential function of the form $y=A[1-\exp(\tau t)]$ used e.g. by Miguel [14] or Iordanskii [15]. Accordingly, even the most simplistic exponential model gives exact prediction at infinite time. At the beginning of the dissolution process, however, this exponential function provides an inaccurate estimation of the actual values, which is admitted also by the authors of this model [15].

### 5.4. Conclusions

A fibrous scaffold was prepared from PHB by wet spinning. The advantage of the technique is that fibers have a regular, cylindrical shape and the diameters of the fibers can be modified by the rate of spinning. The fibers contained a model drug and the kinetics of its release could be controlled by the adjustment of fiber diameter. A novel approach is proposed in the paper for the description of release kinetics, which is based on Fick's laws, and does not involve any simplification or the introduction of empirical constants. The comparison of prediction to experimental results showed good agreement
and proved that the approach offers an exact description of experimental data recorded during the dissolution of a drug from a fibrous scaffold. Although the method allows the estimation of the diffusion coefficient as well, the value obtained is biased because of the large surface to volume ratio of the scaffold. The comparison of the proposed model to empirical or semi-empirical approaches published earlier showed that the latter are less accurate and predict false results at various stages of the release study. The proposed method of fiber preparation and the model makes possible the prediction and control of the release rate of a drug from fibrous scaffolds.

5.5. References

Chapter 6

A novel method for the determination of diffusion coefficients in amorphous poly(3-hydroxybutyrate)\textsuperscript{5}

6.1. Introduction

As shown in Chapter 3 and 4, the degradation of PHB could be carried out in a controlled manner even under conditions corresponding to those of the human body. This beneficial feature of PHB (and in general, of the family of microbial polyesters) opens up new possibilities, e.g. its \textit{in vivo} application [1-9]. Furthermore, PHB can be used also as carrier matrix, as proven by the previous chapter of this thesis, and by a vast number of publications as well [10-15].

In the case of actual \textit{in vivo} applications, however, one inevitably must thoroughly understand both the behavior of the carrier matrix and the kinetics of the dissolution, otherwise the time dependence of the release cannot be predicted, thus the application of such carrier matrices is not safe. As scientists working in the field of controlled release have gathered considerable amount of knowledge and experience on this topic over the past decades, the literature contains numerous techniques aiming at the determination of kinetic parameters that can be used for the quantitative description of the dissolution [16-29].

One of the most important among these parameters is the diffusion coefficient, which can be determined by using a number of methodologies, yet each of these is either cumbersome to implement, or not sufficiently accurate to provide reliable data. As the determination of this parameter was especially important for us, we decided not to use any of the above referenced techniques, but to develop an entirely new approach instead.

6.2. Experimental

6.2.1. Materials

To a preparation of the amorphous carrier films, PHB, and chloroform was used, both of which purchased as a product described in chapter 2.2.1. Ethanol, applied during the dissolution studies was obtained in the same form as described in chapter 5.2.1 The drug applied as model compound for the determination of diffusion coefficient was quercetin, [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4\textit{H}-chromen-4-one], a natural antioxidant found in several fruits and vegetables, and it was purchased from Sigma-Aldrich with ≥95 \% purity.

6.2.2. **Film preparation**

Films were cast onto a glass surface from 3 m/m% chloroform solution of poly(3-hydroxybutyrate). The solution contained quercetin at the concentration of 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml (mg quercetin/ml solution). Subsequently, the films were stored at constant temperature (25 °C) and relative humidity (50 %). The diffusion measurements were done on rectangular strips of 40 x 100 mm dimensions cut from the films.

6.2.3 **Diffusion experiments**

100 ml ethanol was poured into a 500 ml Erlenmeyer flask and then the 40 mm long edge of the film was submerged into it. The other end of the film was fastened at the top of the flask with a paper clamp to keep the film in vertical, upright position throughout the entire measurement. A photo was taken from the film just before dipping its end into the solvent and then further photos were recorded in every 60 minutes over a period of 7 hours. Each film was photographed eight times to capture the temporary state of the concentration gradient along the 10 cm length of the studied PHB film. In order to minimize the influence of systematic and stochastic errors inherent to the nature of the approach, each film was placed in front of the same white sheet in a room having constant lighting conditions.

6.2.4 **Data processing**

The photographs taken from the films were cut to size in order to ensure that each pixel of the image corresponds to a discrete point on the film. The images were then resized to 400 x 160 pixel resolution. A software was developed in MATLAB environment, which converts the hexadecimal color codes of the bitmaps to 400 x 160 x 3 multidimensional matrices containing the RGB (red, green and blue channel) values of the picture. From the RGB values, the software calculates the HSL (hue, saturation and luminosity) values as well. The saturation parameter depends linearly on the amount of quercetin in the polymer. Since the concentration and thus the color gradient develops along the 10 cm length of the film, the pixels in the other, vertical direction are assumed to have the same saturation value.

During signal processing the measured data are inevitably loaded with a stochastic noise, but the signal to noise ratio can be improved further. This is done by the averaging of saturation values along the horizontal coordinate resulting in the minimization of the bias caused by the stochastic noise. As a consequence, the 400 x 160 matrices containing the saturation parameters are reduced to 400 x 1 arrays, in which each element contains the average of 160 values.
6.3. Results and discussion

The results are discussed in several sections. The determination of concentration gradients and the quantification of primary results are presented first, followed by the discussion of release kinetics. Numerical analysis leading to the targeted diffusion coefficient is described in the next section, while the advantages and the drawbacks of the approach are discussed in the last part of the chapter.

6.3.1. Concentration gradients, quantification

The determination of the diffusion coefficient \((D)\) of a small molecular weight compound is based on the development of a concentration gradient in a film, the quantification of the gradient and the calculation of \(D\) by applying Fick's laws. The concentration gradient is visible by the naked eye, if the compound used as probe has a distinct color and discolors the polymer. Photos taken from amorphous PHB films before starting the experiment (Fig. 6.1a) and after 5 hour release (Fig. 6.1b) are shown in Fig. 6.1.

The mere observation of the films in Fig. 6.1b shows that the right (lower) edge of the film became completely transparent, while the left retained its original color, i.e. the concentration gradient hoped for develops indeed during the release experiment. However, the determination of the unknown diffusion coefficient requires an adequate quantitative analysis of the images shown above.

Color gradient was converted into concentration gradient by calibration. Films were prepared with various, known concentrations of quercetin and their color was determined to obtain a calibration curve. In order to improve the signal to noise ratio, the concentration of quercetin was set to rather large values to obtain films with an intense yellow color (Fig. 6.1). However, this approach has its drawbacks as well; at large concentrations of the absorbing molecules the concentration vs. absorbance correlation is not linear (Fig. 6.2).

Due to the nonlinear correlation between quercetin concentration and the calculated saturation values, the calibration curve was approximated with a third order (cubic) polynomial. Saturation values were converted into actual concentrations by using the parameters of the regression curve (1.872, 1.802, -0.02433, 1.092E-4 for the constant, linear, second and third order term, respectively).

Release experiments of different duration were carried out in order to obtain the time dependence of diffusion. Using the calibration correlation discussed above, all of them were converted into concentration gradients, which are presented in Fig. 6.3.
Figure 6.1  Photographs of amorphous PHB films containing 2.0 mg/g quercetin a) before the release test, b) after 5 hours.
Figure 6.2  *Calibration curve for the determination of the quercetin concentration of PHB films from their color.*

Figure 6.3  *Concentration gradients of quercetin in PHB films recorded with a time interval of one hour throughout a period of 7 hours. Initial quercetin content: 22.4 mg/g.*
The gradients clearly correspond to the expectation, the concentration value at the upper end of the film indicates the initial value of quercetin added to the film, while it is zero at the other end, at the spatial coordinate of 100 mm. Fick’s second law is applied to calculate the diffusion coefficient of quercetin in the amorphous PHB film from the time dependence of the concentration gradients shown in Fig. 6.3.

6.3.2. Release kinetics

Quercetin molecules move continuously from the upper end of the film towards the ethanol phase as long as any drug remains in the PHB film. The amount of drug entrapped in the polymer phase at a given moment can be determined from the volume integral of concentration as a function of the spatial coordinates in the following way:

$$m_q = \int_{x_0}^{x_1} \int_{y_0}^{y_1} \int_{z_0}^{z_1} c_q(x, y, z) \, dx \, dy \, dz$$

(6.1)

where \( m_q \) is the total amount of quercetin in the film, while \( c_q \) its concentration at the given location. However, in our case the cross section of the film has an approximately constant geometry along the \( x \) axis, which provides the possibility to simplify the volume integral shown above considerably, i.e.

$$m_q = A \int_{x_0}^{x_1} c_q(x) \, dx$$

(6.2)

The solution of Eq. 6.2 yields the amount of quercetin molecules present in the polymer at a given moment and its time dependence describes release kinetics quantitatively. Quercetin concentration calculated in the way presented above is plotted against time in Fig. 6.4 showing the kinetics of the diffusion of the additive in PHB.

The rate of drug release depends on the amount of drug still present in the polymer, therefore, it is faster at the beginning of the experiment and slows down with time. Diffusion is a first order process and it can be approximated by an exponential function (Eq. 6.3)

$$c_q = C_0 \left[ 1 - \exp \left( -\frac{t}{\tau} \right) \right]$$

(6.3)

where \( C_0 \) is the initial concentration of quercetin in the polymer phase, while \( \tau \) is the time constant of diffusion. Such a function was fitted to the experimental points and is shown by the continuous line in Fig. 6.4. Although Eq. 6.3 provides an acceptable approximation of the measured data, it is not an exact solution, but only highlights the global characteristics of the time dependent release of the drug. For a detailed discussion of the exact representation of dissolution, please refer to section 6.3.3.
The release kinetics of quercetin from PHB is influenced by a number of factors some depending on the physical and chemical properties of the carrier matrix, while others on the drug itself. The properties of the polymer were kept constant in this study, but we investigated the effect of the initial concentration of quercetin on the kinetics of its release from the polymer film.

The time constant of diffusion (τ) is plotted against the initial concentration of quercetin in **Fig. 6.5**. As shown by the figure, release rate depends strongly on the initial concentration of the drug, at larger initial quercetin concentration less time is required for the release of a given amount. This correlation can be attributed to and explained by the first law of Fick stating that the molar flux of a diffusing matter is determined by the concentration difference between adjacent spatial coordinates, larger difference leads to faster diffusion. However, the first law of Fick does not provide any information about the time dependence of the process, the diffusion coefficient can be determined by the fitting of Fick's second law to the quantitative results presented in **Fig. 6.3**.

**Figure 6.4** *Total amount of quercetin dissolved in the matrix polymer plotted against release time. Initial quercetin content: 1 mg/g. An exponential function was fitted to the experimental data to describe kinetics quantitatively (continuous line).*
Figure 6.5  Effect of initial quercetin concentration on its rate of release (1/h) from PHB into ethanol.

6.3.3. Diffusion coefficient

Fick’s second law is usually expressed in the form of a partial second order linear differential equation

\[
\frac{\partial}{\partial t} [c(x,t)] = D \frac{\partial^2}{\partial x^2} [c(x,t)]
\]  \hspace{1cm} (6.4)

which, at the initial and boundary conditions of our experiments, i.e. constant initial concentration profile and permanently zero concentration at one particular end of the investigated film, does not have an analytical solution. On the other hand, Eq. 6.4 can be solved numerically. In order to find an appropriate numerical solution for Eq. 6.4, a software was written in the Origin C development environment.

Since the numerical solution of Fick's second law yields a function with one dependent (concentration) and two independent (spatial coordinate, x and time, t) variables, one might present it as a surface of concentration values over the x and t (position and time) plane as shown in Fig 6.6.
Figure 6.6  *Numerical solution of Fick’s second law calculated by using the initial and boundary conditions corresponding to the release experiments used.*

The $c(x,t)$ surface presented in the figure contains all the information of a release study. On the other hand, it is rather difficult to interpret and/or understand such a three dimensional plot. As a consequence, a number of discrete concentration profiles developing at various times has been selected and they are plotted against the spatial coordinate in Fig. 6.7. The correlations plotted in the figure are very similar to those presented earlier in Fig. 6.3.

The quantitative analysis and the fitting of a function to the correlations of Fig. 6.7 requires the knowledge of the unknown diffusion coefficient. Since $D$ is not known, an iteration procedure must be applied to determine its value. Starting with an arbitrarily selected value of the diffusion coefficient, the iteration is carried out until the fitted function corresponds to the one calculated from Eq. 6.4. The result of the fitting procedure is demonstrated in Fig. 6.8 comparing the measured and calculated concentration gradient for a selected polymer film. As the figure shows, the agreement between the calculated and measured profile is excellent.
Figure 6.7  Two dimensional representation of the calculated $c(x,t)$ surface.

Figure 6.8  Fitting of the calculated concentration gradient (dashed line) onto the measured one (solid line). Initial quercetin concentration: 22.4 mg/g. Release time: 5 hours.
The fitting procedure was carried out for polymer films containing the model drug in various concentrations and the diffusion coefficient of the compound was determined in each case. The values obtained are presented as a function of the initial quercetin content of the films in Fig. 6.9.

![Graph showing diffusion coefficient vs. initial concentration](image)

**Figure 6.9** Effect of the initial concentration of quercetin on its diffusion coefficient in the PHB films used in the experiments.

Under ideal conditions the iteration should have resulted in five equal diffusion coefficients, since according to the principles of the diffusion theory it is expected to be independent of both the dependent (concentration) and the independent (spatial coordinate, time) variables. However, the calculated diffusion coefficients depend slightly on concentration, diffusion increases with increasing amount of quercetin in the films. The deviation from the expected behavior needs further considerations and explanation.

### 6.3.4. Considerations, discussion

One of the possible explanations might be related to the physical ageing of the polymer. PHB, like the majority of the family of poly(hydroxyalkanoates) and most of the aliphatic polyesters, undergoes relatively fast and extensive physical ageing. During
physical ageing a number of physical and chemical parameters of the polymer change considerably with time. One of these parameters is free volume which plays an important role in diffusion. The direct determination of free volume is difficult. Positron annihilation spectroscopy is the most frequently used technique these days [30], but both the theory behind and the measurement itself are rather complicated. On the other hand, observations related to the physical characteristics of the polymer film used for the study might give information about the effect of additive concentration on free volume and the change in the value of the diffusion coefficient with increasing concentration.

In our case, physical ageing could be followed relatively simply by the measurement of the thickness of the film as a function of time and quercetin concentration. The initial ~50 μm thickness of the film decreases below 40-45 μm in several hours, if the film does not contain any quercetin. On the other hand, the thickness of films containing the drug changes only slightly or not at all even after longer times. During physical ageing the conformation of the macromolecules approaches to equilibrium resulting in a reduction of free volume. It has been shown earlier that the physical ageing process of poly(lactic acid) was considerably modified by basically all additives introduced into it including, fillers, wood flour, glycerol, other polymers, etc. [31]. The addition of these second components led to faster cold crystallization and a decrease in the glass transition temperature of the polymer. Obviously, the additives modified the mobility of the molecules resulting in a structure closer to equilibrium. Apparently quercetin has a similar effect on the structure of PHB and physical ageing as well as free volume depends on the amount of the drug added. Increased mobility of the polymer chains results in faster diffusion and larger diffusion coefficients and in the small, but clear increase in its value as a function of quercetin content.

Another issue that must be considered is the reliability of the method proposed and that of the diffusion coefficient determined. Since the diffusion of quercetin in PHB has not been studied yet, we must compare our results to those published in the literature for other compounds. In this case, however, we must keep in mind that sample preparation and measurement conditions as well as the calculation methods were different and all influence the obtained values. The diffusion coefficient determined by us is compared to published data in Table 6.1. Since the rate of diffusion is determined by the size of the diffusing molecule and its physical-chemical properties, an approximate size and the dipole momentum of the studied materials are also included in the table.

One of the molecules the diffusion of which is quite widely investigated in PHB is water. Water is much smaller than quercetin, but it is capable of forming strong hydrogen bonds with the ester groups of the polymer. Iordanskii [18,19], Miguel [21], Sultana [32], Yoon [23] and Yang [27], all determined and published diffusion coefficients. The values are scattering in the wide range of $1.1 \cdot 10^{-11} \text{ cm}^2/\text{s} - 7.0 \cdot 10^{-8} \text{ cm}^2/\text{s}$, but most of the values are found in between $2.3 \cdot 10^{-9} \text{ cm}^2/\text{s}$ and $7.0 \cdot 10^{-8} \text{ cm}^2/\text{s}$. Taking into consideration the differences in the measurement techniques and the applied mathematical methods, the agreement among the results reported is fairly acceptable.
Table 6.1  

Comparison of the diffusion coefficients of different compounds in PHB.
Size and dipole moment are included for reference.

<table>
<thead>
<tr>
<th>Permeant</th>
<th>Size\textsuperscript{a} (Å)</th>
<th>Dipole moment (D)</th>
<th>Diffusion coefficient (cm²/s)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.63</td>
<td>1.78</td>
<td>2.3·10⁻⁹-7.0·10⁻⁸</td>
<td>26, 27, 29-31, 35</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>13.7</td>
<td>1.83</td>
<td>2.0·10⁻⁷-2.5·10⁻⁶</td>
<td>28</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>14.5</td>
<td>2.55</td>
<td>1.7·10⁻⁷-2.6·10⁻⁶</td>
<td>28</td>
</tr>
<tr>
<td>Limonene</td>
<td>8.32</td>
<td>0.44</td>
<td>2.0 ± 0.2·10⁻⁸</td>
<td>36</td>
</tr>
<tr>
<td>Quercetin</td>
<td>11.8</td>
<td>1.43</td>
<td>3.1·10⁻⁸-3.7·10⁻⁸</td>
<td>this work</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Longest edge of the smallest cuboid capable of containing each atom in the molecule calculated with MOPac (Molecular Orbital Package) version 17.0.012e.

Since one of the possible use of PHB is as carrier material in controlled release devices, the diffusion of various active molecules was also determined in this polymer. Bonartsev [20], for example, studied the diffusion of two frequently used drugs (indomethacin and dipyridamole) in PHB. Based on dissolution data, the author calculated the apparent diffusion coefficients with a method which considers only the final part of the dissolution correlation. The value of the diffusion coefficient showed considerable concentration dependence, but depended also on the average molar mass of the polymer and on the thickness of the film. The reported values of 1.7·10⁻⁷ cm²/s - 2.6·10⁻⁶ cm²/s [20], are much larger than those obtained for water [18, 19, 21-23, 27]. Sanchez-Garcia investigated the diffusion of a model drug (limonene) in PHB nanocomposites [28], but determined the diffusion coefficient also in a polymer not containing any reinforcement. The actual value reported by Sanchez-Garcia is 2.0 ± 0.2·10⁻⁸ cm²/s [28], which is rather close to the values determined by us in spite of procedural differences. The similarity of our results to published data confirms that the approach proposed in this chapter yields reasonable diffusion coefficients in a simple way, which is much less tedious than the sorption, permeation or other spectroscopy based experiments often used in practice.

6.4. Conclusions

In this work, an approach is proposed for the determination of the diffusion coefficient of certain drugs in amorphous PHB which can be a reliable alternative to the conventional permeation based measurements. If the drug absorbs in the visible wavelength range, its concentration gradient in the polymer film as well as its time dependence can be analyzed quantitatively by following changes in the color of the film.
Color can be converted into concentration with the help of adequate calibration and thus the dependence of additive concentration on space and time, i.e. the $c(x,t)$ function can be determined relatively easily. The fitting of the numerical solution of Fick’s second law onto the measured values provides directly the targeted diffusion coefficient. The comparison of diffusion coefficients obtained by the proposed novel approach to values published in the literature proved that this new method provides reliable results and requires significantly less time and effort at the same time than the conventional approaches used generally.

6.5. References

Chapter 7

A simple spectroscopic method for the determination of the release kinetics of drugs from PHB

7.1. Introduction

While the last chapter was devoted exclusively to the determination of the diffusion coefficient, a profound investigation of the possibilities inherent to the application of PHB as a carrier matrix would certainly not be complete without an analysis of the entire process of dissolution. The analysis and monitoring of the dissolution can be executed several ways, currently the most popular techniques are based on spectrophotometry [1-17].

Among others, spectrophotometry is used for the determination of enzyme activity [18-25]. In this approach, a model substrate is used, which changes its UV-VIS absorption characteristics when it is converted to a product by an enzyme molecule [26-30]. The process can be monitored by recursively recording the UV-VIS spectrum of the reaction medium.

While this method is currently limited to the field of enzymology, in this work we prove that a similar technique can be applied for the monitoring of the dissolution of an arbitrary drug. Obviously, reaction does not occur during dissolution, but the absorption spectrum changes as the drug traverses from one phase (carrier) to the other (dissolution medium). During this process, the chemical characteristics of the molecules surrounding the drug inevitably change, thus the electron cloud of the drug is distorted differently. A differently distorted electron cloud has different absorption characteristics, which is called in the literature as chromatic shift [31,32]. The use of this phenomenon leads to a novel measurement technique, that is as easy and fast to implement as a routine enzyme assay.

7.2. Experimental

7.2.1 Film preparation

PHB and chloroform were obtained as products that have been previously described in chapter 2.2.1. The polymer was dissolved in chloroform by refluxing the solution at the boiling point of the solvent (62 °C) for 4 hours. The concentration of the solution was 2 m/m%. In order to eliminate contaminations from the solution, this latter was decanted for 24 hours, during which the remnants of the fermentation process being

less denser than the solvent settled on top. Subsequently 0.2 mg/ml fuchsin (Finomvegyszer K. Sz., Hungary), used as a model drug, was dissolved by stirring the solution for approximately 10 minutes at room temperature. The solution prepared was stored in a sealed vial to prevent the evaporation of the solvent and keep concentration constant.

The solution containing both PHB and fuchsin was cast onto the surface of a glass plate, from where the chloroform evaporated almost instantaneously. The process generally required approximately 10 seconds. After the evaporation of the solvent, amorphous PHB films containing fuchsin molecules were left behind. The films were removed from the surface of the glass plate, and then later cut into rectangles of 1 x 3 cm dimensions, a size that fits the cuvette of the spectrophotometer used in the study.

7.2.2 Physical ageing

The results presented in Chapter 6 showed that the physical ageing of PHB films influences the diffusion coefficient determined. Consequently, physical ageing was monitored by the measurement of the thickness of the films in order to consider the process during evaluation. The thickness of the solvent cast films was measured every 10 minutes over an interval of 3 hours.

7.2.3 Dissolution measurements

The specimens cut from the solvent cast PHB films were placed into the cuvette of the UV-VIS spectrophotometer (Unicam UV-500) in a position to be completely perpendicular to the path of the light ray emitted by the photon source of the instrument. Furthermore, the film was positioned halfway between the front- and the backside of the cuvette in order to make sure that the film does not touch the inner surface. After the careful positioning of the film, the dissolution media (Phosphate Buffered Saline, PBS buffer) was poured into the cuvette. The parameters of the buffer, osmomolarity, ion concentrations and pH, were set to match those of the human body, i.e. NaCl (Molar Chemicals Kft.) 137 mmol/dm$^3$, KCl (Fluka GmBH.) 2.7 mmol/dm$^3$, Na$_2$HPO$_4$ (Fluka GmBH.) 10 mmol/dm$^3$, KH$_2$PO$_4$ (Fluka GmBH.) 1.8 mmol/dm$^3$. The filling of the cuvette with the dissolution medium was the starting point of the measurement, from that moment on, the instrument recorded the spectra of the dual-phase system (film, buffer) with a time interval of 2.5 minutes in the wavelength range of 400-700 nm.

Besides the primary dissolution study described above, supporting measurements were also carried out in order to make the model calculations presented in the discussion section of this chapter more reliable and accurate. The first additional measurement was the determination of the ratio of the chemical potential of fuchsin in the polymer and in the aqueous phase. PHB films containing a known amount of fuchsin were prepared in order to determine this ratio. Compared to the primary measurements, a rather large film with length and width of 12 and 8 cm, respectively, was chosen, which contained fuchsin in a concentration of 0.5 mg/ml. This film was placed into a PBS
buffer of 100 ml, and then the thermodynamic system was allowed to reach its equilibrium, i.e. we waited until the concentration of fuchsine became constant in both phases. In order to obtain information about the state of the thermodynamic system, and to determine, if the concentration of fuchsine reached a constant value, the aqueous phase was continuously sampled and quantitatively analyzed using a spectrophotometer. After reaching equilibrium, the amount of fuchsine in the polymer and in the aqueous phase, respectively, was calculated. The ratio of fuchsine in the two phases correlates with the ratio of the chemical potential of fuchsine in the two phases. We have approximated this correlation with a simple reciprocal function and calculated the unknown ratio as a -1st order power of the actually measured ratio of fuchsine amounts.

The other supporting measurement that was required for reliable and accurate calculations was the determination of the rate-determining step of the procedure. One should keep in mind that the diffusion of fuchsine from the polymer phase into the dissolution media consist of three elementary steps. The first is diffusion inside the polymer phase, the second molecular transport through the polymer-aqueous phase boundary and the third diffusion within the aqueous phase. In order to determine the relative magnitude of the rate of these steps, the single cuvette dissolution study was scaled up, and was repeated by using a PHB film with the dimensions of 12x8 cm loaded with fuchsine and a dissolution medium of 2 dm³. Both components were placed into a glass beaker of 2.5 liters, and the behavior of fuchsine was followed by visual observation. Since fuchsine has a strong color, no additional instrumentation was needed, thus the dissolution of fuchsine into the aqueous media could be followed easily and the relative rate of the three processes could be estimated.

7.3. Results and discussion

The results are presented in several sections. The primary results of the experiments, the time dependence of drug delivery are shown in the first section. This is followed by the consideration of the effect of physical ageing and that of thermodynamic factors on the dissolution of the drug. Release kinetics and diffusion are discussed next leading to the determination of the diffusion coefficient of our model compound in the polymer. In addition, some considerations of practical consequences are presented in this section.

7.3.1. Dissolution kinetics

The hypsochromic shift of the model drug selected for the study is relatively strong, thus it can be observed even with the naked eye. Depending on the medium, its presence appears either as red, or purple color. This color difference in the two supporting medium is demonstrated in Fig. 7.1. At this point, we would like to point out that although the effect of hypsochromic shift is not as strong for all chemical compounds, it is always present.
Figure 7.1  Change in the color of the fuchsine model drug upon the transfer from the polymer to the solution phase.

Figure 7.2  Visible spectrum of fuchsine recorded on an aqueous solution of 0.015 mmol/dm$^3$ concentration (solid) and on a PHB film cast from chloroform solution (dashed).
Although the chromatic shift shown above can be observed without any analytical instrumentation, absorbance spectra must be recorded for quantitative analysis. Here we must consider, though, that PHB is a crystalline polymer, and the presence of crystals would bias the results considerably. Consequently, the experiments can be done only on completely amorphous films, the preparation of which is described in the experimental section (see section 7.2.1.). The combined spectrum recorded on the aqueous solution and the polymer film, both containing the model molecule, fuchsine, is presented in Fig. 7.2, which is practically the spectral representation of the color difference observed in Fig. 7.1.

As expected, the wavelength of the peak maximum shifted towards smaller values in the aqueous solution, but also the amplitudes differ considerably in the two media. The aqueous phase contains fuchsine at a concentration of 0.015 mmol/dm$^3$, while the concentration of the drug in the polymer is 26.4 mmol/dm$^3$ fuchsine, i.e. the concentration of fuchsine is considerably larger in the amorphous film. Quite surprisingly, the intensity of absorption is much smaller in the film than in the solution, which means that the adsorption coefficient of the drug is different in the two media. In our case, this spectral characteristic is especially advantageous, since the release of the drug can be monitored quantitatively by the analysis of both the wavelength and the intensity of the absorbance peak. We must also emphasize here that the number of molecules of the drug does not change in the cuvette during the measurement; the experiment is simple and easy to carry out, it does not require any interference.

**Figure 7.3** UV-Vis spectra recorded in the experimental setup with increasing time during the dissolution experiment. Both the size and the position of the absorption peak shift.
The other characteristic of our arrangement is that the spectrophotometer records simultaneously the absorbance of the film and the buffer around it, thus the measured values are the sums of the absorbances attributed to the polymer and the aqueous phase, respectively. Spectra recorded at different times are plotted in Fig. 7.3.

According to the spectra, the location of maximum absorbance moves towards smaller wavelengths, while its intensity increases with time. Since the amount of fuchsine does not change in the cuvette, the changes in the spectra result from the diffusion of the drug from the polymer to the aqueous phase.

The two parameters, i.e. peak height, \( A(t) \), and the wavelength of the maximum, \( \lambda(t) \), are plotted against time in Fig. 7.4. We must mention here that the position of the peak maximum was determined numerically in order to increase accuracy.

![Figure 7.4](image)

**Figure 7.4** Changes in the maximum and intensity of visible absorption during a release experiment. Symbols: (□) intensity (⊙) position of the absorption peak.

According to Fig. 7.4 both parameters change exponentially with time and they approach a constant value. The fitted regression curves shown in Fig. 7.4 can be described rather well with the \( A_{epx}(-\lambda t) \) function. However, it must be pointed out that although the process may be approximated with a simple first order kinetics, its exact mathematical background is somewhat more complicated as will be shown later (see section 7.3.5.).
7.3.2. Physical ageing

Before we attempt to describe and predict the diffusion of the drug from the polymer to the aqueous phase quantitatively, we must consider several factors, which might influence the outcome of the calculations. As mentioned before, one of these factors is physical ageing, which reduces the free volume of the polymer, and thus decreases the rate of molecular transport inside the amorphous film. Macroscopically, this phenomenon results in the reduction of the apparent diffusion coefficient, even though this parameter is defined theoretically as a value depending only on the characteristics of the permeate. We followed physical ageing by the measurement of the thickness of our PHB films. The thickness of two films, one without and one with drug incorporated in it, is plotted against time in Fig. 7.5. Relative thicknesses are plotted in the figure in order to facilitate comparison. The figure clearly shows that physical ageing takes place indeed and it changes the dimensions of the films used for the study of drug release considerably.

![Graph showing changes in relative thickness of PHB films as an effect of physical ageing.](image)

**Figure 7.5** Changes in the relative thickness of PHB films as an effect of physical ageing. Symbols: (□) film with 11.9 mg/g fuchsin, (○) neat film, no drug.

The most important question for modeling, however, is the effect of this physical ageing on diffusion, and whether this effect must be taken into account during analysis or not. Fig. 7.5 clearly demonstrates that the physical ageing of amorphous PHB is rather
fast, the process ends in a few hours. Consequently, one day after the preparation of the film loaded with the drug, its physical state is very close to equilibrium and does not affect molecular diffusion. Accordingly, physical ageing can be neglected and we do not have to consider it during modelling. **Fig. 7.5** also calls the attention to the fact that the presence of the drug does not modify the rate of physical ageing, but it changes its magnitude.

Physical ageing is smaller in the presence of the small molecular weight compound used in the study. This phenomenon might be explained by steric hindrance; the molecules of the drug hinder the movement of PHB segments and occupy space as well, which otherwise would have been filled by the polymer. As free volume is especially cumbersome to measure \[33\], this explanation remains rather tentative and needs further confirmation, but the characterization of physical ageing in amorphous polyhydroxy-alkanoates is way beyond the scope of this study.

### 7.3.3. Thermodynamic considerations, partitioning

Another issue to be considered before modeling is the concentration of the drug in the aqueous and the polymer phases in equilibrium, i.e. the partitioning of the drug between the phases. Theoretically, the concentration of the phases could be measured directly by recording the UV spectra of the film and the solution at the end of the dissolution study separately. However, the very small size of the experimental setup, i.e. 3 ml volume of the solution and 3 x 1 x 0.01 mm size of the film, would have led to considerable experimental error. Accordingly, the experiment has been scaled-up, and the chemical potential of the components was calculated from the results of a measurement in which fuchsine diffused from a film of 8 x 12 cm dimensions into a PBS buffer of 100 ml volume. The results of the quantitative analysis are shown in **Fig. 7.6**.

According to **Fig. 7.6**, the measured concentration of fuchsine is converging towards a value of 7.89 µmol/dm$^3$, from which the partitioning of the drug in the two phases can be determined. The ratio of 4210 to 1 was obtained for the PBS buffer and the PHB film, respectively, meaning that the vast majority of the drug leaves the polymer phase during the release study. Accordingly, the chemical potential of fuchsine is large in the polymer phase at the beginning of the measurement, thus its dissolution into the aqueous phase is thermodynamically favorable with a significant driving force. In thermodynamic equilibrium, only 0.024 % of the drug is located still in the carrier, the rest of it dissolves into the buffer. Partitioning and the determined ratio depend on both the chemical structure of the drug and the carrier phase. Since amorphous PHB is rather apolar, the dissolution of the rather polar fuchsine is thermodynamically favorable and it leaves the carrier.
Figure 7.6  Concentration of the aqueous phase plotted against the time of the experiment. Equilibrium concentration is 7.89 µmol/dm$^3$.

7.3.4. The diffusion process, rates

The transport of a particular molecule of fuchsine from the polymer into the aqueous phase occurs in three consecutive steps: diffusion in the polymer phase, molecular transport through the polymer-aqueous boundary, and then diffusion in the aqueous phase. Since fuchsine is a colored substance we can observe diffusion macroscopically that facilitates considerably the identification of the rate determining step. The experiment was carried out in a large sample holder with the volume of 2.5 dm$^3$. During this scaled-up macroscopic experiment, fuchsine molecules, which entered into the buffer formed a homogeneous solution instantaneously, i.e. a concentration gradient has not developed in the aqueous phase. The red color of the solution surrounding the PHB film containing fuchsine became more and more intensive with time and the color was independent of location, the dissolution medium remained homogeneous throughout the entire experiment. Accordingly, the rate-determining step is either diffusion in the polymer phase, or molecular transport through the surface of the film. However, we have proved already (see section 7.3.3.) that the dissolution of the drug is favorable thermodynamically, thus once a fuchsine molecule reaches the surface of the film, it is expected to dissolve into the buffer practically instantaneously. Consequently, the step that determines the global rate of the entire process is the diffusion of the drug within the polymer phase.
7.3.5. Release kinetics, modeling

The diffusion of a small molecular weight compound in a continuous medium can be described by the second law of Fick, which can be written in the following general form

\[
\frac{\partial}{\partial t} [c(x, t)] = D \frac{\partial^2}{\partial x^2} [c(x, t)]
\]  

(7.1)

Eq. 7.1 has an analytical solution in special cases, but the partial differential equation of Eq. 7.1 can be solved only numerically under the initial and boundary conditions of our arrangement. The initial condition is set by the concentration of the drug in the carrier film, which was 26.4 mg fuchsine/g polymer. We also assume that the distribution of fuchsine is homogeneous in the entire film. The boundary conditions, on the other hand, change with time. The limits of the spatial variable are set by the dimensions of the film. However, the concentration of the solution changes continuously and during the calculation of the time dependence of diffusion, i.e. the concentration profile within the film, we must consider this change. At the beginning of the experiment, the concentration of fuchsine is zero in the buffer. After the start of the measurement, dissolution begins immediately resulting in the appearance of growing amounts of fuchsine in the aqueous phase whose concentration converges to its equilibrium value.

In order to solve Eq. 7.1 under the conditions defined above an algorithm has been written in the Origin C Integrated Development Environment, and the \( c(x,t) \) functions were calculated with it. A three dimensional representation of this \( c(x,t) \) surface is shown in Fig. 7.7. In order to facilitate the interpretation of the profiles presented in the figure, a two dimensional projection is shown in Fig. 7.8.

The figure can be interpreted as a consecutive set of concentration gradients, each belonging to a certain time coordinate. Accordingly, the first correlation corresponds to the initial condition, i.e. the concentration of fuchsine is constant in the polymer and zero in the surrounding medium. The other concentration gradients represent the concentration distribution of fuchsine in the polymer at different times. The concentration of fuchsine increases continuously outside the film, while decreases inside until it becomes the same everywhere in the film. We must mention here that concentrations plotted on the vertical axis of Figs. 7.7 and 7.8 were corrected with the ratio of chemical potentials in order to facilitate viewing. The uncorrected values are enormously different, their ratio is 4210:1, and thus their plotting on the same graph would have been practically impossible. Because of normalization, concentrations are comparable and converge towards the same value.
Figure 7.7  Numeric solution of Fick’s second law with the initial and boundary conditions determined by the arrangement of the dissolution study.

Figure 7.8  Two dimensional representation of the calculated $c(x,t)$ surface.
The model allows also the estimation of the chemical potential of fuchsine in the two phases. At the beginning of the experiments, the chemical potential is large in the film and zero in the solvent. With increasing time, the two approaches each other and converge toward the same value at equilibrium. The time dependence of the chemical potential of fuchsine in the two phases is presented in Fig. 7.9.

![Figure 7.9](image)

**Figure 7.9**  *Time dependence of the chemical potential of fuchsine in the polymer (dashed) and the aqueous phase (solid), respectively.*

The correlations clearly show the characteristics described above, i.e. continuous decrease in the polymer and increase in the aqueous phase. The difference in chemical potential is the driving force of diffusion.

7.3.6. Comparison, diffusion coefficient

We have shown above that the dissolution process occurring in the cuvette of our experimental setup can be described adequately by the numerical solution of the second law of Fick with the appropriate initial and boundary conditions corresponding to our experimental arrangement. We could also estimate the time dependence of the chemical potential of the drug. The next step is the comparison of the prediction of the calculations to the experimental results.
Fick’s second law has a single parameter, the diffusion coefficient (see Eq. 7.1). It was determined by iteration. Based on our previous results presented in Chapter 6, we selected an initial value and started the calculations. The cumulative error of the deviation between the calculated and measured data was minimized and the calculation ended when the difference between the errors obtained in two consecutive cycles was smaller than 0.05 percent of the absolute value of the error computed in the previous cycle. The calculated correlations are compared to the measured data in Fig. 7.10. The agreement is very good thus validating our approach.

![Image of graph](image)

**Figure 7.10** Comparison of model calculations (prediction) to the experimental results. Symbols: the amount of fuchsine (□) in the aqueous and (⊙) the polymer phase; continuous line represents the calculated correlation.

The calculations yielded a diffusion coefficient with the value of $2.16 \cdot 10^{-7}$ cm$^2$/s for the diffusion of fuchsine in the amorphous PHB film used as matrix, which is considerably larger than the value determined for quercetin ($3.2 \cdot 10^{-8}$ cm$^2$/s) (see Chapter 6). The difference might result from two main reasons. The first is rather obvious; the chemical structure of fuchsine and quercetin differs considerably (Fig. 7.11).
The former is polar and has an enormous chemical potential in the polyester phase, while the latter, in spite of its hydroxyl groups, does not dissolve in water significant amounts, thus its polarity is much smaller. Besides polarity, the size of the two molecules is also different, a factor, which also influences the rate of diffusion. Apart from the structure and properties of the chemical compounds used in the experiments, also the experimental arrangements influence the diffusion coefficient obtained. In the case of quercetin, drug molecules had to pass through the entire film of 10 cm length (see Chapter 6). In the present experiment, the entire diffusion path was about 5 µm, several magnitude smaller than in the previous case. In the present case, both the bulk and the interphase are microscopic, thus the influence of the interphase and the bias resulting from it, is much more significant. Consequently, if the determination of the diffusion coefficient is the main goal of the experiments, we recommend the method developed earlier, and described in Chapter 6.

7.4. Conclusions

In this chapter, a method was proposed for the determination of the release kinetics of small molecular weight drugs from amorphous PHB. The method uses the hypsochromic shift of the absorbance of active molecules resulting in changes in the UV-Vis spectra as an effect of changing environment. We created a simple experimental setup, which makes possible the quantitative determination of dissolution in the cell of an UV-Vis spectrometer without any further interference. 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. Excellent agreement was found between the prediction and the experimental results. The approach made possible also the determination of the diffusion coefficient of the model drug in amorphous PHB. The developed method can be used in all polymers and with all drugs, which show sufficiently strong hypsochromic shift during their transfer from the polymer to the solution phase.
7.5. References

Chapter 8

Summary

One does not necessarily have to browse through the entire literature of biopolymers to come to the conclusion 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, PHB, can now be produced even on a large scale, as beside its application, its fermentation has also become an intensively studied area.

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 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.

One of the most important among these trends concerns 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.

No matter how intensively studied PHB actually is, there are still a number of factors that impede microbial polyesters from becoming a leader in the market of biopolymers. The most important of these is the fact, that 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 came to the conclusion 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 empirical data.
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 came to the conclusion 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 empirical data, 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.

Upon this conclusion, 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 mutagenesis, which allows the creation of new gene sequences, and therefore, enzyme molecules, in which one or even multiple amino acids are swapped to a different one. By implementing this technique, we created depolymerase molecules, which had their binding site left intact, but contained an entirely inactive active domain. 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 finishing the studies aiming at the characterization of the decomposition of PHB, we have turned towards more application-oriented areas, and investigated the possible use of PHB as a scaffold. Exclusively for this purpose, a new spinning method has been developed, 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 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 part of this study, we have also shown that the dissolution can be also described and predicted by solving Fick’s second law numerically to describe 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 coefficients – all this without the need of the rather obsolete instrumentation generally used for the acquisition of this data. We also showed, that the monitoring of concentration gradients in amorphous PHB films yields data that correlate closely with those obtained by other methods – a further factor that underlines the reliability of our approach.

The other technique we developed provides a fast and cost effective way to monitor entire dissolution process. As a part of this research, we found that the method of recording chromatic shifts observed on 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 hypsochromic shift which could be used for the quantitative characterization of the dissolution of the drug into the surrounding medium.

The most important conclusions of this thesis can be summarized briefly in the following main points:

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 (Chapter 2).
2. By assuming that the hydrolysis of PHB is a S\textsubscript{N}2 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 the concentration of the monomer could be predicted also in the aqueous solution. Such a model did not exist before (Chapter 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 (Chapter 3).
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 (Chapter 4).
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 (Chapter 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 (Chapter 6).

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 (Chapter 7).
List of symbols

\[ \lambda \] time constant (1/s)
\[ \rho \] density (g/cm\(^3\))
\[ \mu \] dipole moment (D)
\[ A \] pre-exponential coefficient (mol/dm\(^3\))
\[ c \] concentration (mol/dm\(^3\))
\[ D \] diffusion coefficient (cm\(^2\)/s)
\[ [E] \] concentration of the enzyme (mol/dm\(^3\))
\[ [ES] \] concentration of the enzyme-substrate complex (mol/dm\(^3\))
\[ H \] length of the fiber (mm)
\[ J \] diffusion flux (mol/m\(^2\)/s)
\[ k \] reaction rate coefficient (1/s)
\[ l \] thickness of the film (µm)
\[ m_t \] mass of the entrapped drug at \( t \) time coordinate (g)
\[ m_\infty \] mass of the entrapped drug in equilibrium (g)
\[ n \] amount of substance (mol)
\[ p \] proportionality factor (mol/dm\(^3\)/min)
\[ [P] \] concentration of the product (mol/dm\(^3\))
\[ r \] radius (µm)
\[ [S] \] concentration of the substrate (mol/dm\(^3\))
\[ t \] time coordinate (s)
\[ V \] volume (m\(^3\))
\[ x, y, z \] spatial coordinates (µm)
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>DAD</td>
<td>Diode array detector</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DOM</td>
<td>Digital optical microscopy</td>
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<tr>
<td>HIS</td>
<td>Histidine tag</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HSL</td>
<td>Hue-saturation-lightness color space</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
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<tr>
<td>MATLAB</td>
<td>Matrix laboratory</td>
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<tr>
<td>MOPAC</td>
<td>Molecular orbital package</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>NTA</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PHB</td>
<td>Poly(3-hydroxybutyrate)</td>
</tr>
<tr>
<td>PHB-HV</td>
<td>Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)</td>
</tr>
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<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PP</td>
<td>Polypropylene</td>
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<tr>
<td>RGB</td>
<td>Red-green-blue color space</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>UV-VIS</td>
<td>Ultraviolet-visible spectroscopy</td>
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</table>
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**List of publications**

**Papers used for the preparation of this thesis**


**Manuscripts**

1. Polyák, P., Bartha, K., Benke, H.C., Pukánszky, B.: Quantitative determination of release kinetics from fibrous poly(3-hydroxybutyrate) scaffolds, *in preparation*

**Other publications**


### Conference presentations


Nyilatkozat

Alulírott Polyák Péter kijelentem, hogy ezt a doktori értekezést magam készítettem és abban csak a megadott forrásokat használtam fel. Minden olyan részt, amelyet szó szerint, vagy azonos tartalomban, de átfogalmazva más forrásból átvettem, egyértelműen, a forrás megadásával megjelöltem.


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