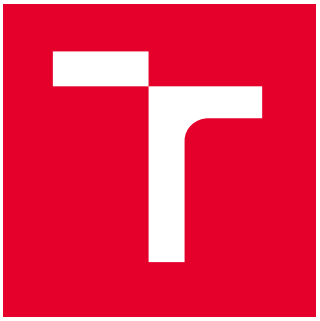

BRNO UNIVERSITY OF TECHNOLOGY

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

FACULTY OF CHEMISTRY

FAKULTA CHEMICKÁ

INSTITUTE OF PHYSICAL AND APPLIED CHEMISTRY

ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

TAILORING TRANSPORT PROPERTIES OF CONTROLLED RELEASE SYSTEMS BASED ON POLYHYDROXYALKANOATES.

MOŽNOSTI CÍLENÉ MANIPULACE TRANSPORTNÍCH VLASTNOSTÍ NOSIČOVÝCH MATERIÁLŮ NA BÁZI PHA.

MASTER'S THESIS

DIPLOMOVÁ PRÁCE

AUTHOR — **Bc. Zarina Apsalikova**

AUTOR PRÁCE

SUPERVISOR — **doc. Ing. Petr Sedláček, Ph.D.**

VEDOUCÍ PRÁCE

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Assignment Master's Thesis

Project no.:	FCH-DIP1827/2022	Academic year: 2022/23
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Title of Master's Thesis:

Tailoring transport properties of controlled release systems based on polyhydroxyalkanoates.

Master's Thesis:

1. Prepare a state–of–the–art literature search focused on: i. application of polyhydroxyalkanoates (PHA) in the field of controlled release of active substances, ii. preparation of different colloidal forms of PHA (e.g. films, gels, foams, particle dispersions).
2. Based on the literature search, propose and test various preparation procedures for PHA–based carrier systems for model active pharmaceutical ingredients.
3. Characterize the prepared carrier materials mainly in terms of their morphology and transport properties.
4. Discuss the obtained experimental results mainly from the point of view of the relationship between the chemical/physical structure and the resulting useful properties of the prepared carrier materials based on PHA.

Deadline for Master's Thesis delivery: 8.5.2023:

Master's Thesis should be submitted to the institute's secretariat in a number of copies as set by the dean This specification is part of Master's Thesis

Bc. Zarina Apsalikova student	doc. Ing. Petr Sedláček, Ph.D. Head of thesis	prof. Ing. Miloslav Pekař, CSc. Head of department

In Brno dated 1.2.2023

prof. Ing. Michal Veselý, CSc.
Dean

ABSTRACT

In this work, in order to investigate the transport properties of different types of carriers, the polyhydroxyalkanoates with 3-hydroxyvalerate, 4-hydroxyvalerate, 4-hydroxyhexanoate, and 5-hydroxyvalerate monomer units were synthesized using the bacterial strain *Aneurinibacillus* (isolates: AH30 and AFN2). Results of gas chromatography (GC-FID) showed that isolates are able to accumulate PHA with different fractions of monomers. Further, the molecular weight and polydispersity index of isolated biofilms isolated were analyzed via size exclusion chromatography (SEC-MALS). Then, the delivery systems were fabricated, using synthesized copolymers and commercial homopolymer poly(3-hydroxybutyrate). In particular, a series of optimization experiments focused on particles, films, and porous monolith preparation. The processing conditions were evaluated including polymer concentration, amount of additive, and isolation procedure. Fabricated carriers were characterized regarding their ability to incorporate and release the active agent (ibuprofen) into the model medium (phosphate saline buffer). Based on the results from characterization methods, it was found that polymer structure and polymer-solvent relationship have a significant influence on the transport behavior of the incorporated agent. Techniques, such as Scanning electron microscopy (SEM), Differential scanning calorimetry (DSC), Attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectrometry and ultraviolet-visible spectroscopy (UV-VIS) were applied as tools to compare and understand the correspondence between transport properties and chemical structure of polyhydroxyalkanoate.

Keywords: copolymer, monomer composition, controlled release system, transport property

ABSTRAKT

V rámci předložené práci, polyhydroxyalkanoáty s odlišnými monomerními jednotkami, jako 3-hydroxyvalerát, 4-hydroxyvalerát, 4-hydroxyhexanoát, a 5-hydroxyvalerát byly připravovány za využití bakteriálního kmenu *Aneurinibacillus* (AH30, AFN2) za účelem porovnání transportních vlastnosti nosičů na bázi izolovaných kopolymerů. Na základě výsledku plynové chromatografii (GC-FID) se ukázalo, izoláty jsou schopné produkovat PHA s různým procentuálním zastoupením monomerů. Dále molekulová hmotnost a index polydispezity izolovaných kopolymerů byly analyzovány pomoci SEC-MALS. Samotné nosičové systémy byly připravovány s využitím izolovaných kopolymerů a komerčního poly(3-hydroxyburyrát). Optimizace byla zaměřena na nastavení podmínek pro tvorbu částic, filmu a porézního systému. Zkoumanými podmínkami byly koncentrace roztoku, množství pomocné látky, a proces izolace nosičů. Vytvořené nosičové systémy byly charakterizovány z pohledu jejich schopnosti uvolňovat aktivní látku (ibuprofen) do modelového prostředí (fosfátový pufr). Na základě provedené charakterizaci bylo stanoveno, že transportní vlastnosti jsou ovlivněny strukturou polymeru a interakcemi v systému polymer-rozpouštědlo. Skenovací elektronová mikroskopie, diferenciální skenovací kalorimetrie, infračervená spektroskopie s Fourierovou transformaci a UV-VIS spektroskopie byly využity jako nástroje pro porovnání a pochopení korespondence mezi chemickou strukturou PHA a jejich transportními vlastnostmi.

Klíčova slova: kopolymer, monomerní složení, nosičové systémy, transportní vlastnosti

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PROHLÁŠENÍ

Prohlašuji, že jsem diplomovou práci vypracovala samostatně a že všechny použité literární zdroje jsem správně a úplně citovala. Diplomová práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího diplomové práce a děkana FCH VUT.

……

Bc. Zarina Apsalikova

Poděkování

Na tomto místě bych chtěla poděkovat vedoucímu diplomové práce doc. Ing. Petru Sedláčkovi Ph.D za odborné vedení práce, inspiraci, cenné rády a realizaci některých analýz. Dále velké poděkování patří Ing. Evě Slaninové Ph.D za pomoc při řešeni experimentální části, poskytnutí cenných konzultaci a motivaci. Dále bych poděkovala Ing. Monice Trudičové a kolektivu laboratoře 242b za jejich ochotu pomoci, příjemné chvíle v laboratoři a věnovaný čas. V neposlední řadě dekuji své rodině za jejich trpělivost a podporu během celého studia.

Contents

1 Introduction

During the past few decades, biopolymers have been widely studied regarding their potential applications in medicine. Among these polymers, polyhydroxyalkanoates have been investigated due to their biocompatibility, nontoxicity, and other beneficial properties. PHA is accumulated under stress conditions by different microorganisms. The properties of naturally synthesized PHA are given by the producing organism, carbon source, and processing approaches. Additionally, material features can be modified by using different methods, such as chemical modification (carboxylation, halogenation, or grafting), or physical modification (blending).

In this context, due to modifiable properties, polyhydroxyalkanoates are auspicious materials for various fields, not only the biomedicine. PHA may be applied in production of films, fibers, hydrogels, nano-, and micro devices. On the one hand, various forms are possibly used as pure materials or copolymers, for example, PHA-based scaffolds, sutures, and meshes. By varying the monomeric units, these devices' degradability and mechanical properties can be controlled. On the other hand, PHA films, particles, gels, etc. can be applied as carriers for active pharmaceutical ingredients.

Generally, drug delivery systems (DDS) are systems used for transporting active substances to the selected site while reducing unpleasant side effects. Thus, the major role of carrier systems is to improve the efficacy of incorporated agents. For the development of carrier systems, or drug delivery systems, it is vital to understand how the release mechanism is affected by various parameters, including those which will be discussed in this thesis. It is also quite important to consider the purpose that the carrier system is designed for. To sum up, as far as it is now well recognized that targeted localization of compounds and their controllable release into the environment can be achieved by using carrier systems, application of PHA in this field has recently become a hot spot in the biopolymers' research. Not only the variety of polyhydroxyalkanoates, but also their tunable physical and chemical properties, make them excellent candidates for applications in carrier systems.

2 Theoretical part

2.1 Active agent delivery systems

The study of recent carrier systems for medicines and active substances has attracted many researchers. Delivery control of active agents is a promising strategy to protect them during transportation, as well as to improve (pharmaco)kinetic properties, when the agent is highly hydrophobic or hydrophilic, non-bioavailable, or hardly-overcoming barriers [1]. In general, these systems consist of two basic components: carrier material and active compound. Besides the main components, stabilizers, plasticizers, or ligands are also used [2]. These additives assist in targeted localization, improvement of stability of the carrier material, or affecting the release profile.

The development of innovative active agent delivery systems is a certain trend. In this context, one part of the reviews focus on the chemical behavior of components, while others investigate the manufacturing technologies or release kinetic and transport processes. For instance, a drug release mechanism from a PLGA-based system was characterized by *Fredenberg et al.* [3]. The authors studied physicochemical processes occurring in the drug delivery systems and the influence of these processes on the release profile.

The delivery of active agents has been explored in diverse fields, such as biomedicine, application in pharmaceuticals, agriculture, intelligent packaging materials, and food industry. Thus, these delivery systems differ in terms of components, physicochemical properties, and way of utilization [3]. However, improving an active agent's efficacy and reducing adverse side effects are the key aims of the development of delivery systems [4].

2.1.1 Carriers of an active compound

There are numerous kinds of delivery systems with unique properties and specific advantages. Some of the well-known types, such as liposomes, inorganic formulations, carbon tubes, and polymer-based delivery systems, are shown in Figure 1. The choice of design depends upon the type of the intended application.

Inorganic-based delivery systems are typically used as transporter of medicines and as contrasting agent in cancer diagnosis and treatment [1]. This type of carrier has unique physical, electrical, magnetic, and optical features. Common inorganic carrier materials include gold particles, iron oxide, mesoporous silica nanoparticles, etc. [11].

As another class of drug delivery carriers, carbonaceous materials (e.g. carbon tubes, fullerenes) provide interesting mechanical, electronic, and magnetic properties [1]. Due to such properties, areas of their use include biosensors, electrode materials, nano-electronics, and, particularly in the field of delivery systems, the carriers of genetic materials, proteins, and immunoactive compounds [1].

Next type of delivery system are lipid-based (nano)particles, which are the most widely used among FDA (The Food and Drug Administration)-approved materials [11]. The lipid-base systems can load water-soluble as well as water-insoluble pharmaceutical agents. The considered systems' critical characteristics such as size, charge, and surface properties, which

can affect the release profile, can be easily modified. Thus, release patterns can be controlled and regulated based on the use of the system [1].

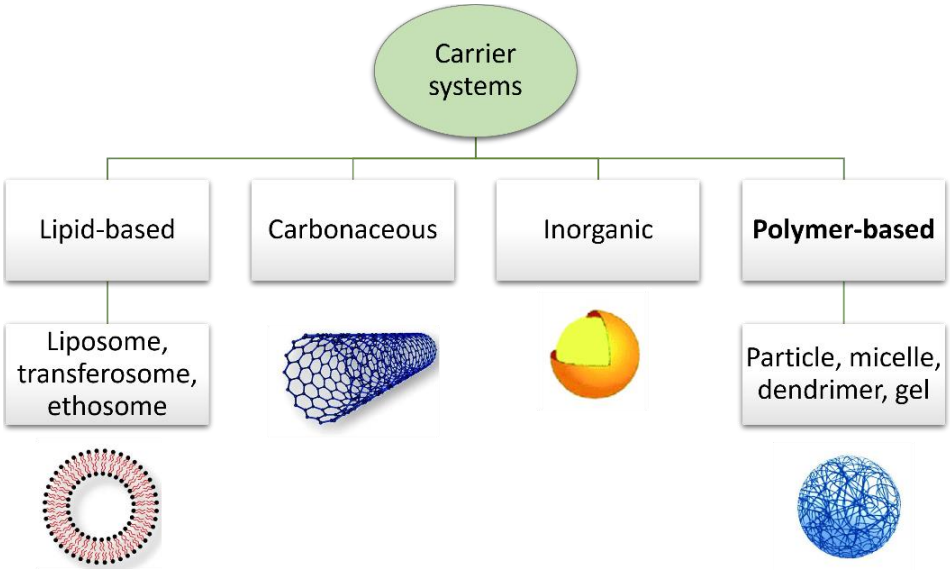


Figure 1: Different carrier systems for active agents (based on [1])

More details on specific benefits of polymeric carrier materials will be discussed in the thesis. Polymers have been intensively studied in the field of active agent delivery systems due to their feasible properties, such as controllable synthesis and processability, structural variance, and variability in physicochemical characteristics [4]. For polymer-based carrier materials, chemical and physical features of the macromolecule, such as glass transition temperature, viscosity, polymer structure, and concentration, are important factors that influence the distribution of active agent, release rate, and stability of the final delivery system [5]. However, there are key characteristics that indicate the aptness of the polymer for carrier systems, such as biocompatibility and nontoxicity Based on degradability, polymers used in drug delivery systems can be biodegradable or non-degradable [6]. Some examples of degradable and non-degradable macromolecules will be described below.

Biodegradable polymers typically have labile bonds (ester, amide, and anhydride bonds) that are susceptible to hydrolysis, forming oligomers and monomers. One of the widely used degradable macromolecules are polyesters, for example, polylactic acid (PLA). The benefits of PLA nanoparticles include low level of immunogenicity, low cytotoxicity, and good mechanical properties [6][7]. Chitosan carriers are also completely biodegradable [6]. Chitosan can be utilized as an antibacterial agent or as an adhesive. In this context, chitosan has also suitable wound-healing properties. Regarding the delivery systems, enzymes, vitamins, and drugs can be encapsulated into chitosan particles or films [1]. Gelatin, albumin, polyglycolic acid (PGA), polyhydroxyalkanoates (PHA), and other degradable polymers are also subjects of numerous investigations. In addition to previously mentioned single-purpose systems, multipurpose delivery compounds have been developed. A combination of different biodegradable carriers leads to a modification of the surface characteristics and physicochemical features [8].

Non-degradable polymers include polyurethanes, polyethylene vinyl acetate, and polydimethylsiloxane [5][6]. For example, for achieving prolonged release profiles hydrophobic drugs can be incorporated into a polydimethylsiloxane (PDMS)-based delivery device. Non-degradable polymers have been applied as transdermal films, drug delivery implant devices, or particulate carriers [6].

Several methods, including emulsification, ionic gelation, microfluidics, electrospinning, and solvent casting, are being used to create polymer-based delivery systems, and each method produces a different final product [4]. The most common types of such final product are polymeric capsules and spheres. Micelles, dendrimers, gels, suspensions, and thin layers have been successfully investigated within these two forms [10].

However, only small number of polymeric carriers are being used in clinical trials, because of the increased risk of aggregation of polymeric particulate carriers. Other types of delivery systems are also limited by several factors, such as permeability, mechanical characteristics, stability, or fast excretion [9].

2.1.2 Applications

Polymer-based delivery systems have been investigated for the incorporation of a wide range of compounds, such as therapeutic agents (antibiotics, hormones, anticancer agents), bioactive substances (essential oils, food additives), and other types of active cargoes (antioxidants, antimicrobial agents, flavors, etc.) [11]. Besides the variability of active agents, there is a diversity in carrier configurations. Different sizes and shapes, e.g., nano-, microparticles, thin films, and gels have been developed. However, there are still many obstacles to overcome, as majority of carrier systems perform well *in vitro*, but fail *in vivo* due to environmental complexity.

Poly(lactic-co-glycolic) acid microspheres containing leuprolide (*Lupron Depot*) is one of the first commercially successful peptide carrier system, whose release rate can be controlled [12]. Another type of drug delivery system are transdermal patches. Commercially available fentanyl patches are applied for chronic pain control [12]. According to the review, fentanyl-based patches (*Duragesic*) have two advantages: controlled release of active substance and self-administrated dosage [12].

Many other drug delivery systems were approved by the Food and Drug Administration (FDA), for instance, *Estraderm, Nitro-Dur, Vivitrol, Doxil*, and others [12]. Several publications focus on delivery systems and commercialization process of these systems, particularly of nanoscale drug delivery (see [10]) and active packaging materials [13]. The main challenges and future development of the delivery systems were also reviewed. Authors highlighted, in order to apply clinical research into practice, that a delivery system must, at minimum, be safe, perform its functionality, be easy to administrate and simple to manufacture [10].

2.2 Factors affecting transport process

Revealing the release mechanism and understanding factors that influence the release profile is essential for the successful development of a carrier system. Based on the release mechanism from the polymer-based carrier, the main groups include diffusion-controlled systems, solvent-

activated systems, erosion (biodegradable) systems, and externally-triggered systems [14]. Generally, in all mentioned systems, one key process (mass transport) is always involved.

2.2.1 Transport Phenomenon

Depending on the delivery system, several processes affect transport and release kinetics. Examples are convection processes, adsorption/desorption, crystallization or drug dissolution, creation of pores, etc. It is not rational to consider all these processes at once, as these combined phenomena lead to complicated mathematical models. Therefore, the solution to such problem is to identify the dominating process [16]. The fundamental transport phenomenon which occurs in the carrier systems during drug distribution is diffusion [14].

Diffusion is defined as a mass transfer of individual molecules of substance from one point to another. Because of the spontaneous flux of matter, the concentration difference between two regions is reduced. This spontaneous movement and collisions between molecules that stand behind this flux are caused by thermal motion [15].

The first laws of diffusion were introduced by the German physiologist Adolf Eugen Fick. His research made a significant contribution to diffusion theory. Fick noticed the analogy between heat transfer and molecular diffusion [15]. Thus, based on analogy, he applied a mathematical model of heat transfer for describing the flux of matter. *Fick's first law* for steady conditions is defined as equation (1)

$$J_i = -D \cdot \frac{\partial c_i}{\partial x} \tag{1}$$

where the diffusive flux J in a one-dimensional system is proportional to the gradient of concentration in the x -direction; the index "i" describes the diffusing substance (i.e., drug, ion, agent). The diffusion coefficient D depends on the parameters related to the solvent and particles [15]. The process occurs in the direction of concentration decrease; thus, the flux of molecules is positive.

For non-steady diffusion, the *second Fick's law* was derived. Equation (2) describes that the concentration as a function of time t in the determined system is proportional to the concentration gradient in the same region of the system [14][15].

$$\frac{\partial c_i}{\partial t} = D \cdot \frac{\partial^2 c_i}{\partial x^2} \tag{2}$$

Generally, the solution of equation (2) can be obtained for a variety of initial and boundary conditions. In the context of carrier systems to characterize mass transport, empirical models based on each of the conditions and mechanisms have been developed (Table 1). Empirical models are established on mathematical functions, which describe the mass transfer pattern.

Table 1: Empirical models of drug release

Model	Expression	Reference
Higuchi	$\frac{M_t}{M_\infty} = k \cdot \sqrt{t}$	[6], [14]
Ritger-Peppas	$\frac{M_t}{M_\infty} = k_t \cdot t^n$	[3], [16]
Zero-order	$\frac{M_t}{M_\infty} = k_d \cdot t$	[6], [16]

As it was discussed previously, mass transfer is affected by transport processes that occur in the system. Furthermore, other multiple factors influence mass transport, especially drug release from polymers.

2.2.2 Structure of carriers and active agents

Understanding the transport behavior and parameters affecting release mechanism are required for the development of the carrier systems. Some of these attributes will be discussed in this chapter.

2.2.2.1 Interactions

An active compound and a polymer can interact at the physical and chemical levels through van der Waals interactions, electrostatic interactions, hydrogen, and covalent bonds. Molecular interactions in the composite system have a major influence on the release behavior as well as they affect carrier formation. There are a number of publications describing the development of polymer-based delivery systems, where physical interactions play a crucial role (Table 2). In order to control the release patterns, the strength and type of interaction influence the rate of transfer of the incorporated agent. Exemplary, *Liu et al*. found that decreasing number of hydrogen bonding interactions increases the release of the drug from the transdermal patch [9].

Table 2: List of the release-controling interactions and polymer-based carrier systems

Interaction	Used polymer	Reference
Hydrogen bond	poly(ethylene oxide), poly(ethylene glycol)	[9], [17]
Electrostatic bond	chitosan	[9]
Hydrophobic effect and van der Waals	poly(ethylene glycol)-b-poly(D,L-lactide)	[18]
π -π stacking	poly(ethylene glycol)-b-(N-[2-benzoyloxypropyl] methacrylamide)	[19]

To sum up, molecular interactions are essential in the formation of self-assembled carrier systems. Furthermore, the release kinetic of an active agent from the carrier matrice is

influenced by interactions in the following way: the stronger the interaction, the slower the release rate of the active compound [9].

From different point of view, release rate of incorporated agent increases due to the movement of the polymer chain. Solid polymers have crystalline and amorphous regions in their structure. Crystalline regions, where intramolecular interactions are strong, make the polymer material compact and tough. Consequently, the compact structure decreases mass transport from the polymeric carrier [41].

2.2.2.2 Structural configurations

In addition to physical and chemical interactions, also the form, in which the active agent is associated with polymer, is exceptionally important for transport process. It has been reported that carriers' shapes affect targeting, circulation, and drug release profile [4]. Various structural configurations of delivery materials are given in Figure 2.

As mentioned in chapter 2.1.1, a combination of different types of carriers is also being used. For example, PLGA nanoparticles with docetaxel were surrounded by lipids [20]. Another example is a system chitosome where chitosan is used as a wall material for liposomes. This system is considered as a promising candidate for modifying the surface features of liposomes [21].

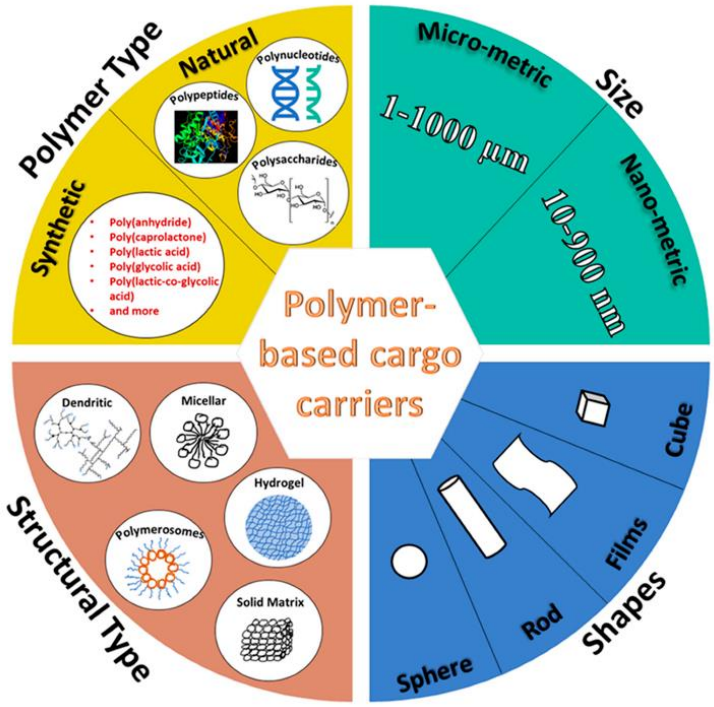


Figure 2: Various types of polymer-based carriers for drug delivery [4]

Polymer particles can be categorized into two main types: matrix (spheres) and reservoir systems (core-shell capsules) [6]. In a matrix system, the drug is uniformly dispersed throughout a polymer. In reservoir system the drug phase is surrounded by a polymer membrane [4][6]. In case of reservoir carriers, the release rate is affected mostly by the thickness and permeability of the polymeric shell. Thus, the release profile is relatively constant. On the

contrary, for the matrix type, the release rate is affected by the concentration gradient, which is associated with Fickian diffusion [15]. A schematic diagram of such polymer particles is shown in Figure 3.

The delivery systems consisting of water-soluble polymer materials with three-dimensionally cross-linked networks are excellent candidates for the development of controlled release systems [43]. 3-D carriers or hydrogels are generally biocompatible due to their high-water content. Release mechanism of an agent from hydrogels can be characterized by empirical Ritger-Peppas model (Table 1) [19]. For these delivery materials, the mesh size plays a crucial role in the mass transport [43].

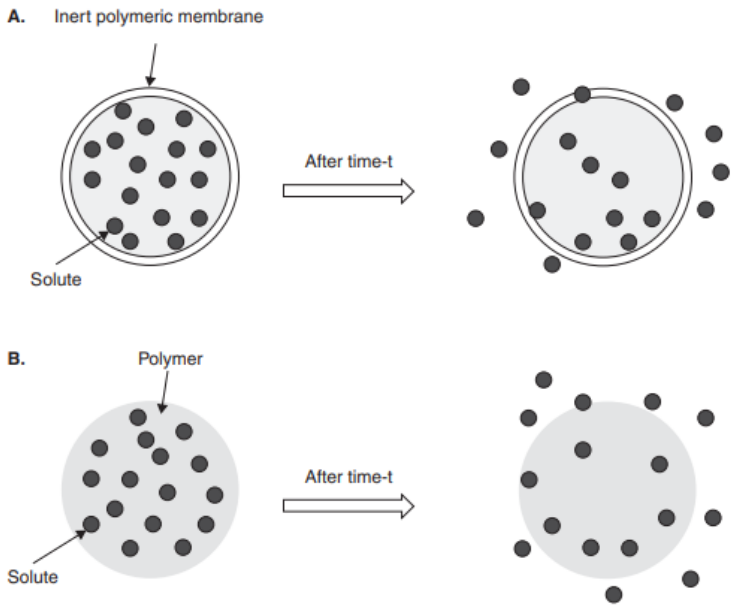


Figure 3: Structural configuration of reservoir and matrix particles and release of active compounds [6]

Other structural configuration of current delivery systems are thin films, i.e. the flexible layers of a polymer. Films are considered as an appropriate application form for targeting drugs and food additives. They should be non-toxic and have applicable mechanical properties. Remarkably, some films are designed to be easily dissolved (oral drug delivery), while others are intended as stable layers (for wound dressing, active packaging) [22] This kind of carrier system is preferably prepared by solvent casting method and hot melt extrusion [9][22]

Depending on the application, carrier composition can be designed as a single carrier, for example, polymeric micro-, nanoparticles; or as a composite of layers of natural or synthetic polymers, for instance, chitosomes, multilayers systems; or functionalized by other materials, such as targeted ligands [1][21].

In addition, there is a variety of active agent configurations. The core or the active substance is encapsulated into solid form, or liquid form, while liquid agents are hydrophobic, hydrophilic, eventually amphipathic [4]. The encapsulation of agent molecules into polymeric carrier allows to overcome poor water solubility of lipophilic agent and increase stability against hydrolytic cleavage of hydrophilic incorporated molecules [18]. Moreover, polymer-based carriers are

able to deliver compounds with different molecular weights such as small molecules, biological macromolecules, and vaccines [11].

2.2.3 General characteristics of the carrier system

The complexity of the transport behavior is discussed in many types of reviews [14][15]. Besides the chemical properties of the components, physical characteristics are also considered as key parameters in delivery system characterization [14].

2.2.3.1 Size

One of the widely studied parameters of carrier systems is a carrier size. This parameter has a significant influence on the distribution of drugs and their circulation time. The final circulation time of a drug-loaded carrier *in vivo* is depended on the excretion of the drug delivery system. *Gao et al*. stated that the particles with active agents smaller than 5 nm are washed out through kidneys (elimination is faster), while carriers larger than 100 nm are mainly washed out through a liver. Generally, carrier systems with their size between 5 and 100 nm have longer circulation times [23] . Long-circulation carriers as well as short-circulation delivery systems can be used in biomedicine. The carrier size influences the loading capacity, the release profile and carrier stability [4].

Some variations in carrier size are given in figure 2. When comparing macro and nanoparticles, the difference in surface area and volume ratio makes the most important difference. The surface-to-volume ratio (*s/v*) determines cohesive energy in the system which is an important factor influencing thermal properties of the delivery system. Based on the (*s/v*) parameter, *Otto et al.* described that nanoscale particles show lower glass transition temperature and transition melting point [24]. Also, it was noticed that the drug solubility and dissolution rate of nanocarriers is higher compared to microparticles [24].

A choice between micro- and nanoscale devices is a complex task as both delivery systems have benefits and shortcomings. On the one hand, it is reported that the rate of drug release would increase by decreasing particle size, because less voluminous carrier disposes of bigger surface area [25][24]. Furthermore, shape of the carrier, amount of the active agent within the carrier, and also its surface properties are considered as critical criteria for the kinetics of the drug release[24][25]. Moreover, several studies have concluded that nanosized particles tend to attract each other creating aggregates [41].

2.2.3.2 Surface properties

The interaction of polymeric carrier and its application is directly influenced by surface properties, which include surface charge, morphology, hydrophobicity, and many others [28].

Surface morphology controls uniform distribution of the active compounds throughout the polymer carrier. The surface analysis includes examining the agent distribution (aggregated or dispersed), and surface texture (porous or rough). Surface properties can be evaluated by scanning electron microscopy (SEM), atomic force microscopy (AFM), and X-ray diffraction crystallography (XRD)[26]. Moreover, the surface analysis provides information about the degradation of the carrier material. It should be noted that the degradability of the polymeric

matrice changes the conditions for mass transport and complicates the mathematical modeling of transport phenomena [15].

A number of physical and chemical parameters, such as surface charge and surface hydrophobicity, stability, drug loading, polymer size distribution play important role in final efficiency of carrier. [10][20]. Detailed characterization of these parameters is crucial for proper understanding of the carrier system's behavior.

2.2.4 Environmental influence

Environmental conditions are important for the drug release kinetics. An evident illustration of how the environment can affect the release kinetics is temperature. Temperature is a good example of a case where environment can influence a mass transport, as fas as the higher temperature increases the mobility of polymer chains. However, the mobility of a chain and structural changes affect pore formations as well as pore closure [3]. Thus, the increasing temperature can accelerate the rate of mass transport, as it was reported in article by *Zhang et.al* [9]. The authors found that temperature affects the release rate of nisin from PVA-based films [9]. In another review, the opposite situation was investigated (see [27]). At higher temperatures, a low release rate was detected [27], because the authors observed the pore-closing phenomenon in their microspheres.

Moreover, there is a group of especially environmentally sensitive polymeric delivery systems. They are known as stimuli-triggered carriers, where *in vivo* release is controlled by specific stimuli, for example, ionic strength, pH changes, magnetism. For instance, *Qiu et al.* worked with pH-sensitive hydrogels made of methyl methacrylate and dimethylaminoethyl methacrylate. In this case, caffeine was incorporated into the hydrogel and released from the carrier at pH 3-5, while at neutral pH, mass transport was not detected [29]. *Dima et al*. investigated a behavior of encapsulated coriander oil in different types of wall materials. The results showed that release was affected by both pH and temperature. Specifically, there was a higher release rate from chitosan particles at low pH (pH 2.5). [30]. For pH-responsive systems, ionizable polymers with pK_a values ranging between 3 and 10 makes a good choice. When ionizable groups, such as carboxyl or amino groups, are linked to the polymer structure, their dissociation causes a conformation change and affects the swelling behavior upon variation of the pH. Conformation changes lead to a controlled release of the model compound from the polymeric carrier [29].

2.3 Polyhydroxyalkanoates

2.3.1 General characteristic

Polyhydroxyalkanoates are a family of sustainable biopolymers, accumulated by certain types of microorganisms as intracellular granules, whereby the PHA content can reach as high as 90% of the cell dry weight [31]. Thus, different organisms can produce PHA inclusions up to various cell volume fractions. The granules are spherical consist of proteins, and polyester [32]. They are synthesized as a carbon and energy reservoir, whenever there is an excess of carbon and a lack of other nutrients (oxygen, nitrogen, phosphorous), or even fluctuating pH of the growth media [33]. Moreover, it has been shown that some bacteria can produce PHA granules without any nutrient limit, for example, *Alcaligenes latus* strains IAM 12,664T [34][33].

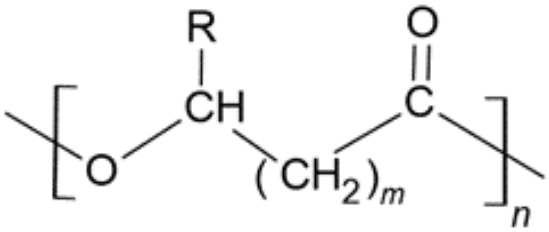


Figure 4: General chemical structure of polyhydroxyalkanoates

From a chemical point of view, polyhydroxyalkanoates are thermoplastic polymers where monomer units are connected by ester bond. Aliphatic polyester PHA consists of a side chain R, which can be a saturated or unsaturated alkyl group, substituted, or branched alkyl group (Figure 4). According to the number of carbon atoms, they are categorized into following types: short-chain length PHA with the monomer unit consisting of 3 to 5 carbon atoms (*scl*-PHA), and medium-chain length with monomers of 6 to 14 carbon atoms (*mcl*-PHA). Short-chain length PHA consists the most often of monomeric units of 3-hydroxybutyrate, 4-hydroxybutyrate, or 3-hydroxyvalerate. The second type, medium-chain length PHA typically involves aside from the 3-hydroxybutyrate also 3-hydroxyoctanoate or 3-hydroxydecanoate. Nevertheless, the particular monomeric structure of the PHA depends on the carbon substrate, microbial strain, media composition, and cultivation modes and conditions [35][36].

2.3.2 Microorganisms used for PHA production

Polyester accumulation has been shown in several microorganisms primarily to provide storage of carbon and energy. The most widely used industrial strain for PHA production is *Cupriavidus necator* which accumulates poly(3-hydroxubutyrate) up to 90% of the cell dry mass [47][46]. Some investigations demonstrated that *C. necator* H16 can synthesize copolymers containing 3-hydroxyvalerate, 4-hydroxybutyrate, 3-hydroxypropionate, or 3-hydroxyhexanoate [47]. Additionally, *C. necator* H16 synthesizes polymer PHA from waste fluxes produced from food processing industries, such as waste frying oil, or coffee oil [48]. Application of waste carbon sources in general leads to more cost-effective production of PHA [49].

Another attractive option for reducing the production cost of biopolymer synthesis is utilizing extremophilic microorganisms [53]. Extremophiles produce PHA under adverse environmental conditions, such as high pressure, temperature, or salinity [54]. Consequently, PHA synthesis does not require completely sterile conditions. Thermophilic *Aneurinibacillus* sp. H1 is a promising candidate for the accumulation of PHA polymers with a high molar fraction of 4-hydroxybutyrate and 3-hydroxyvalerate. Recently, *Pernicová et al.* described the ability of *Anerinibacillus* sp. H1 to accumulate PHA copolymers and terpolymers under different cultivation conditions, such as temperature, carbon sources, and concentrations of carbon sources [51]. In the second part of this study, *Sedláček et al*. pointed out the attention on the material characterization of polyesters produced by *Anerinibacillus sp* H1 [50]. Based on instrumental analysis, such as infrared spectroscopy, X-ray diffractometry, and thermoanalytical methods, it was found that the properties of solvent-casted films can be tuned by the monomeric composition as well as the cultivation process.

Among the other extremophilic organisms producing PHA are *Bacillus shackletonii*, *Schlegelella thermodepolymerans*, archaeon *Haloferax mediterranei* [52][53]. Further examples of extremophiles are presented in Table 3

Table 3: Various extremophiles synthesized polyhydroxyalkanoates (adapted from [55])

Division of extremophiles	Microorganism	Carbon source	PHA content
Halophiles	*Halomonas* TD01	Glucose + yeast extract	82% of P(3HB-*co*-3HV)
	Bacillus megaterium H16	Glucose	39% of P(3HB)
	Natrinema pallidum	Starch	75,5% of P(3HB-*co*-3HV)
Thermophiles	*Bacillus shackletonii* K5	Glucose	72,6% of P(3HB)
	Cupriavidus sp. S-6	Gluconate	49% of P(3HB)
	Chelatococcus daeguensis TAD1	Glucose + glycerol	80% of P(3HB)

2.3.3 Physicochemical properties

Polyhydroxyalkanoates have a high potential for numerous commercial applications, due to variety of properties, such as good resistance to UV rays, hydrophobicity, and thermoprocessibility. PHA polymers are more soluble in chlorinated solvents like dichloromethane or chloroform [33]

Generally, material characteristics of *scl*-PHA include high glass transition temperature, relatively high melting point (more than 180 °C) [37][36]. They are brittle and rigid, due to high crystallinity (between 60 and 70%) [37][36]. In the field of biomedicine, their material properties are well applied for hard tissue engineering [37][36]. Medium-chain length PHAs have lower glass transition temperatures and are amorphous, elastomeric polymers. However, their applicability is limited due to low mechanical strength. Stiffness, elongation at break, and tensile strength are some of the mechanical characteristics of these polymers [33]. The stiffness of a polymer is mostly determined by Young's modulus. According to *Masood*, PHAs' Young's moduli range from the extremely ductile *mcl*-PHA (0.008 MPa) to the far stiffer *scl*-PHA ($3.5 \cdot 10^3$ MPa). The level of a material's stretchability prior to breaking is indicated by elongation at break. The PHAs provide a wide range of elongation at break values (between 2 % and 1000 %) [31]. Tensile strength is the total amount of force needed to pull a material before it breaks; with PHAs, this typically ranges from 8.8 to 10^4 MPa [31]. Table 4 represents thermal and mechanical properties of various PHAs.

In general, incorporation of another monomers into the stiff P(3HB) chain leads to an improvement in the physical properties, especially in terms of increased flexibility, and decreased crystallinity. Such a good example is copolymer P(3HB-*co*-3HV) which has a higher elongation to break and tensile strength, comparable to P(3HB). Or another one is P(3HB-*co*-4HB) with increased elongation at break, decreased melting point, and glass transmission rate. Thermal properties are critical factors that determine polymer processibility and applicability. Copolymer P(3HB-*co*-4HB) is comparatively more flexible, and stretchable, and has a larger processing window than P(3HB) [32][56].

Table 4: Properties of PHA homopolymers and copolymers (adapted from[56])

Types of PHA	Thermal properties		Mechanical properties		
	T_m (°C)	T_g (°C)	Young's modulus (MPa)	Tensile strength (MPa)	Elongation at break (%)
Homopolymers of *scl*-PHA					
P(3HB)	178	4	3500	43	5
P(4HB)	61	-47	180.9	13.8	696.6
Copolymers of *scl*-PHA					
P (3HP-*co*-25 mol% 4HB)	62.7	-31.3	14.5	1.7	962.9
P (3HP-*co*-38 mol% 4HB)	63.5	-36.1	4.4	0.9	1661.0
Copolymers of *mcl*-PHA					
P (16 mol% 3HD-*co*-3HDD)	77.6	-32.5	103.1	5.2	88.3
P (44 mol% 3HD-*co*-3HDD)	74.9	-43.0	2.0	5.9	188.3

2.3.4 PHA-based delivery systems

Because of the wide range of advantageous material features, biodegradable PHAs are auspicious materials for active agent delivery systems. PHA have been used for the delivery of a number of substances such as antibiotics, hormones, vaccines, and anesthetics. These polyesters were also used to encapsulate anticancer agents, such as docetaxel, ellipticine, and doxorubicin. [30][32]. Drugs can be incorporated in a PHA homopolymers, copolymers, or PHA blends. Moreover, both hydrophobic and hydrophilic drugs can be incorporated into hydrophobic PHA carriers [26][25]. It is interesting that PHA carriers might be processed into porous matrices, capsules, spheres, gels, fibers, and thin films [57].

Table 5: Applications of PHA-based delivery systems (adated from [57])

PHA-based carrier	Structure	Incorporated agent	Application
P(3HB)	microsphere	Tetracycline	Increase the cell viability
P(3HB-*co*-3HV)	nanoparticle	Ellipticine	Increase drug cytotoxic effect
P(3HB-*co*-4HB)	nanoparticle	Doxorubicin	Drug carrier, effective against HeLa tumor cells
P(3HB-*co*-3HHx)	nanoparticle	5-Fluorouracil	Controlled release
P(3HB-*co*-3HV)/PLA	microsphere	Ibuprofen	Prolong the drug release
P(3HB-*co*-3HV)/Guar gum	film	Curcumin	Antimicrobial wound dressing

in general, active substance release rate from copolymer PHA, such as P(3HB-*co*-3HV), is higher than from the corresponding homopolymer. In addition, a chain length of copolymers influences the hydrophobicity of a carrier, which also affects the release profile [42][41]. Besides, it was investigated, that drug delivery P(3HB) microspheres prepared by employing low molecular weight PHA (65 kDa) with drug 5-fluoro-2′-deoxyuridine showed better release capacity as compared to others with higher molecular weight (135 kDa, 450 kDa) [42][41].

Mohanasundram et al. discussed in review, due to PHA chain length, the *scl*-PHA is the better choice as a carrier material than mcl-PHA since polymer is degraded by surface erosion [45]. Carriers made of *scl*-PHA tend to release drugs in a biphasic pattern regulated by diffusion [44]. However, medium-chain length polyhydroxyalkanoate provides controlled release due to its low melting point [45]. Furthermore, copolymer blends of *mcl*-PHA and *scl*-PHA showed tunable features for controlled release of drugs [44]. To sum up, mass transfer from PHA-based carriers can be easily controlled by varying the chemical structure [44] [45].

Another direction of research focused on utilizing PHA as carrier matrices for pesticides. PHA systems, including granules, microparticles, and films, can be used for soil applications due to degradability in different environments. Moreover, PHAs are applied as carrier materials in the food packaging industry. Active agents have been added to PHA films and PHA-coated papers to improve quality, shelf life, and sensory properties of the packaged food [64].

2.3.5 Techniques for synthesis of PHA-based carrier system

The active agent can be encapsulated in a polymeric sphere or a capsule. Alternatively, the active substance can be dissolved or dispersed in the polymer. In both cases, high encapsulation efficiency, stability of the loaded agent, and low burst release are required [43].

In a detail, formulation techniques, such as *in situ* polymerization, dialysis, nanoprecipitation, and emulsion solvent evaporation (i.e., oil-in-water (O/W) single emulsion, and water-in-oil-in-water (W/O/W) double emulsion), have been investigated to produce PHA particles [32]. Widely spread approach for producing PHA particles is emulsion solvent evaporation as the processing parameters of this method are easy to control and enabling the encapsulation of both hydrophobic and hydrophilic compounds. Generally, an active agent is dissolved or emulsified in the oil phase, and then emulsified in the continuous aqueous phase, while the solvent evaporates. Finally, the particles are collected by centrifugation [32]. Another form of the PHA-based carrier system was made via solvent casting method (film), compression molding (multilayers), or electrospinning (fibers) [31]. The choice of preparation method depends on the nature of the incorporated agent, as well as depends on the specifications of potential application.

2.3.6 Properties of PHA carriers

In general, the properties of delivery systems based on PHA depend on various factors, such as composition of the monomeric subunits of PHA, molecular weight, drug loading method, and form and size of the carrier system. However, PHA-carriers possess unique features (Figure 5) which make them useful for various medical applications, such as controlled release systems, porous materials for tissue engineering, or antimicrobial wound dressing [41].

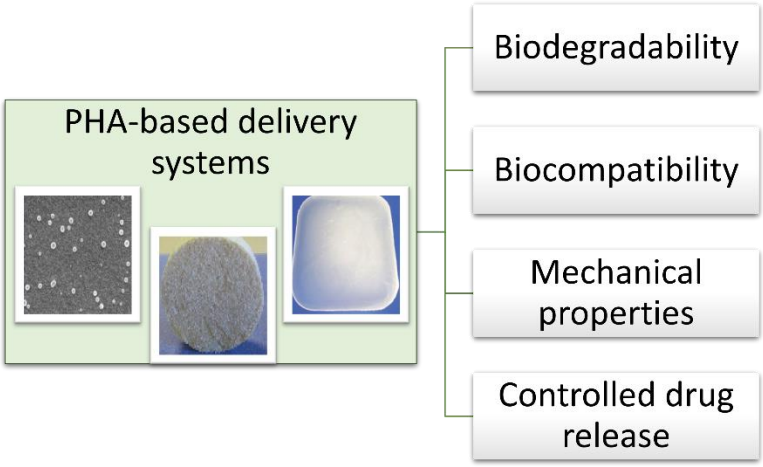


Figure 5: Key highlights of PHA-based carriers (nanoparticles, porous material, biofilm)(based on [41][74])

PHA is known to be degradable under *in vivo* and *in vitro* conditions. Moreover, their degradation products are non-toxic and can be eliminated by the body. Further, PHA-based drug carrier does not exhibit detrimental interaction with blood constituents [42][41] [43]. Degradation of polymer is influenced by monomeric composition and the amorphous and crystalline phase of the polymer. For example, varying content of 3-hydroxyvalerate monomeric subunit in P(3HB-*co*-3HV) affects the crystallinity, porosity, and molecular weight of the macromolecule as well as its ability to degrade [42][41]. Thus, to accelerate the degradation process, these can be controllably modified by incorporation of monomeric units or blending with other materials. *Qu et al*. investigated *in vivo* biodegradation of P(3HB) and the copolymer with a fraction of 3-hydroxyhexanoate, and reported increased degradation rate

for P(3HB-*co*-3HHx) compared to homopolymer P(3HB) [42][41].. Blending with other materials is a good strategy, as it helps to increase porosity and reduce crystallinity [42][41]. In order to study the degradation of PHA, *Freier et al*. designed P(3-hydroxybutyrate) films [58]. After a year of incubation in a buffer solution (pH 7.4 at 37°C), the molecular weight of P(3HB) films was reduced by half [58]. The authors also summarized that the blending of the poly(3-hydroxybutyrate) films with atactic P(3HB) accelerated the degradation process [58]. Regarding biocompatibility of PHA, *Kose et al.* investigated homopolymer P(3HB) with many cells, such as chondrocytes, osteoblasts and fibroblasts, and concluded that PHA is biocompatible without any adverse side effects [42].

Polyhydroxyalkanoate delivery systems are designed for both controlled release and targeted drug delivery [44]. Due to these systems, the frequency of drug administration can be reduced. In general, particles based on *scl*-PHA tend to release the drugs in a biphasic pattern by diffusion since *scl*-PHA degrade slowly [44]. Furthermore, the active agent release rate increases by increasing the amount of incorporated drug. Additionally, a combination of PHA with additives optimizes the carrier's efficiency, as was described in the paper by *Elmowafy et.al* [59]. The authors designed P(3-hydroxybutyrate-*co*-3hydroxyvalerate)-TPGC nanoparticles, and a more sustained release of the docetaxel from polymeric nanoparticles was observed [59]. *Bidone et al.* fabricated microspheres based on a blend of highly crystalline P(3HB) and amorphous poly(D,L-lactide)-*b*-PEG or gelatin [44]. More controlled release of ibuprofen was detected for carriers based on blends with higher homopolymer P(3HB) content [44]. Furthermore, as described previously, drug release rate and release patterns correspond to size, form, and physicochemical properties of the particle.

Detailed characterization of the physicochemical properties of PHA has already been provided in chapter 2.3.3. Nevertheless, it should be pointed out again that material properties depend on the monomeric composition of the polymer. Consequently, drug release can be affected by the chemical structure of PHA. Many publications have investigated different methods to change and improve the mechanical properties of PHA carriers (e.g. [56][60]). One study focused on scaffolds based on P(3HB-*co*-3HHx) and blend P(3HB-*co*-3HHx)/P(3HB). It was found that the blend of copolymer and homopolymer P(3HB-*co*-3HHx)/P(3HB) with 60*wt*% of copolymer had the highest percentage increase for elongation at break rates [61].

Another paper investigated materials prepared from the same type of copolymer P(3HB-*co*-3HHx) with different porosities and compared their strength since mechanical strength is a crucial requirement for PHA-based multifunctional scaffolds (see [60]). These composite materials can be used for structural support, tissue regeneration, and for active agent delivery [60].

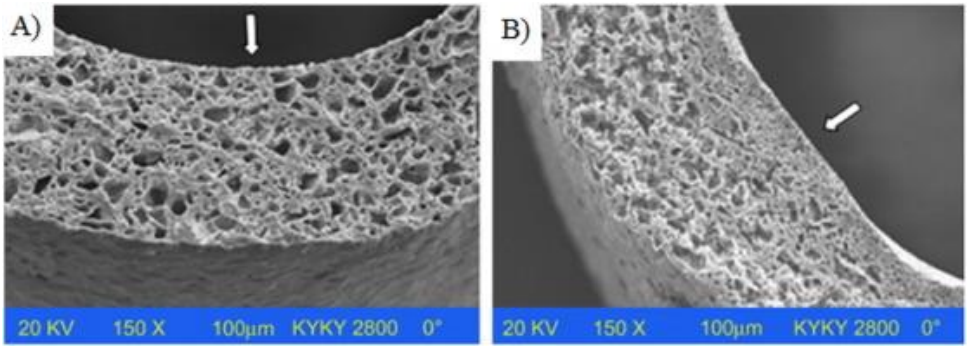


Figure 6 P(3HB-co-3HHx) conduits under SEM. A) uniform wall porosity. B) non-uniform wall porosity [60]

A study carried out by *Volant et al*. demonstrated that the mechanical properties of various PHA particles correspond to their crystalline content [62][61]. The authors fabricated microbeads of P(3HB-3HV), P(3HB-3HHx) and PLA by emulsification process. In the result, P(3HB-*co*-3HV) samples gave a high Young's modulus as compared to the P(3HB-*co*-3HHx) [62][61]. Moreover, diameter and morphological surface were investigated. The size and surface morphology of particles was found to be related with the polymer nature [62]. The SEM images (reproduced in Figure 7), show the size and surface roughness of microbeads P(3HB-*co*-3HHx). particles have smaller diameters, whereas PLA carrier is rather smooth [62].

Overall, polyhydroxyalkanoates possess attributes beneficial not only for drug delivery and and biomaterial applications. Because of this fact, PHA is widely studied biopolymer and its applied research follows numerous directions, including material engineering, environmental technology or food industry [31][32][73].

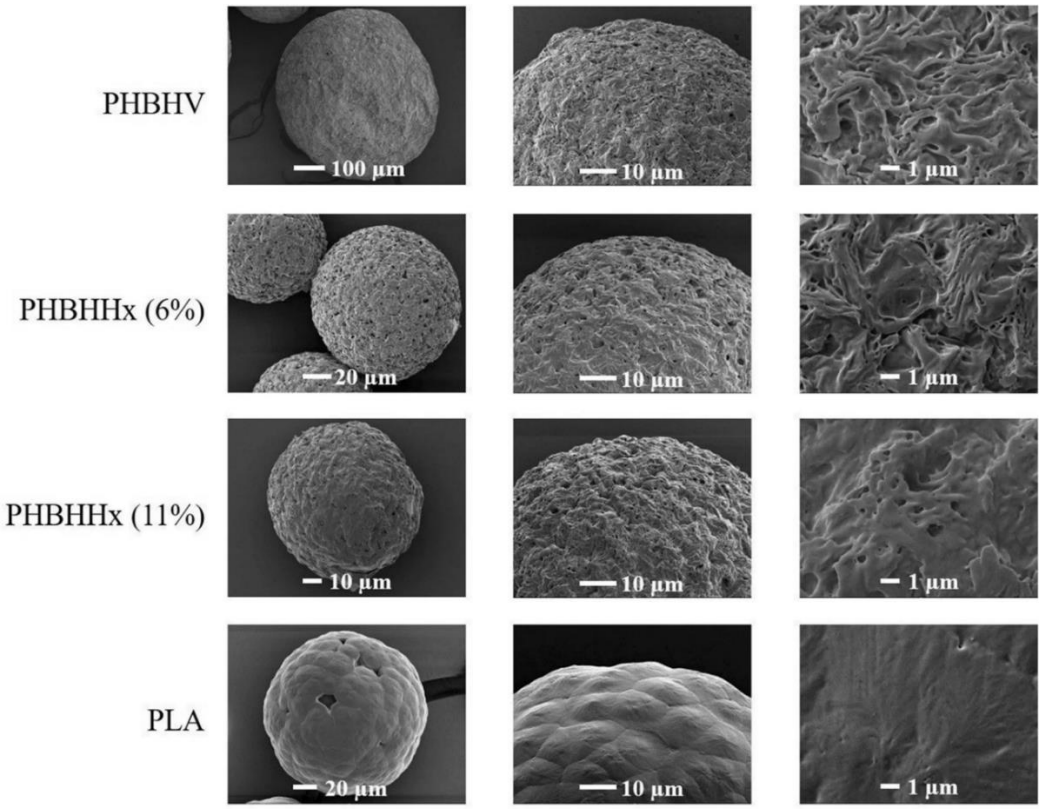


Figure 7 SEM images of P(3HB-co-3HV), P(3HB-co- 6%, 3HHx) , P(3HB-co-11%,-3HHx) and PLA carriers [62]

2.4 State of the art in PHA based drug delivery systems

Numerous investigations have shown that PHA materials have a significant potential to be applied in drug delivery applications. Generally, drugs are encapsulated in carrier material to increase their bioavailability, prolong their half-life, or improve their transport characteristics.

Peng et al. created hydrophilic insulin-phospholipid complex-loaded P(3HB-*co*-3HHx) nanoparticles for controlled and sustained release via the emulsion-solvent evaporation method. When compared to the insulin solution, ready-to-use particles demonstrated a greater bioavailability [38][37].

A novel targeting drug delivery system was developed by *Zhang et al*. (see ref. [39]). Authors deal with poly(3-hydroxybutyrate-*co*-3-hydroxyoctanoate) as the drug carrier, folic acid (FA) as the targeting ligand, and doxorubicin (DOX) as the incorporated anticancer agent. The shape of nanoparticles was spherical, with size distribution from 200 to 300 nm. Studied carriers showed therapeutic efficacy in the inhibition of tumor growth [39].

Gentamicin has been encapsulated into P(3HB) spheres and coated onto Bioglass scaffolds to apply in bone tissue engineering by *Francis et al*. (see [40]). A solid-in-oil-in-water emulsion method was used for P(3HB) carrier preparation. Gentamicin release rate was determined by liquid chromatography-mass spectrometry (LC-MS). The drug release from Bioglass composite scaffold was slow and controlled. Authors summarize that multifunctional scaffolds containing encapsulated drugs have the potential to provide structural support, enhance cell attachment and provide controlled delivery of drugs [40]

Another type of carrier, i.e. P(3HO-*co*-3HD-*co*-3HDD) films, was prepared using the solvent-casting method by *Gregory et.al* in [37]. Films were modified to create polydopamine layers, which showed increase of surface free energy, enhanced cell viability, and promoted neovascularization when implanted *in vivo* [37].

Gonta et al. made an active food packaging system using P(3HB) [31]. Antimicrobial agents (benzoic acid and Silbiol) were immobilized into the PHA films and PHA-coated paper surface. Both systems demonstrated antimicrobial activity [31]. Similarly, *Xavier et al.* developed P(3HB) films incorporated with the antimicrobial agent vanillin (4-hydroxy-3-methoxy benzaldehyde) in [31]. Vanillin was added to the P(3HB) solution at different concentrations (20, 40, 50, 80, 100, or 200 μg). Then films were prepared by the solvent-casting evaporation method. Compared to P(3HB) film, the active package film based on P(3HB)-vanillin showed higher percentage of elongation at break (2.09%), while 0.91% for P(3HB) film. However, a reduction in tensile strength and Young's modulus of P(3HB)-vanillin film has been reported. Based on the agar diffusion assay, the minimum inhibitory concentration against bacteria was ≥80 μg/g P(3HB) [31].

Application of PHAs as drugs delivery biomaterials due to diverse features and high biocompatibility is quite attractive. The primary difficulty is developing PHA-based carrier systems while improving their material characteristics without affecting their biodegradability and biocompatibility. Moreover, microbiologically produced polyhydroxyalkanoates demonstrate significant potential for a range of various applications, including agriculture: controlled release of pesticides, plant growth regulators and herbicides; biomedicine: bone plates, surgical sutures and films, implants, in tissue engineering; others: hygiene items, packagings, medical surgical clothing, biofuels [31][46][56][74]. Controlled release systems based on polyhydroxyalkanoates have been designed by using a variety of strategies, for example, combination of different preparation methods, different polymers blending, using copolymers and additives. It is clear that searching for new strategies and use of existing ones to fabricate novel type of carriers is continuous, thanks to PHA in delivery systems is a theme with space for creativity and great potential.

3 Experimental part

3.1 Materials and equipment

3.1.1 Bacterial strain

The bacterial strain used in the experimental part was *Aneurinibacillus* (isolates AH30 and AFN2). Thermophilic *Aneurinibacillus* strain was indicated as a promising candidate to utilize different types of lactones to produce PHA with diverse monomer composition and material characteristics [63].

3.1.2 Chemicals

Ammonium chloride	Lach-Ner, Czech Republic
Ammonium ferric citrate	Fluka, Germany
Benzoic acid	Lach-Ner, Czech Republic
Calcium chloride dihydrate	Lach-Ner, Czech Republic
Chloroform	Lach-Ner, Czech Republic
Dimethyl sulfoxide	Lach-Ner, Czech Republic
Disodium hydrogen phosphate dodecahydrate	Lach-Ner, Czech Republic
Glycerol	Lach-Ner, Czech Republic
Ibuprofen	Sigma-Aldrich, Germany
Isopropanol	Penta, Czech Republic
Magnesium sulfate heptahydrate	Lach-Ner, Czech Republic
Nutrient broth	HiMedia, India
Poly(3-hydroxubytyrate)	Biomer, Germany
Sodium dihydrogen phosphate dihydrate	Lach-Ner, Czech Republic
Sodium hydroxide	Lach-Ner, Czech Republic
Yeast extract	HiMedia, India
Tween 20	Sigma-Aldrich, Germany
Sodium dodecyl sulfate	TCI, Belgium
γ-valerolactone	Sigma-Aldrich, Germany
γ-hexalactone	Sigma-Aldrich, Germany
δ-valerolactone	Sigma-Aldrich, Germany

3.1.3 Instrumentation and apparatus

Analytical balance	Boeco, Germany
Balances	Kern EW 630-3NM
Centrifuge	Hermle, Germany
Centrifuge	Maneco, Czech Republic
Differential scanning calorimeter	TA Instruments, USA
FTIR spectrophotometer	Thermo scientific, Waltham, MA, USA
Gas chromatograph with FID detector	Thermo Scientific, Trace 1300
Incubator shaker	Heidolph, Czech Republic
Laminar flow box	Aura mini, Bioair
Magnetic stirring	MERCI, Czech Republic
Nanophotometer	Implen, UK

pH meter	XS instruments
Scanning electron microscope	Carl Zeiss NTS, Germany
SEC with MALS detector and differential refractometer	Agilent technologies, DAWN HELEOS II, Wyatt technology, OPTILAB T-rEX, Wyatt technology
Thermostat	Bibby Scientific
Vortex	Benchmark Scientific, USA

3.2 Polyhydroxyalkanoate synthesis

3.2.1 Preparation of complex and mineral media

A complex medium and mineral medium was used for the growth of *Aneurinibacillus*. Nutrient broth complex medium was prepared in a volume of 50 ml at the concentration of 25 g/l in 100 ml Erlenmeyer flasks. Flasks containing Nutrient broth were sterilized. Then, mineral medium was prepared according to list of chemicals and concentrations (Table 6). Following the mixing of components, 250 ml Erlenmeyer flasks with 100 ml of prepared production medium were sterilized. Trace element solution (TES) was added to the 100 ml of production at the concentration of 1 ml/l. The composition of the trace element solution is listed in Table 7

Table 6: Composition of production medium

Chemicals	Concentration (g/l)
$Na_2HPO_4 \cdot 12\ H_2O$	9
$KH_2\ PO_4$	1.5
NH_4Cl	1
$MgSO_4 \cdot 7\ H_2O$	0.2
$CaCl_2 \cdot 2\ H_2O$	0.015
$FeNH_4$ citrate	0.0012
Yeast extract	0.5

Table 7: Compostion of trace element solution (TES)

Chemicals	Concentration (g/l)
EDTA	50
$FeCl_3$	8.3
$ZnCl_2$	0.84
$CuCl_2 \cdot 2\ H_2O$	0.13
$CoCl_2 \cdot 6\ H_2O$	0.1
$MnCl_2 \cdot 6\ H_2O$	0.016
H_3BO_3	0.1

3.2.2 Inoculation

The bacteria were maintained in cryotubes at –80 ℃ in 10% glycerol. The thawed bacterial culture was inoculated into 50 ml of Nutrient broth in a laminar flow box. The cultivation was carried out in 100 ml Erlenmeyer flasks for 24 hours at 50 ℃ and stirred at 190 rpm. After cultivation in a complex medium, the mineral medium (in 250 ml Erlenmeyer flask) was inoculated in the laminar flow box with 10 % inoculum culture. Glycerol and lactones (γ-valerolactone, γ-hexalactone and δ-valerolactone) were used as carbon sources at a concentration of 4 g/l. The main precursor, in this case glycerol, was added before sterilization.

Table 8: List of substrates and isolates for inoculation

Carbon source	Strain
Glycerol+γ-valerolactone	AH30
Glycerol+γ-hexalactone	AFN2
Glycerol+δ-valerolactone	AFN2

3.2.3 Cultivation in mineral salt medium

The production medium with inoculum culture was transferred to an incubated shaker. The cultivation was carried out for 72 hours at 50 ℃ under constant shaking of 190 rpm. All cultivations were performed in a high number of Erlenmeyer flasks (20–30).

3.3 Determination of biomass and PHA

3.3.1 Gravimetric analysis

After cultivation, 10 ml of bacterial suspension from each flask was placed in centrifuge tubes. Following the first centrifugation (6,000 rpm, 5 min), the biomass was washed with distilled water and centrifuged again (6,000 rpm, 5 min). The biomasses were left in a thermostat at 70 °C until dried to constant weight. The prepared samples were determined gravimetrically in duplicate.

3.3.2 Gas chromatography

The amount and monomer composition of synthesized polyhydroxyalkanoate in the biomass was analyzed by gas chromatography with a flame ionization detector (GC-FID). The method used to PHA quantification is called methanolysis. The dried biomass (from 5 to 11 mg) was mixed with 1 ml of chloroform and 0.8 ml of internal standard solution (5 mg/ml benzoic acid in 30% sulfuric acid methanol) in crimp neck vials. The prepared samples were heated in a thermoblock for 3 hours at 94 ℃. After esterification, the solution was transferred into screw-top vials with 0.5 ml of 50 mM sodium hydroxide, and the sample was shaken. Following the neutralization, the lower organic phase was pipetted into small screw-top vials with 0.9 ml of isopropanol. The samples were assayed via GC-FID. All samples were analyzed in duplicates.

3.4 Extraction of PHA copolymers from biomass

Polyhydroxyalkanoate copolymers were isolated from dry biomass via solvent extraction method. Approximately 200 mg of microbial biomass was mixed with 10 ml of chloroform in Pyrex tubes. The tubes were heated at 70 ℃ for 12 hours. Lastly, the mixture was filtered using

paper filter and poured onto Petri dishes. PHA was separated from the solvent by evaporating chloroform at laboratory temperature.

3.5 Characterization of isolated PHA copolymers

The size-extrusion chromatography with a multi-angle light scattering detector (SEC-MALS) was used to determination of molecular weight and polydispersity index. Approximately 3 mg of solvent-casted films were dissolved in 1.5 ml of chloroform. After dissolution at 70 °C, the mixture was filtered through a nylon syringe filter with 0.45 μm pore size, and the final solutions were analyzed via SEC-MALS.

3.6 Preparation of the delivery systems

The experimental methods to fabricate PHA drug delivery systems were chosen according to the available literature, for example [65][66][67]. However, optimization of conditions for individual formulation is necessary step to develop reproducible method for production of polymeric carriers. For this reason, processing conditions were optimized using copolymer P(3HB-*co*-4HB) and commercial homopolymer P(3HB).

3.6.1 Optimization of PHA carriers' production

3.6.1.1 PHA – based particles

Particles composed of polyhydroxyalkanoate were fabricated via solvent-exchange method. Various combinations of processing parameters were tested. Fundamental steps of the process include the preparation of an oil phase and an aqueous one, incorporation of the model agent, particles formation, and particles isolation.

Step 1: PHA solution preparation

The first step consists of the dissolution of the P(3HB-*co*-4HB) in the solvent. In this experimental part, polymers were dissolved in dimethyl sulfoxide (DMSO) at 70 °C to obtain a transparent solution. After dissolving, the polymer mixture was allowed to cool at laboratory temperature. 1 ml of organic medium was added dropwise to a water phase using an automatic pipette.

Step 2: Non-solvent phase optimization

Several aqueous phases were tested to achieve optimal conditions which would lead to a uniform, stable drug-loaded particles. Various mixtures of ethanol and water (ethanol content 30 %*v/v*, 50 %*v/v*, 70 %*v/v*, 80 %*v/v*, 90 %*v/v*) were tested as an aqueous medium. In addition, water solutions of surfactant were prepared, such as sodium dodecyl sulphate (2.2 mg/ml, 4.4 mg/ml), and Tween 20 (0.1 %*v/v*, 1 %*v/v*), to compare the influence of aqueous phase formulation on particles properties. The list of tested water phases is shown in Table 9

Table 9: Water medium for particles fabrication

Solution	Concentration of anti-solvent	Processing condition
Ethanol in water	30 %*v/v* 50 %*v/v* 90 %*v/v*	Not specified
Ethanol in water	70 %*v/v* 80 %*v/v*	Heat/cool, stirring
SDS in water	0.22 %w/*v* 0.44 %*w/v*	Stirring
Tween 20 in water	0.1 %*v/v* 1 %*v/v*	Stirring
Tween 80 in ethanol	1 %*v/v*	Not specified

Step 3: Incorporation of the model drug

The third step of developing delivery systems is the incorporation of the model drug (in this work acidic form of ibuprofen). An active agent was dissolved in the polymer solution or in the solvent (dimethyl sulfoxide), e.g., in an organic medium. In this series of experiments, the concentration of ibuprofen was 0.3 mg/ml and 3 mg/ml. In another case, the model drug was dissolved in ethanol (water phase) at different concentrations (0.005 %*w/v*, 0.01 %*w/v*, 1 %*w/v*, 3 %*w/v*).

Table 10: Preparation of drug-loaded formulation

Phase for ibuprofen dissolution	Formulation	Concentration of ibuprofen (mg/ml)
Organic	Polymer solution, solvent	0.3 3.0
Water	80% ethanol	0.0395 0.0789 7.89 23.67

Step 4: Isolation of drug-loaded particles

The last step focuses on the separation process. A frequently used method for particles isolation is centrifugation. Thus, centrifugation was carried out at various revolutions per minute and centrifugation times (3,000 rpm during 3 or 5 minutes, 4,000 rpm during 3 or 5 minutes). The second tested method was decantation. In this work, gravity separation was suggested as a

suitable method for the isolation of polymer-ibuprofen particles. In addition, the pellet was filtered using a fabric filter. After each isolation, particles were allowed to dry at room temperature.

3.6.1.2 PHA – based films

Polymer films loaded with ibuprofen were prepared via solvent casting method. In brief, PHAs were dissolved in two different solvents – chloroform and dimethyl sulfoxide, at 60 °C and 70 °C, respectively. The final concentration of polymer in solution was always 4 *w/v*%. A series of PHA homopolymer/copolymer blends with different compositions (weight ratios 75/25, 50/50, 25/75) were tested. After the complete dissolution, the mixtures were poured into Petri dishes, and the solvent was allowed to dry at laboratory temperature.

The drug-loaded films were fabricated by dissolving ibuprofen in 4 *w/v* % polymer-solvent mixtures to form a homogenous solution at the concentration of 0.3 mg/ml of ibuprofen. Based on the thesis (in ref. [65]), 0.3 mg/ml of ibuprofen was determined as an optimal value for drug release analysis.

Table 11: Composition of solvent-casted films

Polyhydroxyalkanoates	Ratio(*w/w*)	solvent
P(3HB)/P(3HB-*co*-4HB)	0/100	Chloroform
	0/100	Dimethyl sulfoxide
	25/75	Chloroform
	25/75	Dimethyl sulfoxide
	50/50	Chloroform
	50/50	Dimethyl sulfoxide
	75/25	Chloroform
	75/25	Dimethyl sulfoxide

3.6.1.3 PHA – based porous monoliths

The porous PHA material was fabricated using dimethyl sulfoxide as a solvent. P(3HB) commercial powder and P(3HB-*co*-4HB) foil biosynthesized in this work at a different weight ratio (75/25, 25/75, 0/100) were dissolved in DMSO at 70–80 °C. The resulting highly viscous mixture was allowed to cool at laboratory temperature. The pieces of transparent PHA were cut with a cork borer and were washed with 130 ml of distilled water. The final white solid was dried in a thermobox at 70 °C. For the preparation of PHA delivery systems, the 3 mg ibuprofen was dissolved in 1 ml of DMSO, then polyhydroxyalkanoate was added to produce 4 *w/v* % solution of the polymer.

3.6.2 Fabrication of PHA carriers

Based on previous experiments, it can be concluded, each type of studied drug delivery system has its various controllable parameters, which influence the stability, efficiency, and material characteristics of final carrier systems. In the final protocol, synthesized polyhydroxyalkanoates P(3HB-*co*-3HV-*co*-4HHx), P(3HB-*co*-3HV-*co*-4HV) and P(3HB-*co*-5HV); and commercial homopolymer P(3HB) were used for designing PHA delivery systems.

The fabrication of particles was carried out in several stages, as was mentioned previously. Firstly, 3 mg of active agents was dissolved in DMSO. The second step involved the polymer dissolution in DMSO-ibuprofen solvent at 70 ℃ to form 2 %*w/v* polymer solution. The obtained solution was added to 20 ml of 1 % Tween 20 solution to form transparent uniform composites. After 20 min, white stable particles were isolated, washed with distilled water and allowed to dry. Pure particles without ibuprofen for negative control were prepared following the same procedure.

For film production, copolymer and copolymer/homopolymer at a weight ratio of 25/75, 75/27, 100/0 were dissolved in chloroform to 4 *w/v* % concentration of the polymer solution. The active agent (ibuprofen) was added to the polymer solution to obtain a 0,3 mg/ml final mixture concentration. The polymer solution was transferred to Petri dishes and allowed to evaporate at laboratory temperature.

The last tested type of carrier is PHA porous monolith. The main step involved the incorporation of ibuprofen into PHA. 3 mg of model agent was mixed with DMSO. Poly(3-hydroxybutyrate P(3HB) and isolated copolymer were dissolved in DMSO mixture at 70–80 ℃. The weight ratios of polymers in these experiments were 25/75 and 75/25. Then, solutions were cooled at laboratory temperature and lastly, the same size pieces of transparent samples were poured into 100 ml of distilled water. After an hour, distilled water was used to determine the drug release.

3.7 Characterization of drug delivery systems

3.7.1 Scanning electron microscopy

In order to study the surface morphology of the PHA carriers, scanning electron microscopy (SEM) was performed at a voltage of 5 kV. The samples were gold-coated in a coater (Polaron) and examined using Zeiss EVO LS 10 scanning electron microscope.

3.7.2 Fourier transform infrared spectroscopy

The controlled release systems and the negative control materials were subjected to FTIR with the built-in single-reflection diamond attenuated total reflectance (ATR) crystal. Each sample was scanned with a resolution of 4 cm^{-1} in the range of 400-4000 cm^{-1} (16 scans per one spectrum).

3.7.3 Differential scanning calorimetry

Temperature-modulated differential scanning calorimetry was used in order to compare the melting-crystallization behavior of the films based on copolymers and PHA blends. The analyzed samples were equilibrated at 200 ℃ and after that cooled down to -80 ℃ at a cooling rate of a 2 ℃/min and a temperature modulation of 0.3 ℃ every 60 s. Following the equilibrium step (5 min at -80 ℃), the samples were heated to 200 with the heating rate of 2 ℃/min and the temperature modulation of 0.3 ℃ every 60 s. This heating scan was used for characterization of the thermal properties of the polymer.

3.8 Drug release assays

3.8.1 In vitro drug diffusion studies

The release study of ibuprofen from the PHA membranes and particles was carried out in phosphate buffer saline (pH 7.4) at 37 ℃ and 100 rpm in horizontal shaker. The model medium

was prepared by dissolving $Na_2HPO_4 \cdot 12\ H_2O$ (13.864 g), $NaH_2PO_4 \cdot 2\ H_2O$ (1.761 g) in 1 l of distilled water. Films and particles were analyzed as follows. Squares of films of equal dimension were cut. An initial films and particles were weighed using an analytical balance and then were placed in PBS at 37 ℃. The buffer was collected at fixed times, absorbance of collected release medium was scanned using a UV-VIS spectrophotometer, and then the measured sample was returned into the release medium. To monitor ibuprofen release from porous PHA material, the absorbance of distilled water was measured using UV-VIS. All samples were analyzed in duplicate between 200 and 800 nm.

4 Results and discussion

4.1 PHA copolymers synthesis

In this work, the carbon sources utilized for copolymer synthesis were γ-valerolactone, γ-hexalactone δ-valerolactone and glycerol at the concentration of 4 g/l. It is known that the utilization of different carbon sources during PHA cultivation, for example, lactones, results in the introduction of various monomeric units. These subunits in the molecular chain of P(3HB) decreases the degree of crystallinity of homopolymer. Consequently, produced PHA copolymers possess promising material characteristics for biomedical applications. The sample identification is listed in Table 12.

4.1.1 PHA production and analyses

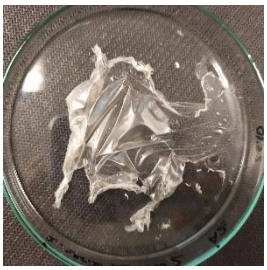
The cultivation was performed in a higher volume to isolate a sufficient amount of biomass. After cultivation, optical density of bacterial suspension was measured using nanophotometer at 630 nm. Further, the biomass was separated from suspension, washed with distilled water, centrifuged and allowed to dry to a constant weight in thermobox at 70 °C. Dry cell weight was determined gravimetrically in parallel samples. 5-11 mg of dried biomass was used to determine the monomer composition of copolymers by gas chromatography with a flame ionization detector. It was observed that the fraction of 4HV, 4HHx, and 5HV monomers in structure was relatively high (between 20 and 40%) while proportion of 3HV varied from 1% to 3%.

4.2 Basic characterization of isolated polymer

The extracted biofilms were also analyzed via gas chromatography. For clarification in the following chapters, copolymers: P(3HB-*co*-32.5%-5HV), P(3HB-*co*-3.9%-3HV-*co*-7.9%-4HHx), P(3HB-*co*-1.7%-co-3HV-*co*-23.4%-4HV), P(3HB-*co*-1.5%-3HV-*co*-33.0%-4HV) will be utilized for further experiments. According to results (Table 12, Table 14), a combination of AH30 with glycerol and γ-valerolactone was identified as the most suitable since the highest yield of dry biomass and highest PHA content were obtained (Table 12). Moreover, surface homogeneity and flexibility of the extracted solvent-casted film was observed. The more complication synthesis was observed when cultures growth on γ-hexalactone and glycerol. Despite the fact, that the high fraction of 4HHx in polymer was achieved, the cultivation must have been performed several times, due to the low PHA content in biomass.

The molecular weight and polydispersity index of solvent-casted films were analyzed by size-exclusion chromatography with multiangle light scattering and differential refractometer (SEC-MALS). The lower molecular weight was detected for polymer synthesized by isolate AFN2 in combination with precursor δ-valerolactone (about 44 kDa). *Řeháková et al.* reported that *Aneurinibacillus* is capable of synthesis of copolymers with molecular weight ranging from 51 to 143 kDa [63]. *Takeharu* discussed in his review paper that molecular weight of polyhydroxyalkanoates is affected by fundamental factors: the concentration of PHA synthase, catalytic activity of PHA synthase, simultaneous degradation of PHA during synthesis and occurrence of the chain transfer reaction [68]. It should be pointed out, PHA content in biomass, PHA composition, and as a result, molecular weight of polymer differs with respect to the individual cultivation The SEC-MALS data with other properties and visual illustration of synthesized PHA are shown in Table 12.

Table 12: Produced polyesters and basic properties

Bacterial strain	AH30	AFN2	AFN2
Main carbon source		glycerol	
Carbon source	γ-valerolactone	γ-hexalactone	δ-valerolactone
Sample identification	GVL	GHL	DVL
Extracted PHA film			
3HB (%)	74.9±1.3	88.1±0.2	67.5±2.4
3HV (%)	1.7±0.1	3.9±0.2	
4HV (%)	23.4±1.4		
4HHx (%)		7.9±0.5	
5HV (%)			32.5±2.4
PHA (%)	44.1±0.6	20.8±0.6	38.8±0.8
CDM (g/l)	3.9±0.3	1.7±0.1	3.34±0.4
Mw (kDa)	84.09±0.47	67.79±1.06	44.26±0.38
PDI	1.55±0.05	1.60±0.06	1.67±0.02

4.3 Controlled release systems based on synthesized PHA

In this work, produced PHAs containing unusual monomeric units were used for delivery systems designing. As was discussed previously, PHA copolymeric carriers possess beneficial properties since the release of incorporated substance is controlled by material characteristics of carrier material, which can be affected by process variables, including polymeric formulation and fabrication method. Ibuprofen was tested as a model drug since is known as soluble in solvents used in the experimental part, e.g., chloroform, dimethyl sulfoxide and ethanol. Also, successful application of ibuprofen in carrier systems were discussed in [65].

4.3.1 Optimization of fabrication parameters

This chapter deals with aspects affecting carriers' formation. The number of performed experiments studying these aspects and unsuccessful results showed procedures complexity, especially when microbial polyhydroxyalkanoates are used. Nevertheless, failed methods indicate that fabrication is affected by obvious parameters, for example, polymer solution concentration, but also by seemingly trifling conditions, such as time for cooling of polymer solution.

4.3.1.1 Particulate delivery systems

In the first series of experiments, ethanol was utilized as an aqueous medium and polymer dissolved in DMSO as an organic phase. By using this approach stable polymeric flocs were prepared. From Figure 8 (a) it can be seen that a decrease in ethanol concentration caused a decrease in number of particles, and there was no significant influence of ethanol percentage on the particles shape. Interestingly, the slower rate of polymer adding into water phase causes the greater number of flocs. Although nonuniform particles were stable in aqueous phase during a long-time interval and get aggregated after centrifugation under different operating conditions (Figure 8 (b)). Thus, the isolation of particulate carriers was later carried out by decantation, i.e. freely under the action of the gravity Figure 8 (c).

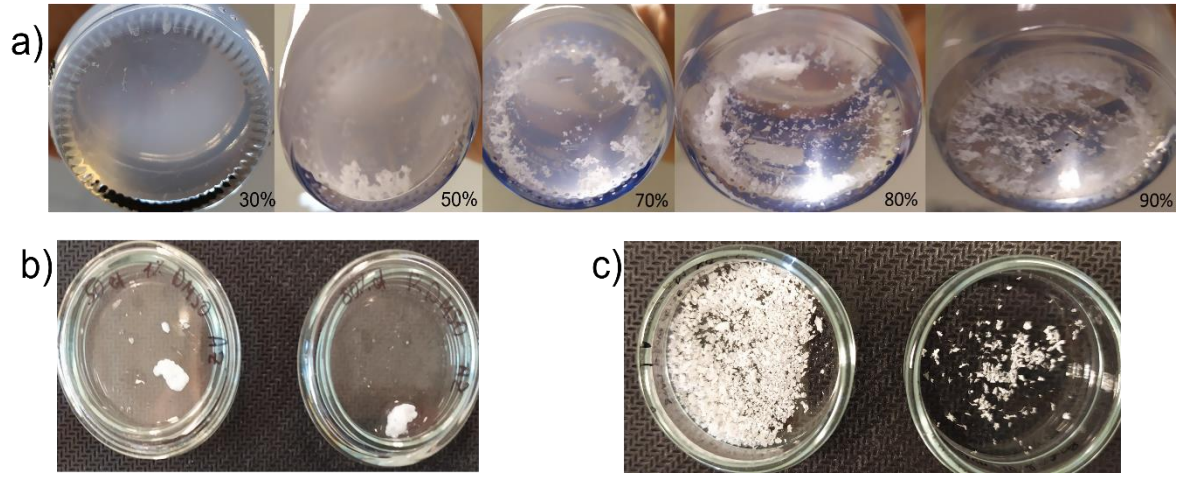


Figure 8: Procedure for preparation of particulate carriers. a)influence of ethanol concentration on flocs formation; b)particle aggregates isolated by centrifugation (3000rpm, 3min); c)particles isolated by decantation

For further optimization experiments, 80 %*v/v* ethanol was chosen as the aqueous phase. In brief, ibuprofen was dissolved in 20 ml of 80 % *v/v* ethanol. Then 1 ml of the polymeric solution of P(3HB-*co*-4HB) in DMSO was added dropwise. Lastly, particles were separated from the aqueous medium and allowed to dry at room temperature. The preparation procedure yielded nonuniform particulate systems which were visible under standard conditions. It can be highlighted; the color of the supernatant was nontransparent and the new particles formation in the remining solution was observed (Figure 9 (a)). Additionally, in the cold medium more flocculated particles were observed. To sum up, mentioned simple method can be successfully applied for fabrication of differently sized particles.

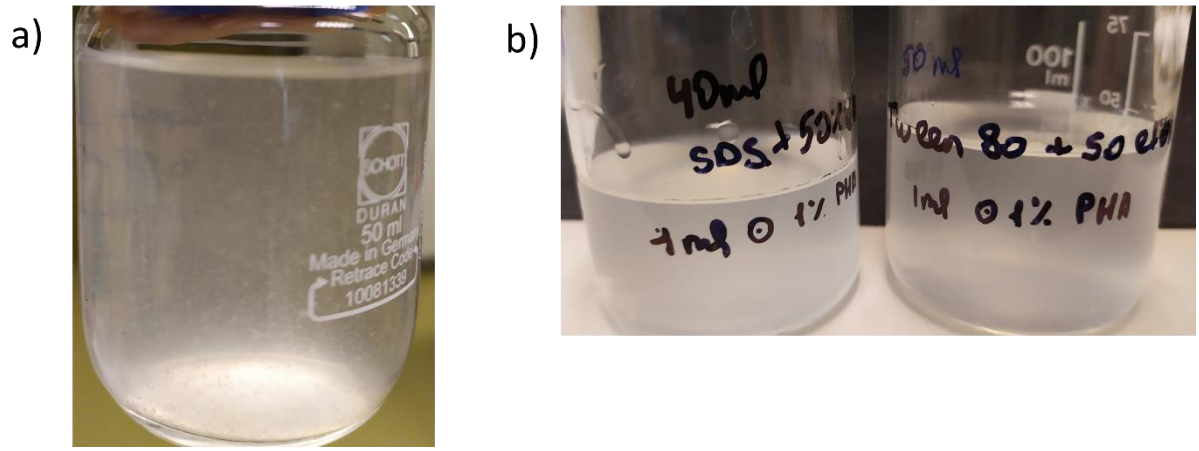


Figure 9: Fabrication of polymeric particles. a)supernatant after 15 hours from fisrt separation; b)preparation of carriers with stabilizers

Another series of experiments involved the utilization of detergents (SDS and Tween 20). Separate, stable and uniform particles were obtained for samples fabricated when 1 % Tween is used as an aqueous medium. Moreover, the size and number of particles can be easily controlled. The process of particles preparation is illustrated in figure 9. It was also found that viscosity of polymer solution has significant influence on the yield of this procedure. Thus, the optimal concentration was not found, due to the fact, that after an addition of various polymer solutions to the aqueous medium, different results were observed even when the same conditions were used (P(3HB), P(3HB-*co*-4HB), P(3HB-*co*-3HV-*co*-4HV) and P(3HB-*co*-5HV); concentration 4 *w/v* % for all of samples). The particles are displayed in figure 10. To summarize, detailed rheological characterization of polymer solution, with respect to the monomer composition and molecular weights of the polymer, can be a useful tool to understand more about the factors affecting particles fabrication.

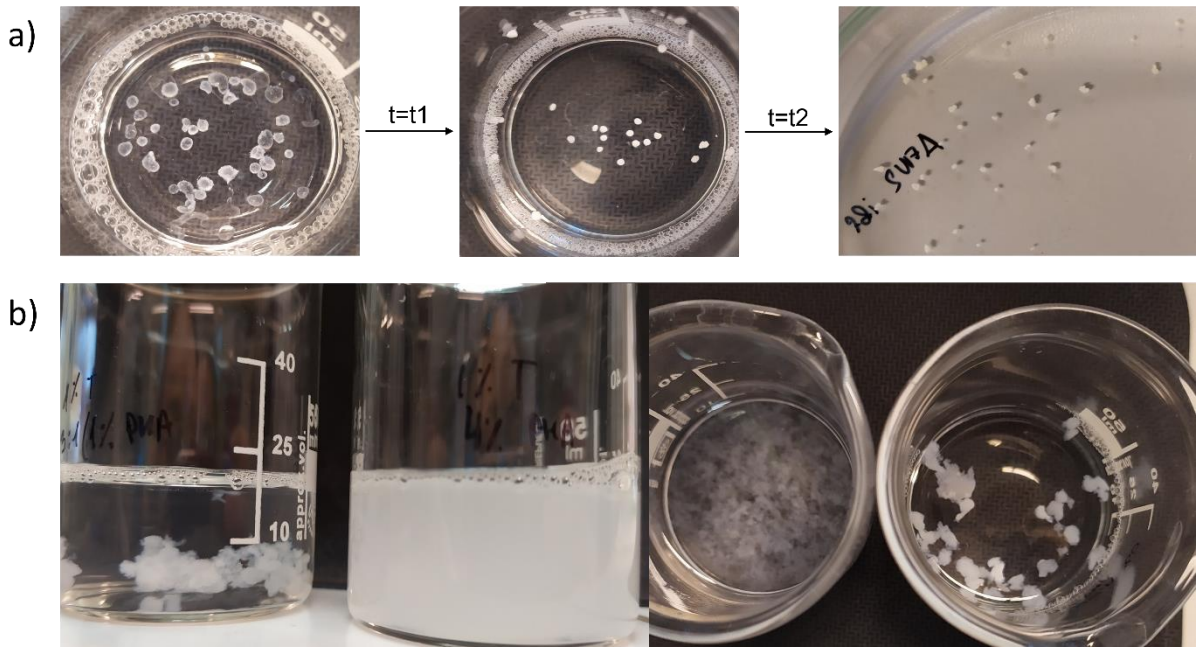


Figure 10: Procedure for particle preparation. a) phase separation of DMSO-copolymer system, b) optimization procedure for various polymers

4.3.1.2 Polymeric films

Solvent-casted films containing ibuprofen were prepared as follows: the copolymer and homopolymer PHA with various ratios (25/27,75/25,100/0) were dissolved in the particular solvent (chloroform or DMSO), and then ibuprofen was dissolved in the polymer solution. The solution was then poured into a Petri dish and allowed to dry. 4 *w/v* % polymeric membranes with drug concentration of 0.3 mg/ml were washed with distilled water.

Since DMSO is not as good solvent for polyhydroxyalkanoates as chloroform, the higher temperatures and longer time were applied for this (70-80 ℃ warm water bath, for 60-90 minutes for dimethyl sulfoxide; 60 ℃ warm water bath, for 40-60 minutes in case of chloroform). The solvent-casted films differ in the terms of surface homogeneity, and mechanical features. Morphology of prepared films can be influenced by the solvent used for polymer dissolving or by polymer-solvent interaction. The evaporation of solvent with higher boiling point (in this situation DMSO, melting point 189 ℃) is slower comparable to chloroform. As the solvent evaporate slowly, more porous structure was obtained (Figure 11). Additionally, the solvent-polymer interactions affected film properties. It can be assumed, in the studied formulation (DMSO-PHA), dimethyl sulfoxide is at ambient temperature "poor" solvent for polyhydroxyalkanoate. Also, in several studies was mentioned PHA insolubility at laboratory temperature for system DMSO-PHA [67]. Thus, because of polymer-solvent relationship in studied formulation, polymer chains prefer to interact with each other, which can lead to polymer contraction, and as a result, to the formation of pores. Membranes prepared at high temperature using DMSO as a solvent were less translucent than chloroform films. It can be attributed to light scattering at the semicrystalline structure. The surface of chloroform-PHA films was smooth, homogeneous, and thin biofilm were flexible. To sum up this part of work, the choice of components has a significant impact on the material properties, such mechanical strength, flexibility, and surface morphology.

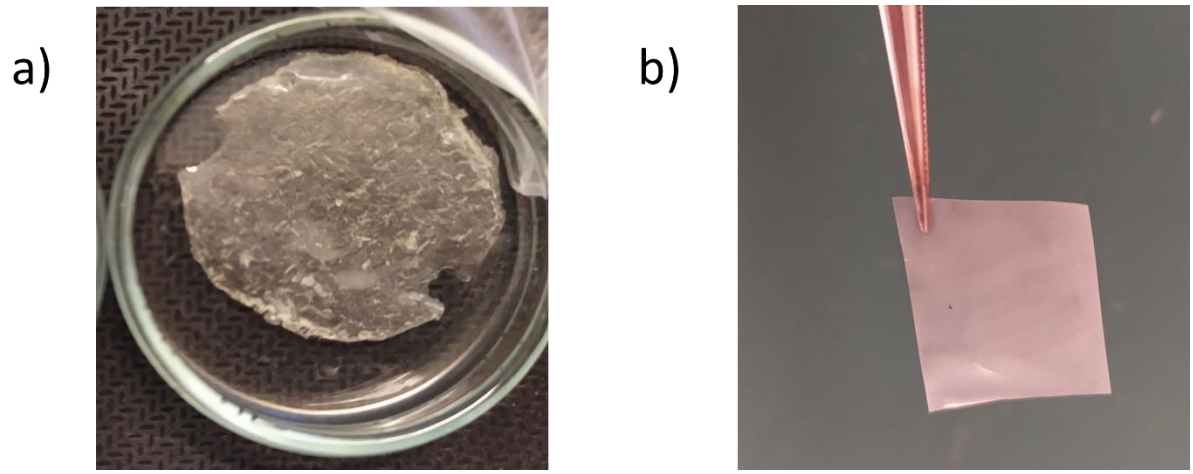


Figure 11: PHA films prepared using different solvent. a) dimethyl sulfoxide; b) chloroform

4.3.1.3 Polyhydroxyalkanoate porous matrices

Polymeric gels were prepared by mixing 3 mg ibuprofen with dimethyl sulfoxide. Then copolymers and homopolymer P(3HB) in ratios 25/75, 75/25 were dissolved in this solution at 70-80 ℃. The most interesting observation in this case was the phase separation of polymer

mixture (illustrated in Figure 12). In the first step, viscous liquid of polyhydroxyalkanoate is transparent. After quenching at laboratory temperature, the viscous polymer mixture became gelatinized and cloudy. This intermediate can be classified as a gel. The last step is exchange of DMSO with another solvent, for example water or ethanol, resulting in production of white porous solid.

In this process, there are several factors affecting the properties of final material. First of all, the time t_1 shown in Figure 12, which represents time for creation of new bonds between polymer chains. Thus, microstructure of the porous material depends on the interactions occuring in the system. Furthermore, the amount of the second solvent (water, ethanol, etc.) and time t_2, represented time of solvents exchange, can affect the pore size and distribution.

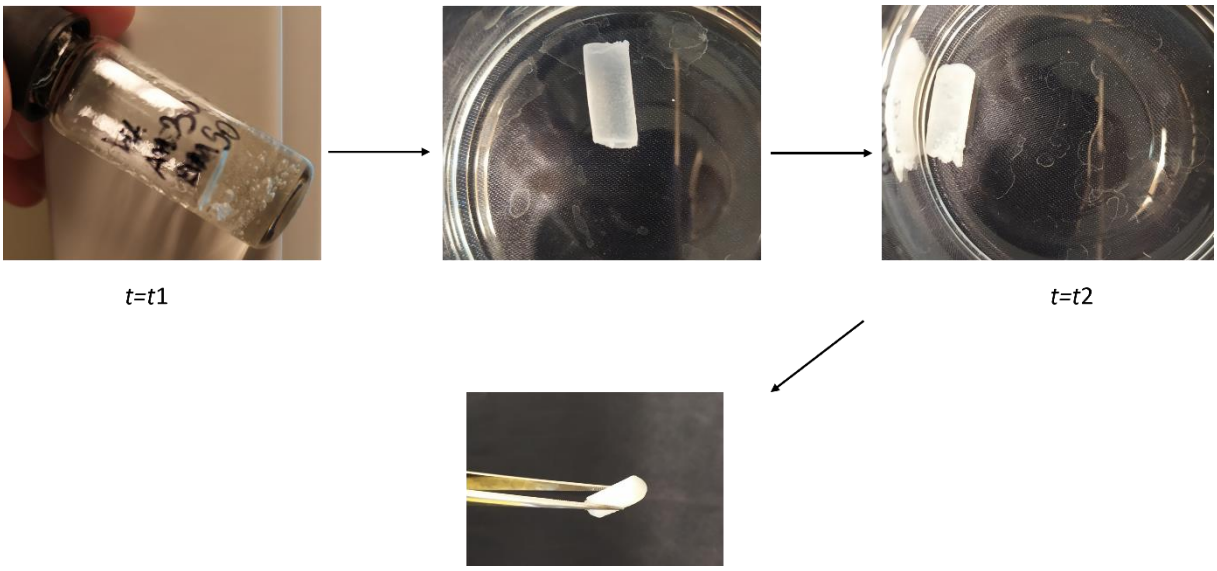


Figure 12: Stages of porous material preparation

The main problem of this carrier is the fact, that ibuprofen tends to release into second solvent since dimethyl sulfoxide must be exchanged with water (or ethanol). However, porous monoliths possess many attractive attributes for different applications, for example, scaffolds.

4.3.2 Designing carriers based on isolated PHA

Isolated copolymers used in the final protocol behaved differently in their ability to form composite carriers with incorporated ibuprofen. A suitable concentration of polymers was found after a series of screening experiments. The most challenging aspect of the optimization process was utilizing DMSO, since there are just a few studies that have reported combination of polyhydroxyalkanoates and dimethyl sulfoxide for carriers' designing, for example [67].

Polyhydroxyalkanoates with various monomer composition used to preparation procedures are displayed in Table 13. The choice of polymer was based primarily on results of cultivation, for the reason that sufficient amount of isolated film is necessary for the preparation procedure. Generally, 200 mg of dried biomass yielded approximately 70-80 mg foils, and the lowest amount of foil (40 mg) was extracted from biomass produced by AFN2 (glycerol + γ-hexalactone).

Table 13: List of used polymers in experimental part

Type of system	Polymer formulation	
	solvent	polymer
PHA films	DMSO	P(3HB-*co*-32.5%-5HV) and P(3HB)
		P(3HB-*co*-3.9%-3HV-*co*-7.9%-4HHx) and P(3HB)
	$CHCl_3$	P(3HB-*co*-1.7%-co-3HV-*co*-23.4%-4HV) and P(3HB)
		P(3HB-*co*-32.5%-5HV) and P(3HB)
particles	DMSO	P(3HB-*co*-1.7%-co-3HV-*co*-23.4%-4HV)
		P(3HB-*co*-32.5%-5HV)
porous material	DMSO	P(3HB-*co*-1.5%-3HV-*co*-33.0%-4HV)* and P(3HB)

**monomer composition were analyzed by GC-FID only in biomass*

4.4 Characterization techniques of controlled release systems

4.4.1 Surface morphology

The surface morphology of prepared delivery systems was studied with a Zeiss scanning electron microscope. All samples were dried and coated with gold to avoid charging.

The parameters affecting PHA films and porous monoliths were detailly described previously, therefore surface characteristics of this types of delivery systems were expectable. Size and pores distribution correspond both with the solvent used for polymer dissolution, and with the P(3HB) amount in initial formulation. Crystallinity of homopolymer causes changes in the surface as follows: the more percentage of P(3HB) is in the blend, the more holes in the structure are obsessed, illustrated in Figure 13. In contrast, films prepared using DMSO had a patchy appearance and created gels when P(3HB) was added into initial formulation. Due to this, release studies were focused primarily on solvent casted films prepared with chloroform solution.

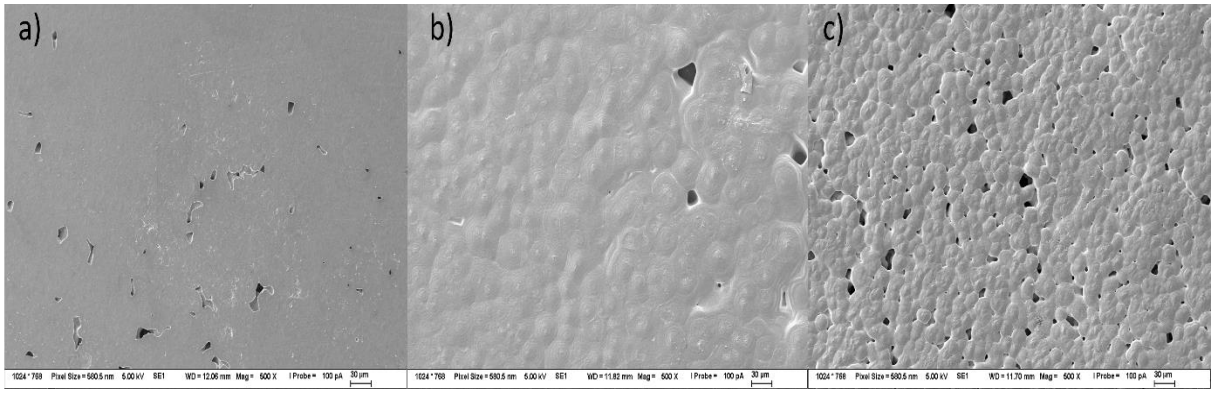


Figure 13: SEM images of GVL film (film identification according to Table 12). a) pure copolymer; b) blend GVL/P(3HB) 25/75; c) blend GVL/P(3HB) 75/25. Magnification ×500 for all samples

SEM images of porous PHA materials showed the morphology was microporous with highly heterogeneous distribution of pore sizes. Based on different contrast in one sample, porous PHA had three-dimensional structure. Porosity and tortuosity of this material can be considered important and had the potential not only in controlled release systems, but in the process of adsorption. One study deal with porous carriers for drug delivery and their applications, including controlled release of drug, improvement of surface affinity, or solubility improvement [69].

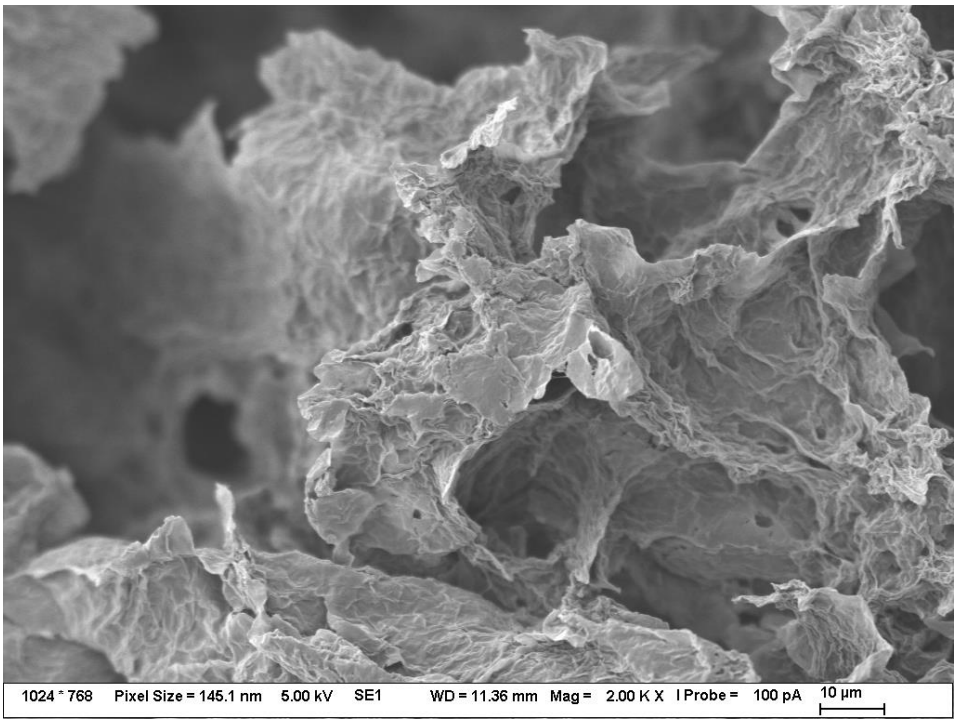


Figure 14: Porous PHA monolith, solvent for phase separation water magnification ×2000

The morphology study on prepared particles provided some surprising insights. As was mentioned, flocs prepared via first method (when ethanol was applied) were nonuniform and differently sized. Analyze of the surface characteristic shows that one analyzed particle consists of aggregated nanoparticles with similar shape and size, as seen in Figure 15. Consequently, different fractions of carriers can be designed by this simple method. Even though isolation of

particles and preventing of the aggregation are open issues. Detailed images of particulate delivery systems fabricated via emulsification with Tween 20 are shown in the same figure. Overall, it was found, that morphology of samples fabricated using surfactant was similar: roughness with randomly distributed holes. Also, it is possible, that pores can interconnect to form channels which accelerate drug release.

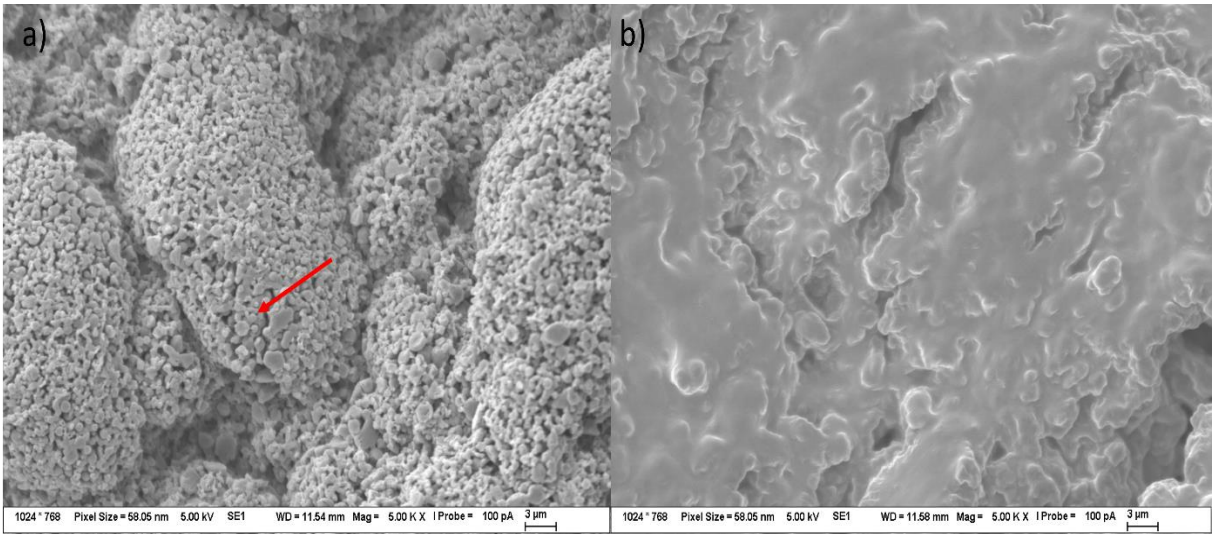


Figure 15: SEM images of particles. a) carriers prepared with ethanol; b) carriers prepared with Tween 20. (magnification ×5000 for both samples)

4.4.2 Characterization of the carrier systems by molecular spectroscopy

Fourier transform infrared spectroscopy was used to confirm the incorporation of model agent into the cargo material. In brief, ibuprofen, pure polymeric particles, biofilms and drug-loaded carriers were analyzed in the range 4000 to 400 cm^{-1}. The characteristic peaks that are commonly observed in the spectra of polyhydroxyalkanoates corresponds to the functional groups of polymers. A peak abroad at 1725 cm^{-1} attributes to the C=O vibration of the ester carbonyls. Further, the vibration band at 1180 cm^{-1} corresponds to the amorphous PHA domains in the homopolymer P(3HB), and the crystalline phase attributes to values 1276 cm^{-1} and 1227 cm^{-1} [50][63]. Generally, long carbon chains in copolymers affects the spectrum patterns associated with stretching band C-H (2800–3000 cm^{-1}) and bending vibrations (1350–1460 cm^{-1}) CH_2 in the polymer [50].

ATR FTIR spectrometry is a powerful tool to investigate the structural motifs of the different types of polyhydroxyalkanoates. In case of drug delivery systems, spectra provided limited information about chemical structure of PHA since the model agent disposes of own absorption. The characteristics peaks of ibuprofen include: the hydroxyl group at 1231 cm^{-1}, C-O stretching at 1184 cm^{-1}, the multiply spectral lines at around 2900–3000 cm^{-1}, and peaks at 1715 cm^{-1}, 2955 cm^{-1} for carboxylic acid group [70]. According to subtracted spectra of ibuprofen in particulate carriers, the model agent was successfully incorporated when particles were fabricated using first technique (ethanol is a water medium) (samples from 1 to 3 of copolymer P(3HB-*co*-4HB) in Figure 17). Interestingly, ibuprofen in flocs was detected even if the experimental procedures differed in terms of step sequence, for example ibuprofen dissolution of model agent in ethanol (1.sample) or in DMSO (2.sample). In contrast, the analyzed particles

fabricated via second method did not provide identifiable peaks in the regions for ibuprofen detection. Additionally, ibuprofen appearance was not confirmed for solvent-casted films. However, data collected from UV-VIS analysis showed strong peak at 220-223 nm when ibuprofen released from biofilms and particulate carriers. It can be supposed, concentration of ibuprofen is not sufficient for characterization with IR spectroscopy, due to the fact, that fabrication process involves finishing steps, such as washing, or solvent exchange during time *t* (Figure 10a). Also, the model drug can be hardly detectible since information is collected from the surface of the analyzed material and, in case of particles, ibuprofen can be surrounded by polymeric layer. Further, it can be explained by the phase separation of solvent-polymer. Due to the interactions between two components, especially when solvent is "poor" for PHA, the polymer-rich and polymer-poor zones appear. Local segregation leads to uneven distribution of the polymer, and as a result, to uneven distribution of incorporated drug in the mixture. *Anbukarasu et.al* explained similar situation, where phase separation on the P(3HB)/acetic acid system was caused by interactions between solvent and macromolecule. Authors concluded, local segregation can affect properties such as thermal, mechanical, and crystallization behavior [71]. Presence of poor and rich zones in studied systems can be confirmed by one observation. Ready-to-use films with the same area was weighted before drug release analysis. Release studies were carried out in duplicate, however, weight of the same-sized samples differed, eventually for ibuprofen-loaded films. In addition, assumption about local segregation can be confirmed by the SEM images of DMSO-PHA casted films. As seen (Figure 16), the samples contain two regions which differ in terms of their translucency, morphology, and, probably in terms of their mechanical and transport properties. Also, the third dimension of samples was not controlled, thus polymer and model drug disappeared unequally.

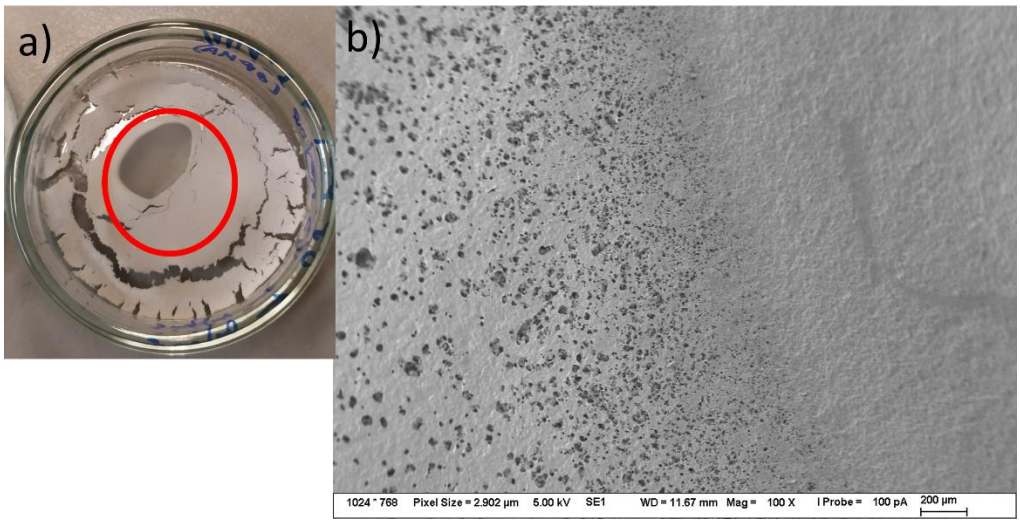


Figure 16: P(3HB-co-4HB)-DMSO solvent-casted film. a) illustration of two regions, b) SEM image of P(3HB-co-4HB)-DMSO film, magnification ×100

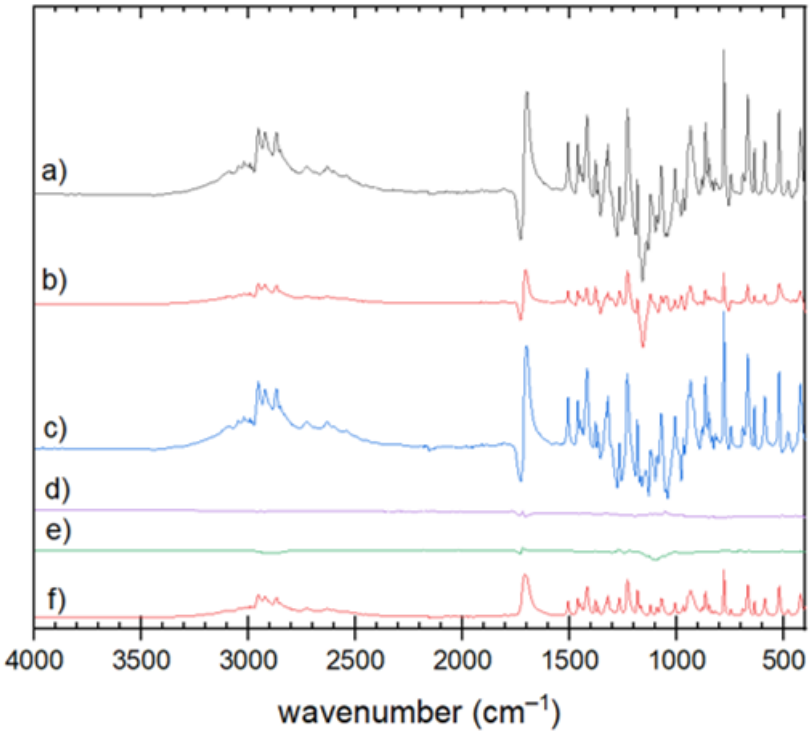


Figure 17: Results of subtraction of the FTIR spectra of the carrier with and without ibuprofen, respectively. a) P(3HB-co-4HB) preparation with ethanol (IBU dissolved in ethanol); b) P(3HB-co-4HB) preparation with ethanol (IBU dissolved in DMSO); c) P(3HB-co-4HB)/P(3HB) (50/50) blend preparation with ethanol (IBU dissolved in ethanol); d) DVL preparation with Tween 20; e) GVL preparation with Tween 20; f) spectra of pure IBU

4.4.3 Thermal properties

Further, solvent-casted films were subjected to differential scanning calorimetry, in order to investigate the melting-crystallization behavior of samples. In this work, the blends of polyester/P(3HB) in ratios 25/75,75/25 100/0 were analyzed with DSC (Table 13). Generally, peak at about 170 °C showing melting endotherm of the P(3HB). Further, double melting peak is common in PHA materials. *Nováčková et al*. in review also explained, incorporated monomer subunits exhibit double melting peaks [72]. -

As expected, differences in the melting behavior of casted films were detected. It is clearly seen, with increasing content of homopolymer in samples, the melting point of film increases. Interestingly, termogram DVL, AFN2 (Figure 29) contains strong peak for melting point since *Řeháková et al* reported, that melting endotherm for copolymer containing 5HV is hardly detectable. In terms of glass transition peak in termogram, this thermal parameter indicates presence (and relative content) of amorphous phase in material [63].

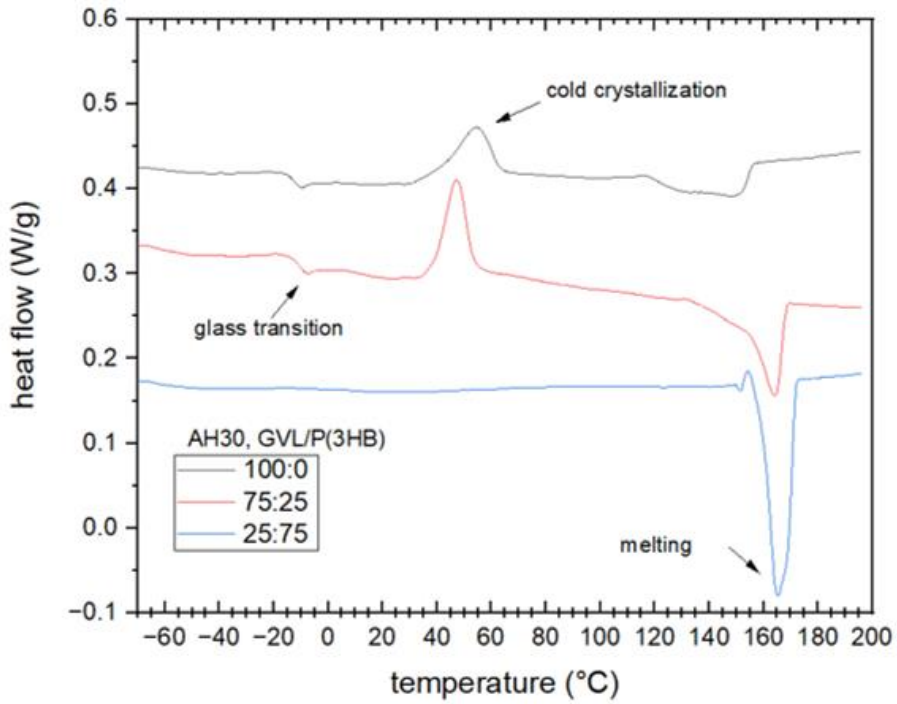


Figure 18: Combined DSC curves for GVL/P(3HB) blends prepared in chloroform

Result of DSC suggested that analyzed solvent-casted films are semicrystalline. Moreover, thermal properties were dramatically changed when poly(3-hydroxybutyrate) was used as a second polymer in initial mixture. From the measured curves and reported ones, it was found, that termograms do not match. However, processing procedure can affect the thermal properties of polymeric material as in this work isolated film was redissolved to form 4%*w/v* solution. Also, the higher temperature was used for preparation procedure of DMSO solution. Thus, only DSC analysis provides limited information and additional techniques are necessary for complex characterization, for example thermogravimetric analysis, and X-ray diffraction.

4.5 Transport properties of PHA delivery systems

The main issue of this work was to compare the transport properties of delivery systems based on polyhydroxyalkanoates. The model drug (ibuprofen) was successfully incorporated into polymeric particles and films. Firstly, optimization procedures were carried out using copolymer P(3HB-*co*-4HB) and commercial P(3HB). Based on screening experiments the optimal amounts of model medium and carrier materials were found. To measure ibuprofen release from carriers, a UV-VIS absorption spectroscopy was used. The absorbance of the model drug in PBS was scanned at different time intervals, and differences in transport properties can be seen in the release profiles represented in Figure 19 and Figure 21. As seen, rapid drug release from carriers of each sample was observed at the beginning of experiment. Comparing the release profiles of ibuprofen from the films, it can be seen that the release rate increases with decreasing P(3HB) percentage in the formulation. Blend prepared with P(3HB-*co*-1.7%-co-3HV-*co*-23.4%-4HV) and P(3HB) (25/75) showed the lowest release (Figure 19b), and the higher release rate was observed for copolymer P(3HB-*co*-32.5%-5HV) illustrated in Figure 20. Surprisingly, it was found, there is no significant differences in release profile of DVL films, when P(3HB) was added into formulation (Figure 19a)

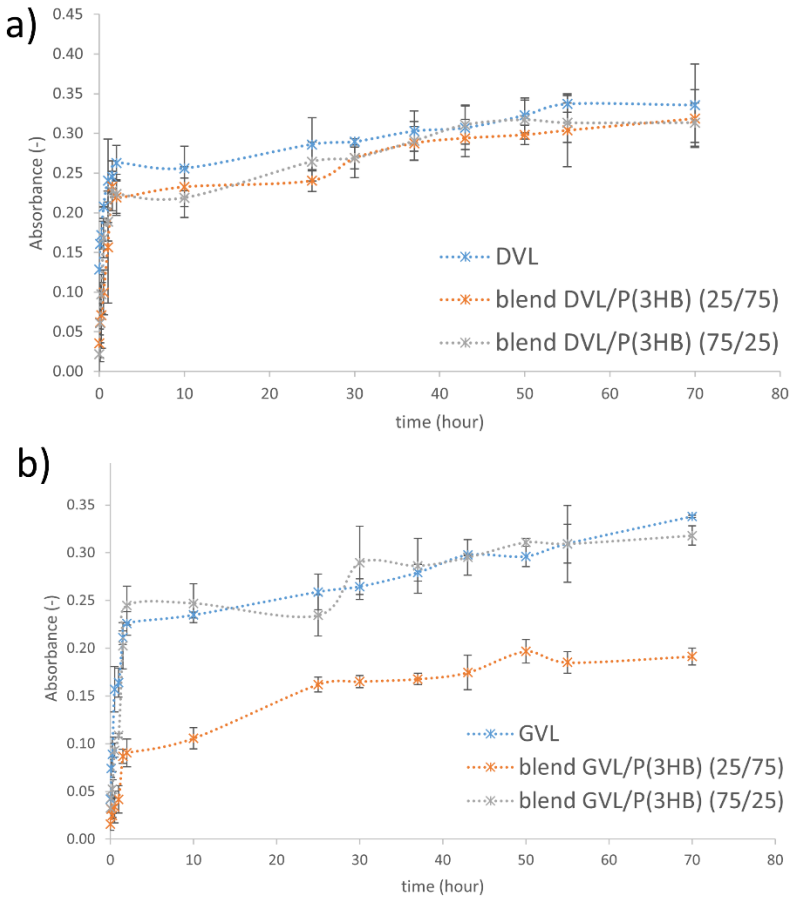


Figure 19: Time evolution of absorption peaks. a) blends of DVL/P(3HB); b) blends of GVL/P(3HB)

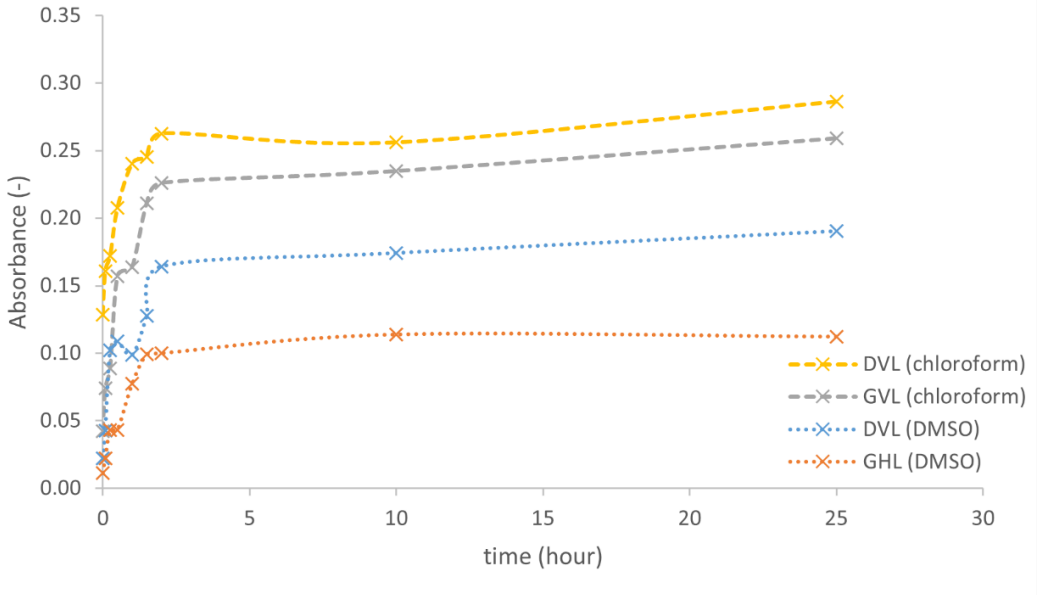


Figure 20: Combined time evolution of absorption peaks for films

Further, it was assumed for particulate carriers, that the faster release rate should be detected for copolymer P(3HB-*co*-32.5%-5HV), since percentage amount of 3HB is lower comparable to copolymer P(3HB-co-1.7%-*co*-3HV-co-23.4%-4HV). Such a release profile behavior was

appeared (Figure 20). Moreover, as seen from figure, ibuprofen release from DVL is slower for the film prepared using DMSO. Taking into consideration the fact that the copolymer was dissolved in DMSO, the solvent can have an impact on the crystallinity of films. As was mentioned in the experimental part, dissolution was carried out at a higher temperature. Thus, the slow cooling allows for more organized structure since enough time is available for rearranging polymer chains into well-defined crystalline regions. The similar situation was observed when the crystallinity of P(3HB) casted films increased with increased processing temperature.[71]. This indicates that transport properties can be controlled by various factors, including copolymer composition, and thermodynamics of polymer/solvent systems. The restrictive consideration for PHA/DMSO samples is the deformation of UV-VIS spectra after 20 hours (Figure 35), which indicates the possibility of polymer disintegration. Because of this, the graph of time evolution of absorption peaks for all samples prepared with DMSO were represented for interval between 0 and 25 hours and, as seen, with larger deviation (Figure 21). Totally, it can be concluded in this section, chemical structure of PHA used for film fabrication and the ratio copolymer/P(3HB) affected the release of ibuprofen. Despite the fact, that data are in agreement with the expectation, the are several limitations. Residual dimethyl sulfoxide in the samples can change position of the band maximum for ibuprofen (at 223 nm), since absorption peak for DMSO was detected at wavelength between 208 and 210 nm. Another limitation was described above, the deformation of absorption spectra can be caused by scattering on the polymer chains released into the analyzed medium.

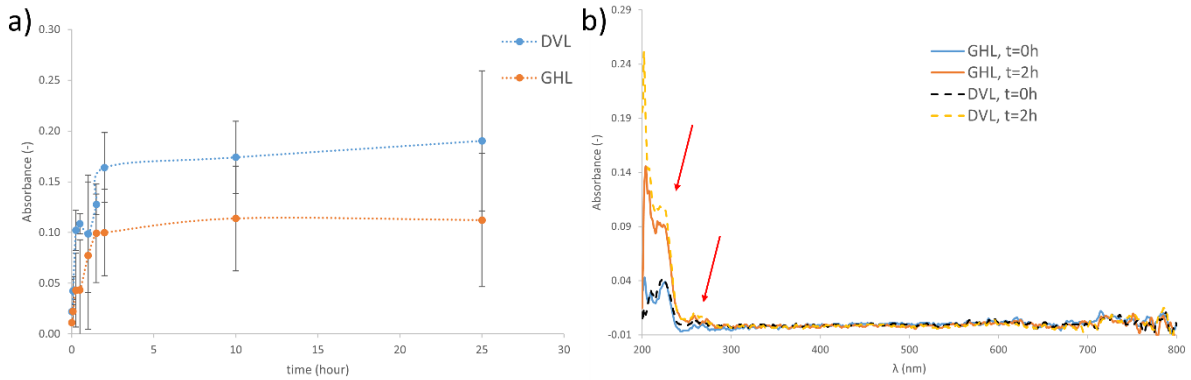


Figure 21: UV-VIS spectra of DMSO films. a) time evolution of absorbance; b) raw data

The release of the ibuprofen from particulate carriers was observed using the same method: the model medium was analyzed via UV-VIS spectroscopy. As seen in Figure 22, incorporated agent release faster from P(3HB-*co*-32.5%-5HV), particles than from carriers prepared from P(3HB-*co*-1.7%-co-3HV-*co*-23.4%-4HV). The same tendency was observed for polymeric films. Furthermore, it was defined there is no correlation between peaks at 208 and at 223 nm, while intensity around 265-270 nm increases with time. Additionally, drug release from particles prepared by first method which was tested during optimization, was also investigated. The data are provided in supplementary materials. Based on drug release analyses, it can be summarized that ibuprofen was successfully incorporated into polymeric particles and its release rate can be affected by carrier composition. However, in order to get more detailed understanding, the characterization of formulation and prepared particles is necessary.

Parameters, such as encapsulation efficiency, size distribution, or stability are the most studied of them.

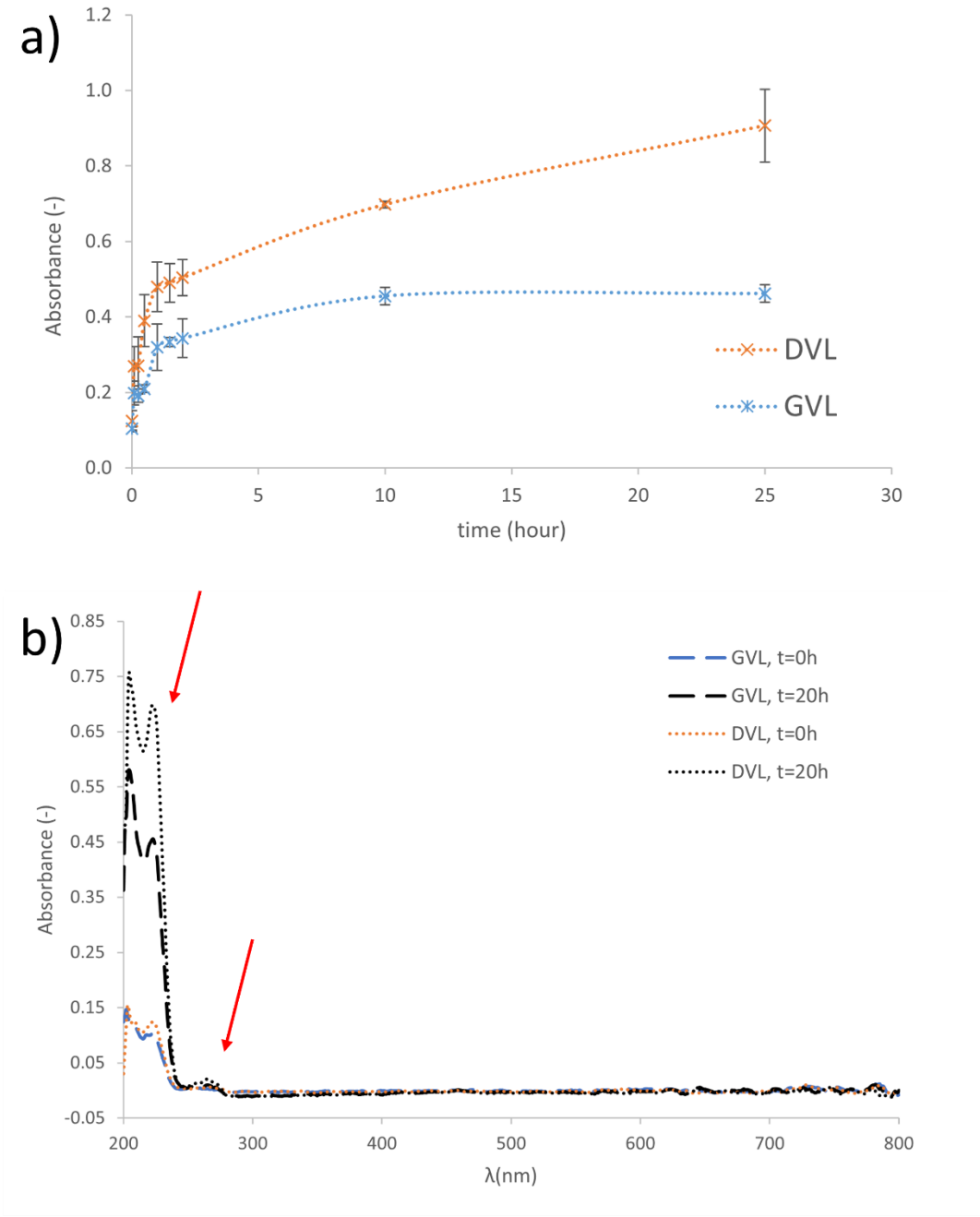


Figure 22: UV-VIS spectra of DMSO particulate carriers. a) time evolution of absorbance; b) raw data

Despite the fact, that the porous monoliths based on PHA possess numerous promising attributes, the transport property is a one limited consideration since the results show drug release from carrier at the beginning of experiment (Figure 23). This type of carrier exhibited burst release of incorporated ibuprofen into the water (during solvent separation at Figure 12). Further examination of the ibuprofen concentration in release medium (water) did not show any changes in UV-VIS raw spectra. On the other hand, there is possibility to create novel multifunctional material, for example, incorporation of drug-loaded particles into porous

matrices based on PHA. The most attractive thing in this case is regulation of material characteristics, such as porosity of system, surface morphology, mechanical properties and so on. One option to modify this material is blending PHA with polyvinylalkohol, which, according to preliminary experiments also performed in this work, has proven to be an option to improve mechanical features and surface roughness.

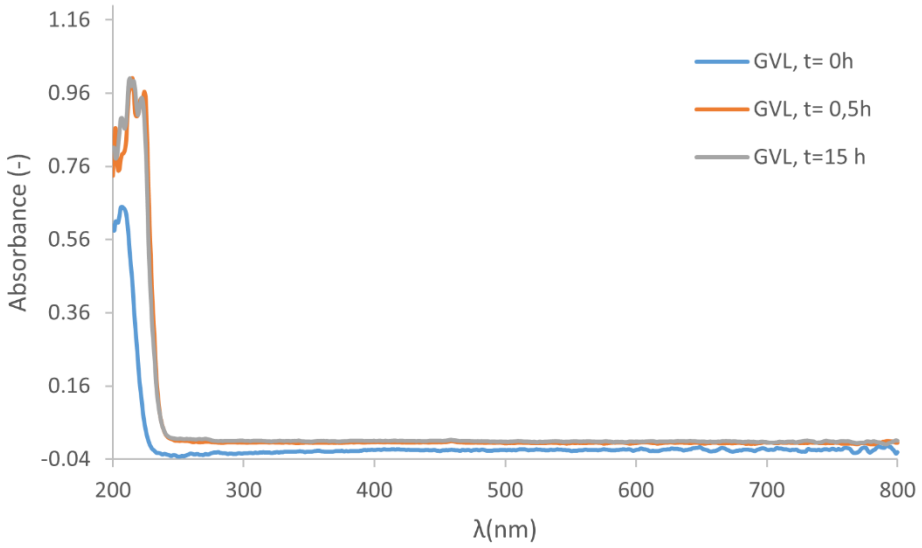


Figure 23: Raw spectra of release medium at different time for porous material

To sum up, delivery of active agent can be controlled by various factors. Several insights into were obtained: chemical structure of polymer plays an important role in material properties, while polymer thermodynamics is a key aspect. Minor changes in polymer formulation have significant effect in analyzed system. Thus, each of described carriers should be clarified on an individual basis with regard to polymer/solution status.

5 Conclusion

The aim of this work was to investigate the material properties of different carriers fabricated using microbial polyhydroxyalkanoates. The thermophilic *Aneurinibacillus* is capable of production of polymers containing various monomeric subunits, such as 3-hydroxyvalerate, 4-hydroxyvalerate, 4-hydroxyhexanoate, and 5-hydroxyvalerate. The amount of accumulated PHA was quantified by gravimetric method. From sediment samples, the monomeric composition of copolyesters were analyzed by gas chromatography. Based on results, isolate AH30 showed capability to synthesize the highest amount of polymer. The extracted PHA copolymers were characterized by GC-FID and SEC-MALS.

The synthesized PHA biofilms and commercial P(3HB) powder were utilized for preparation of controlled release systems. Thus, next part of experimental section was focused on optimization procedures for fabricating carrier systems. Different polyesters such as (P(3HB-*co*-3HV-*co*-4HV), P(3HB-*co*-3HV-*co*-4HV), P(3HB-*co*-5HV)) and blends of P(3HB)/(synthesized PHA) were tested as carrier matrices for model active agent. According to thermal analysis (differential scanning calorimetry), blending of P(3HB) with copolymers produced materials with manipulated crystallinity. Consequently, blending can be applied to tailor transport properties of polymeric delivery systems since these factors correspond. Above that, chemical structure, in particular, fraction of monomeric subunits, has influence on the chain mobility in polymer systems.

Particles, films, and porous monoliths were characterized in terms of their transport properties. Collected UV-VIS spectra revealed that ibuprofen was successfully incorporated and released into model medium. Samples based on polymer P(3HB-*co*-5HV) demonstrated faster release of ibuprofen when comparable to other polymers. Moreover, this copolymer has been found as a unique candidate for application in delivery systems, due to its processability. All type of tested carriers was fabricated with copolymer P(3HB-*co*-5HV). In contrast, copolymer P(3HB-*co*-3HV-*co*-4HV) can be utilized for PHA-based porous material fabrication since copolymer creates viscous liquid when dissolved in DMSO even if lower concentration was used. The last PHA tested in this work was P(3HB-*co*-3HV-*co*-4HHx). Although results of analysis were presented in experimental part, polyester DHL was not characterized in detail. Combination of AFN2 and γ-hexalactone revealed poor PHA production (21%). Because of this fact, applicability of P(3HB-*co*-3HV-*co*-4HHx) in terms of carrier materials remains to perspective direction for future investigations.

Particulate carriers made of GVL and DVL were prepared via solvent-exchange method Several different processing conditions were tested to achieve uniform and stable carriers. After optimization, drug-loaded particles were analyzed with SEM and FTIR. Results of ATR-FTIR confirmed that the ibuprofen was incorporated into carrier when ethanol used as an aqueous medium. Instead, UV-VIS spectroscopy detected model agent in both analyzed samples. SEM images of the particulate carriers exhibited the differences in morphology as a consequence of implementing various preparation conditions. Morphological evaluation of films and gels was also investigated. The gradual increase of pores number and increase of their size were observed when polymers were dissolved in DMSO. Contrary, the chloroform-casted polymer films showed no significant roughness even if ibuprofen was added for initial formulation. Changes

in surface morphology of porous matrices were also monitored. Interestingly, rugosity of the sample was controlled by several factors including polymer composition and formulation, phase separation, and solvent properties for phase separation. Analysis of ibuprofen release into PBS from porous material failed. However, ibuprofen was detected in “exchange” medium.

In this study, PHA-based carrier systems were characterized. Drug-loaded system formation and its release profile are dependent upon various aspects. The crucial factors affecting the transport properties are the molecular structure of polymer and polymer conformation. The monomeric unit and its amount are important parameters to control the crystallinity of the material and the release rate of incorporated drug. Further, preparation procedure is another important point. As was observed in experimental part, optimization procedure is necessary to achieve suitable conditions for individual system. In addition, polymer-solvent relationship and its behavior have significant impact on the processability of polymer formulation and on the properties of the final material. Characterization of interactions between polymer chains and environment is important for polymer processing.

In conclusion, polyhydroxyalkanoates can be fabricated into various forms, including particle dispersion, gels, films, and porous monoliths. Since properties of PHA are tunable, material characteristics of final material can be also controlled. Taking into consideration biocompatibility and degradability, PHA show great potential for use in various biomedical applications, such as drug delivery, implants, or tissue engineering.

6 References

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7 Appendix

Table 14: GC-FID results of cultivation (for biomass)

GVL	3HB%	3HV%	4HV%	4HHx%	5HV%	PHA (g/l)
	73.0 ± 5.1	1.6 ± 0.2	25.4 ± 5.3			4.2 ± 0.5
	64.9 ± 0.9	1.5 ± 0.05	33.5 ± 0.8			4.7 ± 0.2
	92.1 ± 0.5	1.6 ± 0.4	6.3 ± 3.3			4.4 ±-0.3
GHL						
	85.3 ± 1.7	3.5 ± 0.1		11.1 ± 1.8		2.3 ± 0.6
	88.9 ± 0.2	2.7 ± 0.5		8.3 ± 0.3		1.2 ± 0.2
	94.5 ± 0.1	1.2 ±0.4		4.3 ± 0.5		
DVL						
	69.5 ± 2.3				30.5 ± 1.3	3.2 ± 0.1
	67.5 ± 2.4				30.5 ± 2.4	2.0 ± 0.4
	82.2 ± 1.1				24.1 ± 0.9	2.8 ± 0.1

Table 15: Optical density and amount of isolated biomass

	DVL	GVL	GHL
1.OD_{630}	4.636	4.118	2.145
2.OD_{630}	4.318	3.65	1.854
1.biomass (g)	0.6463	0.356	1.048
2.biomass (g)	0.8603	0.4843	0.677

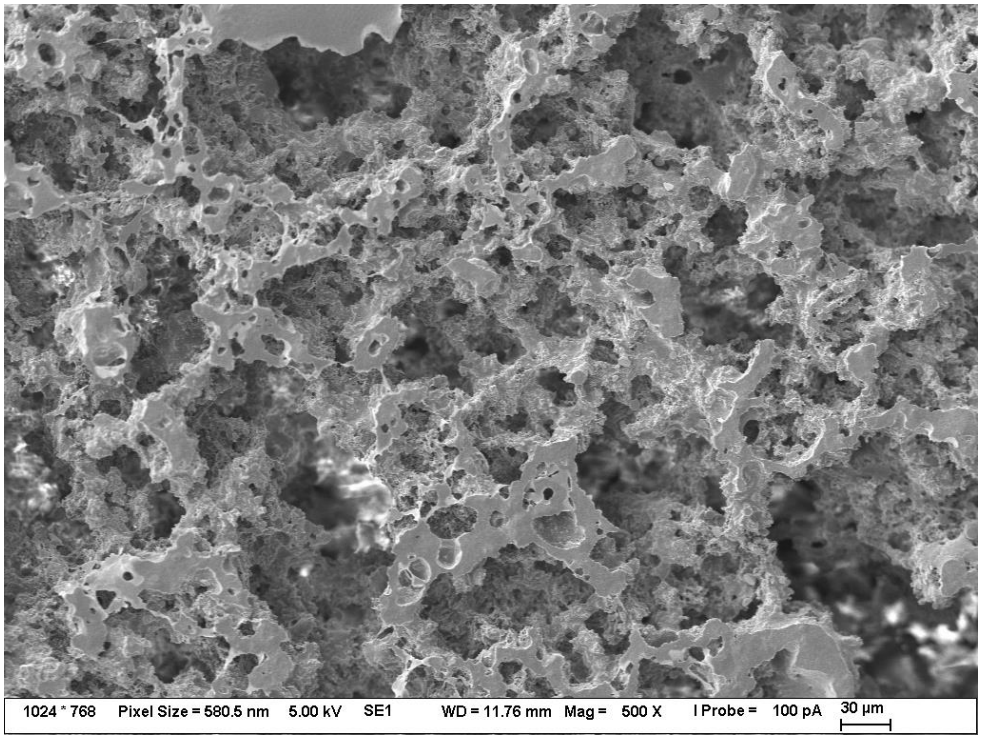

Figure 24: SEM image of P(3HB-co-4HB) film

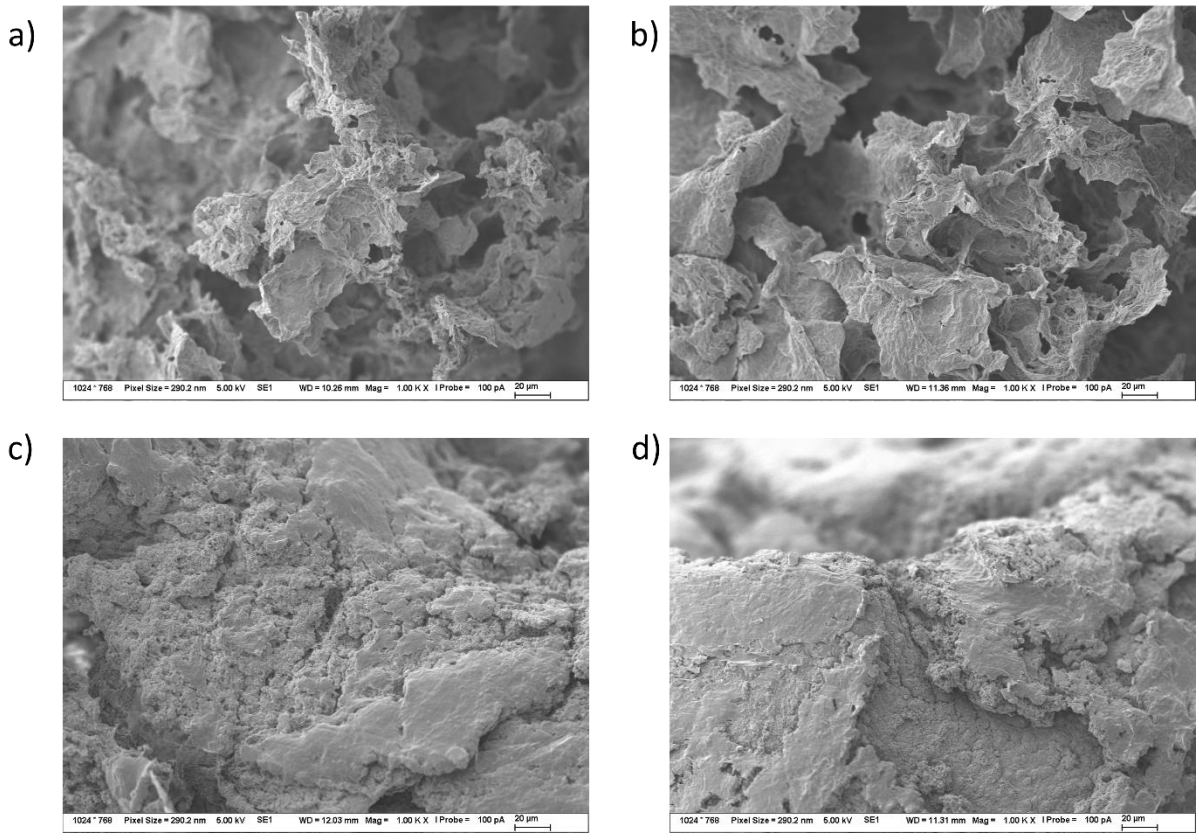

Figure 25: SEM images of porous materials. a) P(3HB-co-4HB) washed with ethanol; b) P(3HB-co-4HB) washed with distilled water; c) GVL with IBU; d) pure GVL. ×1000

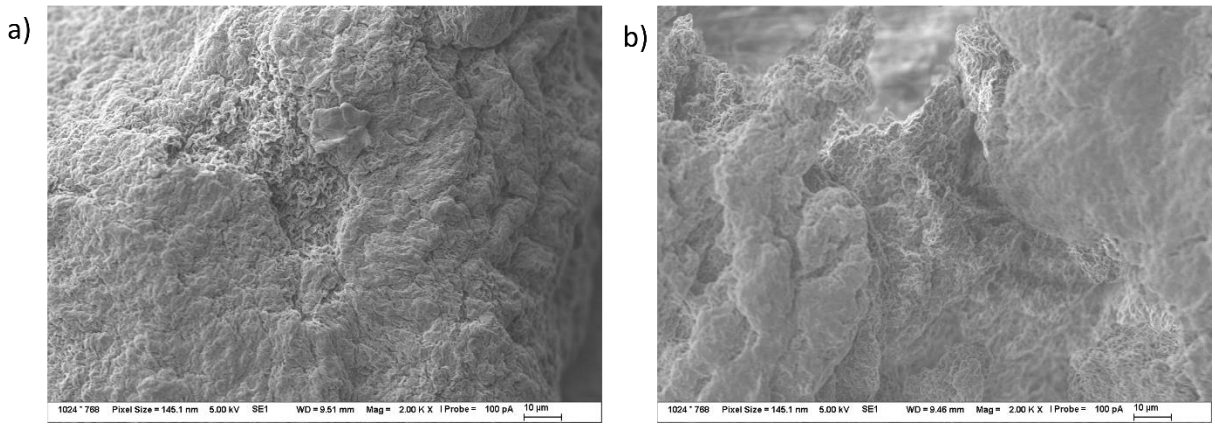

Figure 26: SEM images of porous materials. a)GVL/P(3HB) (75/25); b)GVL/P(3HB) (25/75). ×2000

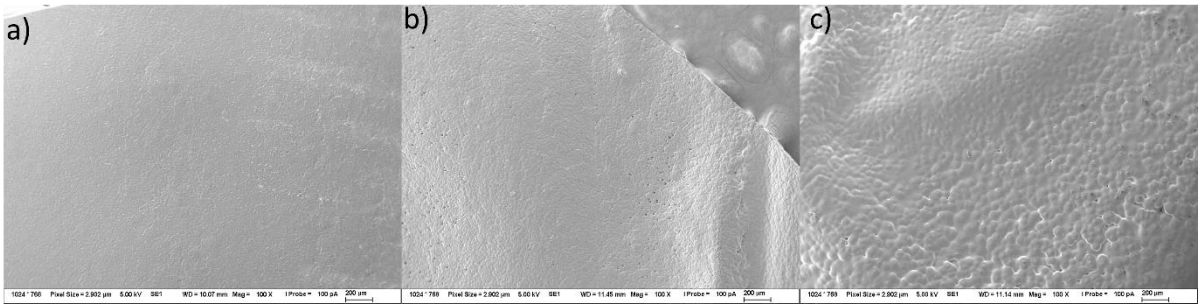

Figure 27: Solvent-casted films of DVL in chloroform. a) DVL/P(3HB) (100/0); b) DVL/P(3HB) (75/25); c) DVL/P(3HB) (25/75); ×100

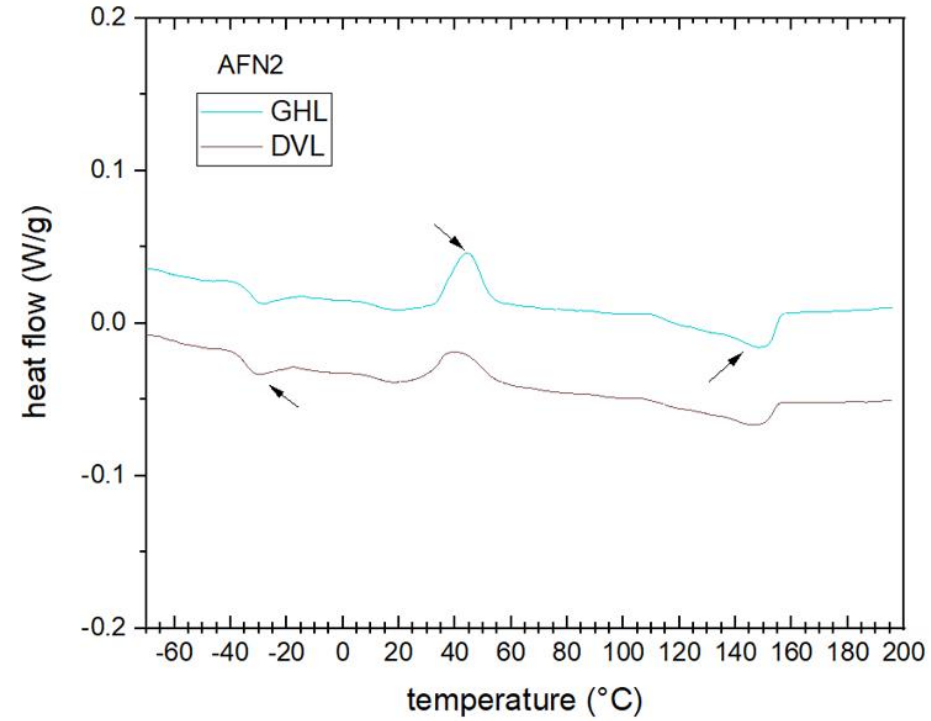

Figure 28: Combination plot of DSC curves for GHL-P(3HB-co-3HV-co-4HHx) and DVL-P(3HB-co-5HV)

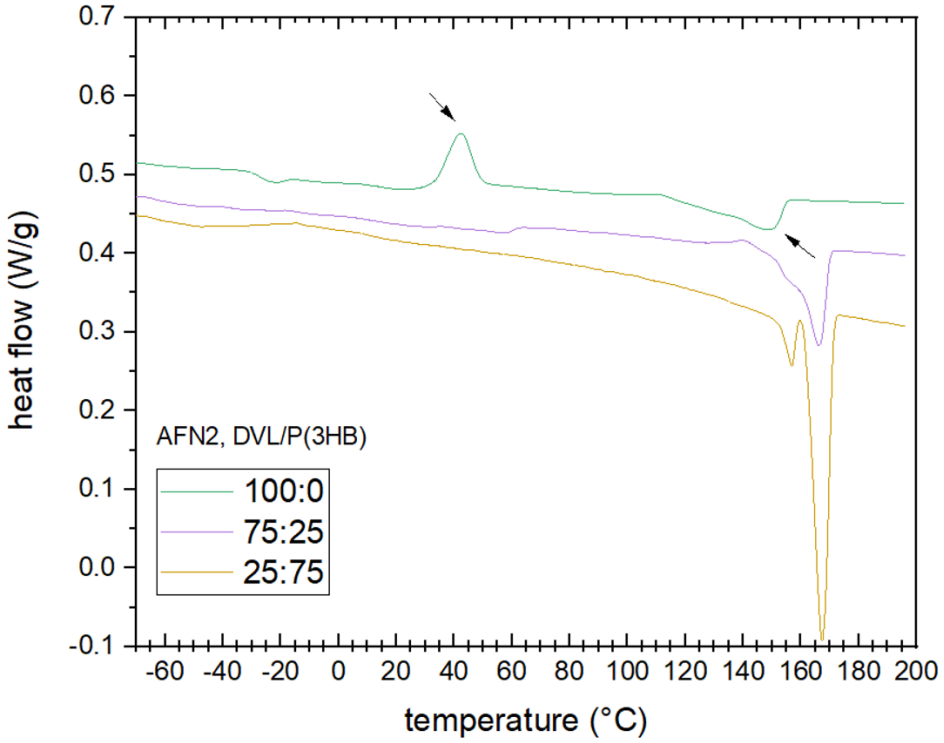

Figure 29: Combination plot of DSC curves for blends P(3HB-co-5HV)/P(3HB)

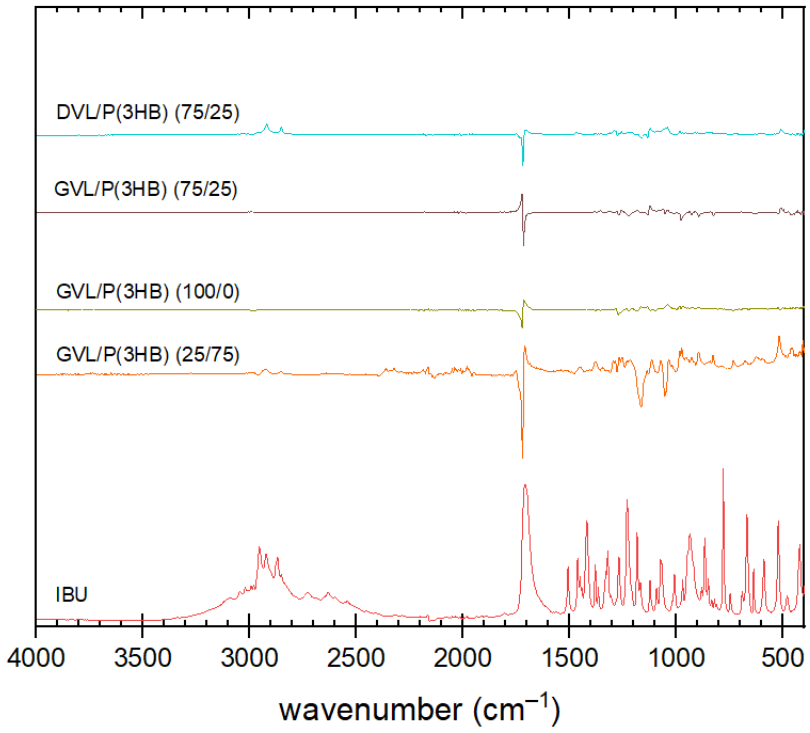

Figure 30: Results of subtraction of the FTIR of the carrier (PHA-chloroform films) with and without ibuprofen

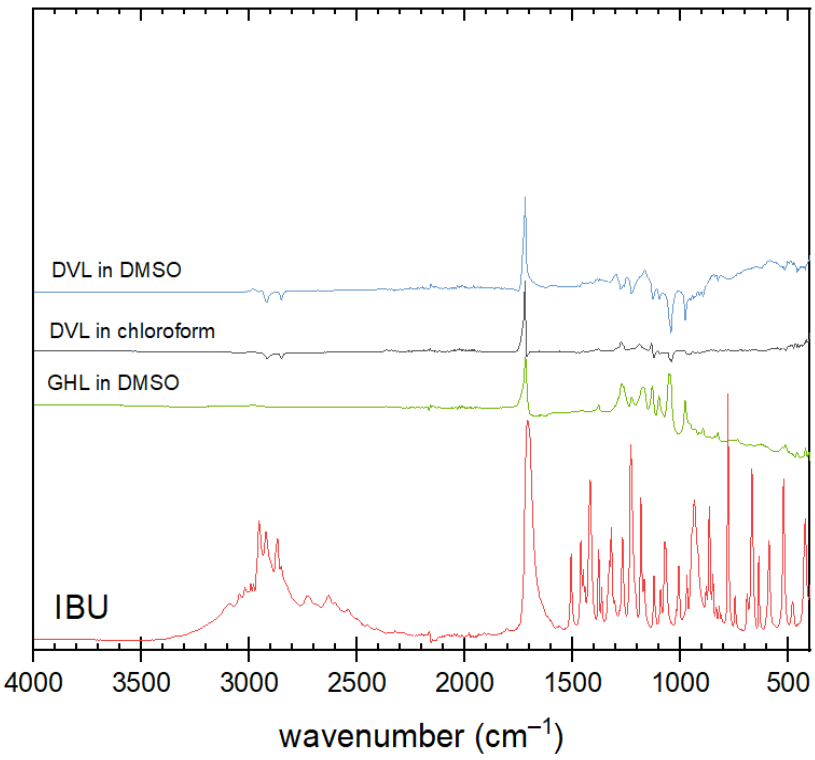

Figure 31: Results of subtraction of the FTIR of the carrier (PHA-DMSO films) with and without ibuprofen

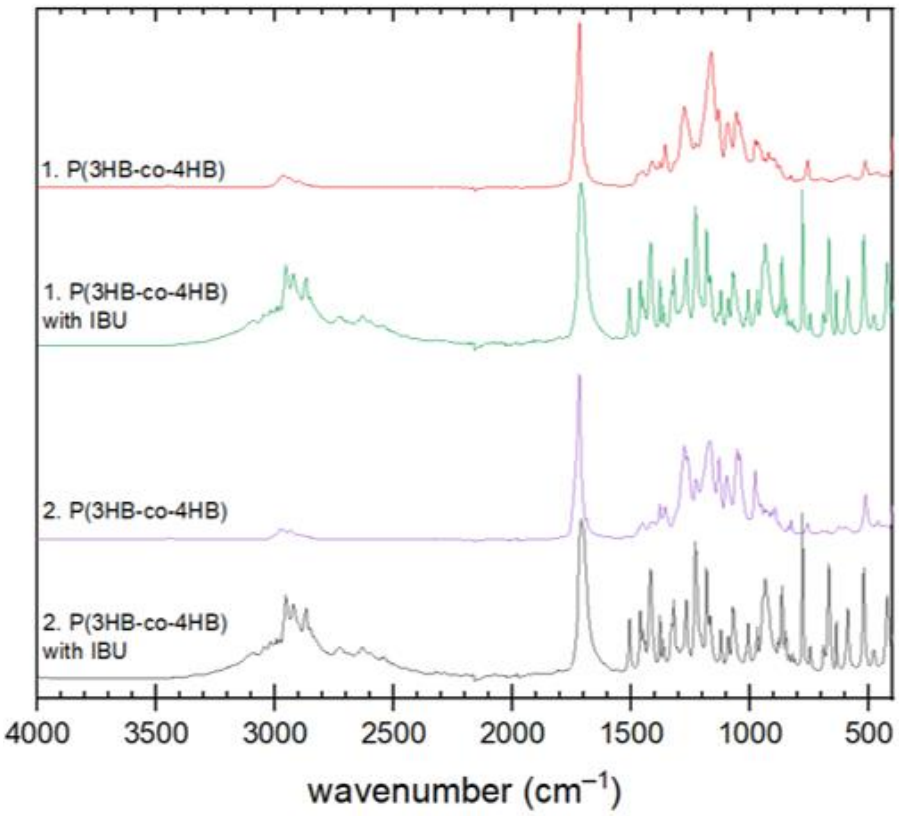

Figure 32: FTIR spectra of pure and ibuprofen-loaded particles prepared with ethanol

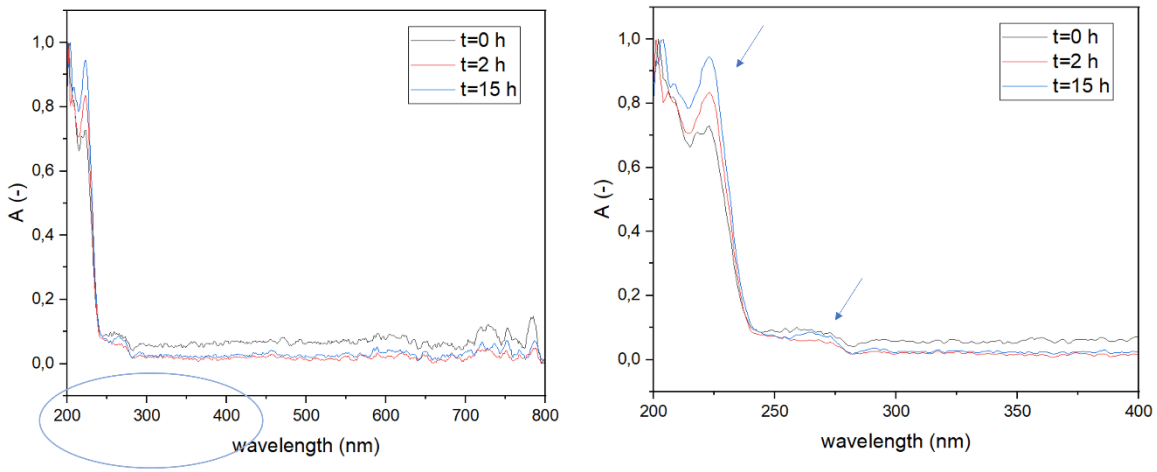

Figure 33: Time evolution of absorbance for particles DVL

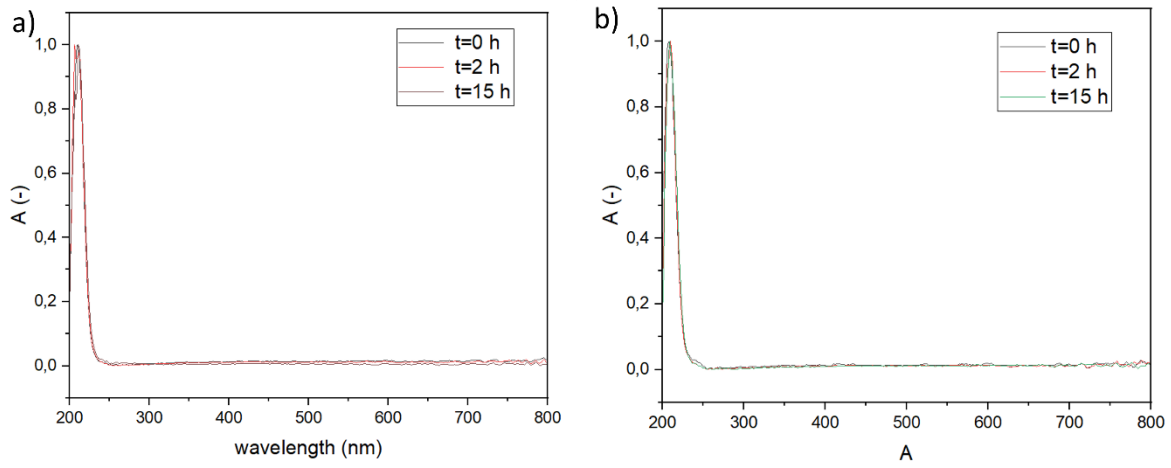

Figure 34: Time evolution of absorbance -negative control particles a) DVL, b) GVL

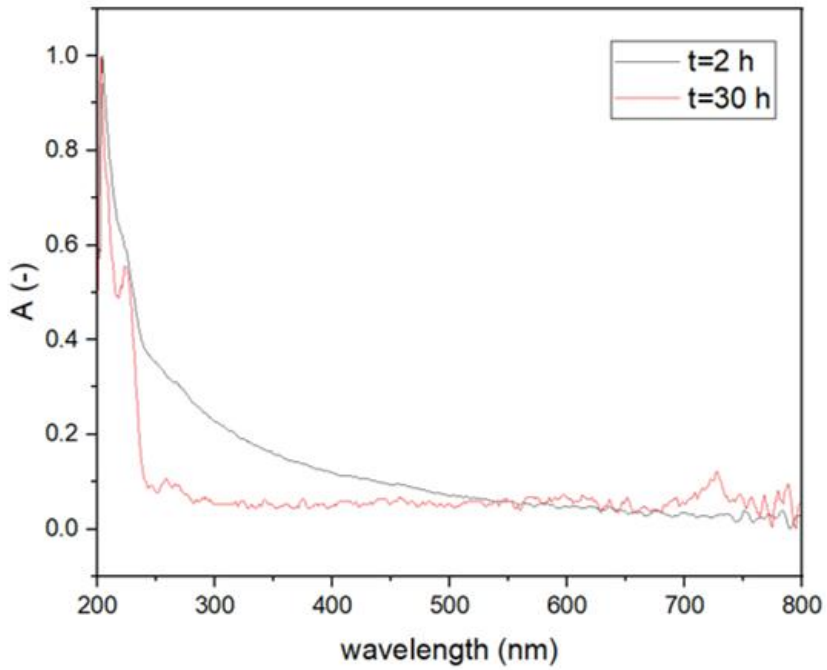

Figure 35: Raw spektra for DVL-DMSO samples at different times

8 List of abbrebiations

3HB	3-hydrobutyrate
3HV	3-hydroxyvalerate
4HHX	4-hydroxyhexanoate
4HV	4-hydroxyvalerate
5HV	5-hydroxyvalerate
ATR-FTIR	Attenuated Total Reflectance Fourier-Transform Infrared
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DSC	Differential scanning calorimetry
DVL	δ-valerolactone
FDA	The food and drug administration
GC-FID	Gas chromatography with flame ionization detector
GHL	γ-hexalactone
GVL	γ-valerolactone
PBS	phosphate buffer saline
PHA	Polyhydroxyalkanoate
PID	Polydispersity index
SEC-MALS	Size Exclusion Chromatography with multiangle light scattering
SEM	Scanning electron microscopy
UV-VIS	Ultraviolet–visible spectroscopy