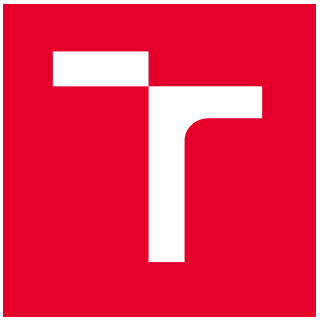

BRNO UNIVERSITY OF TECHNOLOGY

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

FACULTY OF CHEMISTRY

FAKULTA CHEMICKÁ

INSTITUTE OF CHEMISTRY AND TECHNOLOGY OF ENVIRONMENTAL PROTECTION

ÚSTAV CHEMIE A TECHNOLOGIE OCHRANY ŽIVOTNÍHO PROSTŘEDÍ

EFFECT OF ENZYMES ON THE DEGRADATION OF THERMOSENSITIVE COPOLYMERS FOR CONTROLLED RELEASE OF DRUGS

VLIV ENZYMŮ NA DEGRADACI TERMOCITLIVÝCH KOPOLYMERŮ PRO ŘÍZENÉ UVOLŇOVÁNÍ LÉČIV

BACHELOR'S THESIS

BAKALÁŘSKÁ PRÁCE

AUTHOR — **Anna Mária Dávidíková**

AUTOR PRÁCE

SUPERVISOR — **doc. Ing. Lucy Vojtová, Ph.D.**

VEDOUCÍ PRÁCE

BRNO 2023

BRNO UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMISTRY

Assignment Bachelor's Thesis

Project no.:	FCH-BAK1877/2022	Academic year: 2022/23
Department:	Institute of Chemistry and Technology of Environmental Protection	
Student:	**Anna Mária Dávidíková**	
Study programme:	Chemistry and Technology of Environmental Protection	
Field of study:	no specialisation	
Head of thesis:	**doc. Ing. Lucy Vojtová, Ph.D.**	

Title of Bachelor's Thesis:

Effect of enzymes on the degradation of thermosensitive copolymers for controlled release of drugs

Bachelor's Thesis:

1. Literature on the given topic
2. Synthesis of thermosensitive hydrogels
3. Testing degradation in different environments in the presence of enzymes
4. Characterization of degradation products using HPLC, kinetics of degradation using GPC/MALS
5. Evaluation and conclusion

Deadline for Bachelor's Thesis delivery: 22.5.2023:

Bachelor'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 Bachelor's Thesis

Anna Mária Dávidíková student	doc. Ing. Lucy Vojtová, Ph.D. Head of thesis	Head of department
In Brno dated 1.2.2023		prof. Ing. Michal Veselý, CSc. Dean

ABSTRACT

Thermosensitive hydrogels used for drug delivery systems are of great interest these days. They serve as a drug vehicle that transport drugs to a target place and then they serve as a drug depot in the body. Hydrogels can be tuned to be able to encapsulate various drugs, to be sensitive to various external stimuli, or to degrade slower (maintain the drug longer in the body).

To tune hydrogels, we need to know their degradation process and their behaviour in different degradation media; thus, we can predict how they will behave.

In this proposed thesis, the degradations of various thermosensitive hydrogel solutions were studied in different degradation media. The theoretical part of this work provides a short review of thermosensitive hydrogels, their characterisation, properties, use for drug delivery systems, and degradation. Firstly, a triblock thermosensitive copolymer PLGA-PEG-PLGA that consists of poly(D,L-lactic acid-*co*-glycolic acid)-*b*-poly(ethylene glycol)-*b*-poly(D,L-lactic acid-*co*-glycolic acid) was synthesized via living ring-opening polymerization under an inert atmosphere and characterized via proton nuclear magnetic resonance spectroscopy and gel permeation chromatography. This copolymer was further used for hydrogel water solutions in 10, 15 and 20 wt. % concentrations. Hydrogel samples prepared at 37 °C were degraded in various media (UPW (ultrapure water), NaCl, PBS (phosphate-buffered saline), and PBS with enzyme) in the incubator for 30 days. The sample removal was set for 1, 2, 3, 7, 9, 14, 16, 21, 23 and 30 days except for the enzyme solution where we expected quick degradation. Degraded hydrogels were analysed via gel permeation chromatography and degradation media via liquid chromatography with mass spectroscopy. Gravimetric analysis and measurement of pH over time were also part of the hydrogel behaviour study. The last aim of this work was to evaluate the collected data and see whether the used enzyme affected the PLGA-PEG-PLGA hydrolytic degradation kinetics. The results proved a minor acceleration in the degradation by the enzyme. These results will serve as a foundation for further degradation studies.

KEYWORDS

hydrogels, PLGA-PEG-PLGA thermosensitive copolymer, degradation, enzyme, ^{1}H NMR, GPC/SEC analysis, LC-MS analysis

ABSTRAKT

Termocitlivé hydrogély, ktoré sa používajú ako nosiče liečiv v dnešnej dobe zažívajú obrovský záujem v spoločnosti. Používajú sa ako spôsob dopravy liečiva na predom určené miesto a po dosiahnutí určeného miesta slúžia ako dlhodobé úložisko liečiv v tele. Vlastnosti hydrogélov môžu byť upravované tak, aby dokázali naviazať rôzne druhy liečiv, aby boli citlivé na rôzne vonkajšie impulzy a aby degradovali pomalšie, čo predĺži životnosť liečiv v tele.

Aby sme vedeli, ako upraviť vlastnosti hydrogélov a ako sa budú správať, musíme poznať ich degradačný proces a chovanie v rôznych degradačných médiách. Práve preto sú degradačné štúdie veľmi dôležitou súčasťou štúdií hydrogélov.

Táto bakalárska práca je zameraná na sledovanie degradácie rôznych roztokov termocitlivého hydrogélu vo viacerých degradačných médiách. Teoretická časť práce zahrňuje krátky prehľad o tom, čo sú to termocitlivé hydrogély, ich charakterizácia a popis vlastností spolu s použitím hydrogélov ako nosičov liečiv. Ďalej sa teoretická časť venuje degradácií hydrogélov celkovo ako aj degradácií konkrétne použitého hydrogélu v tejto bakalárskej práci. V prvom rade bol syntetizovaný živou polymeráciou za otváraním kruhu v inertnej atmosfére termocitlivý PLGA-PEG-PLGA kopolymér, tvorený poly(mliečnou-co-glykolovou kyselinou)-b-poly(ethylen glykolom)-b-poly(mliečnou-co-glykolovou kyselinou). Následne bol kopolymér charakterizovaný na protónovej nukleárnej magnetickej rezonancii a na gélovej chromatografii. Z nasyntetizovaného kopolyméru boli pripravené tri vodné roztoky hydrogélu o rôznych koncentráciách a to 10, 15 a 20 hm %. Vzorky pripravených hydrogélov za teploty 37 °C, boli degradované v rôznych degradačných roztokoch (UPW (ultra čistá voda), NaCl, PBS (fosfátový pufr) a roztok PBS s enzýmom). Degradačná štúdia bola hlavným cieľom tejto bakalárskej práce. Jednotlivé vzorky boli odobrané v konkrétne stanovené časy a to po 1, 2, 3, 7, 9, 14, 16, 21, 23 a 30 dňoch. Pri vzorkách s enzýmom bola predpokladaná rýchlejšia degradácia vzoriek, tak tieto odbery boli nastavené po 1, 2 a 3 dňoch. Ďalším cieľom tejto práce bola analýza odobraných vzoriek hydrogélov po degradácií na gélovej chromatografii a analýza degradačných médií na kvapalinovej chromatografií s hmotnostným spektrometrom. Počas degradácie vzoriek bola prevedená taktiež gravimetrická analýza a pri odoberaní degradačných roztokov bolo merané ich pH. Následne boli všetky získané dáta vyhodnotené a bolo sledované, či enzým urýchľuje PLGA-PEG-PLGA hydrolytickú degradačnú kinetiku. Výsledky poukazujú na to, že enzým má nízky vplyv na rýchlosť degradácie. Výsledky tejto práce poslúžia ako základy pre budúce degradačné štúdie.

KĽÚČOVÉ SLOVÁ

hydrogély, PLGA-PEG-PLGA termocitlivý kopolymér, degradácia, enzým, ^{1}H NMR, GPC/SEC analýza, LC-MS analýza

DÁVIDÍKOVÁ, Anna Mária. *Vliv enzymů na degradaci termocitlivých kopolymerů pro řízené uvolňování léčiv.* Brno, 2023. Dostupné také z: https://www.vut.cz/studenti/zav-prace/detail/148177. Bakalářská práce. Vysoké učení technické v Brně, Fakulta chemická, Ústav chemie a technologie ochrany životního prostředí. Vedoucí práce Lucy Vojtová.

DECLARATION

I declare that the bachelor thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the bachelor thesis is the property of the Faculty of Chemistry of the Brno University of Technology, and all commercial uses are allowed only if approved by both the supervisor and the Dean of the Faculty of Chemistry BUT.

..……

Student´s signature

Acknowledgement

I would like to express my deepest gratitude to my supervisor assoc. prof. Ing. Lucy Vojtová, Ph.D. for her time and professional guidance and support. The gigantic thank you belongs to Ing. Klára Lysáková for her patience with me, her support, help, and time. Special thank you should also go to Ing. Jana Brtníková, Ph.D., Ing. Ludmila Mravcová, Ph.D., and Ing. Jan Fučík for help with measurements, and Mgr. Jan Žídek, Ph.D. for advice with calculations. CzechNanoLab project LM2018110 funded by MEYS CR is gratefully acknowledged for the financial support of the measurements at CEITEC Nano Research Infrastructure. Last but not least, I could not have undertaken this journey without support from my family and my friends.

TABLE OF CONTENT

1. INTRODUCTION

Hydrogels are highly appropriate materials for biomedical applications, mainly due to their tissue compatibility which is caused by high water content and biodegradability, that is caused by synthesis from biodegradable polymers. With their tuneable properties, hydrogels find their applications in tissue engineering, wound dressing, contact lenses and target drug delivery [1].

Hydrogels for drug delivery are able to encapsulate drugs and transport them to the desired tissue, organ, or cell. At a target site, hydrogels serve as a matrix for the storage of drugs. With subsequent hydrogel degradation, the progressive release of loaded drugs through diffusion occurs. Encapsulation of drugs in hydrogels reduces their solubility and prolongs their lifespan [2], [3].

Thermosensitive hydrogels react to the change in temperature as an external stimulus and undergo a sol-gel transition. Such a feature is used in drug delivery systems; thus a hydrogel can be applied via injection to a target place. With such an application, numerous undesirable effects can be reduced. Understanding the degradation process is crucial for understanding the behaviour of hydrogels and for tuning their properties. *In vitro* experiments that study polymer degradation do not provide information on how a polymer will behave inside the human body. In this thesis, the degradation kinetics of synthesized PLGA-PEG-PLGA thermosensitive copolymer used for drug delivery were studied. In addition, the degradation in phosphate-buffered saline was performed with abundant enzyme lipase, to get closer to possible degradation in the human body [4].

The degradation of hydrogels was studied during the set time by the gravimetric analysis and via the gel permeation/size exclusion chromatography. Degradation products will be analysed via the size exclusion chromatography and the ultra-high pressure liquid chromatography equipped with mass spectroscopy.

2. THEORETICAL PART

2.1 Thermosensitive hydrogels

Hydrogels are macromolecular polymer gels that have a three-dimensional network that is formed by crosslinking of polymeric chains. Cross-linking can be either chemical (irradiation, photopolymerization, etc.) or physical (temperature, pH, pressure, etc.). The structure is obtained by the hydrophilic groups present in a polymeric network after hydration in an aqueous medium. They can absorb up to 99% of water, which is a similar environment to natural tissue. Hydrogels can be made of natural or synthetic polymers, are biocompatible, highly flexible, and viscoelastic, and perform good permeability [1], [5]–[8].

A group of hydrogels containing both hydrophobic and hydrophilic blocks undergo a sol-gel transition (or reverse thermal transition) in response to a stimulus, in our case an increased temperature. Sol refers to the free-floating mixture of hydrophilic polymer segments and individually floating micelles in an aqueous solution. The gel stage occurs after the sol reaches the critical gelation temperature (CGT). Hydrogen bonds between hydrophilic segments of the polymer become weaker and hydrophobic bonds start to occur. Hydrophobic molecules tend to decrease their exposed surface in an aqueous solution, thus forming micelles. Micelles are described as a core-shell aggregate structure. Micelle aggregates with a hydrophobic core have a great advantage for drug delivery because they can solubilise water-insoluble drugs [6], [9]–[11].

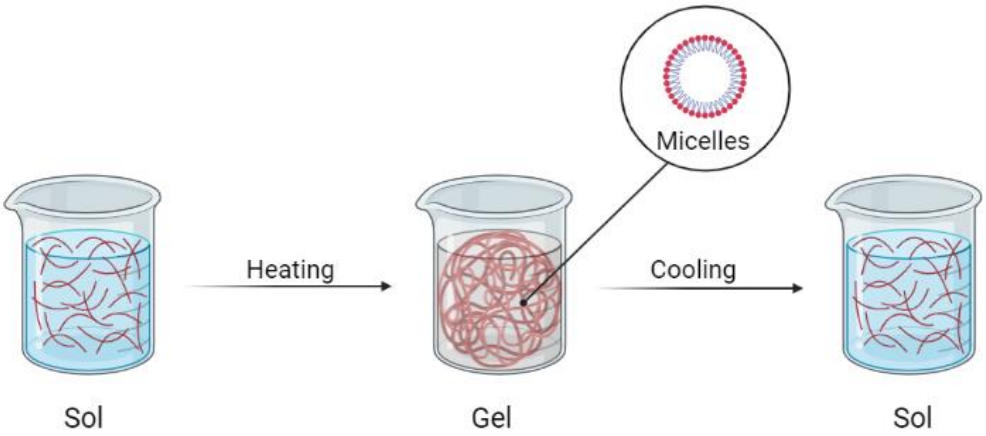

Figure 1: Sol-gel-sol transition of hydrogels with temperature as a stimulus [12].

The properties of thermosensitive copolymers can be tuned by different ratios and sequences of monomers, molecular weight, concentration, and chemical composition. Due to their properties, hydrogels are nowadays largely used in the fields of tissue engineering and regenerative medicine (TERM), such as drug delivery, contact lenses, wound regeneration, cell therapy, diagnostics or biosensors, electronics, and soft robotic components [13]–[15].

2.2 Hydrogels for drug delivery

Because of the unique physical properties and the possibility of tunning, hydrogels are widely used for drug delivery applications. The amphiphilic nature of thermosensitive hydrogels provides the possibility to deliver a wide range of hydrophilic and hydrophobic molecules, including drugs and bioactive molecules such as growth factors and cells. Drug delivery

systems (DDS) use hydrogels that are biocompatible and biodegradable. Biocompatibility is achieved by a high concentration of water and a strong physiochemical similarity to the extracellular matrix. Biodegradability can be promoted through several pathways, such as enzymatic, hydrolytic, or environmental [5], [6], [16].

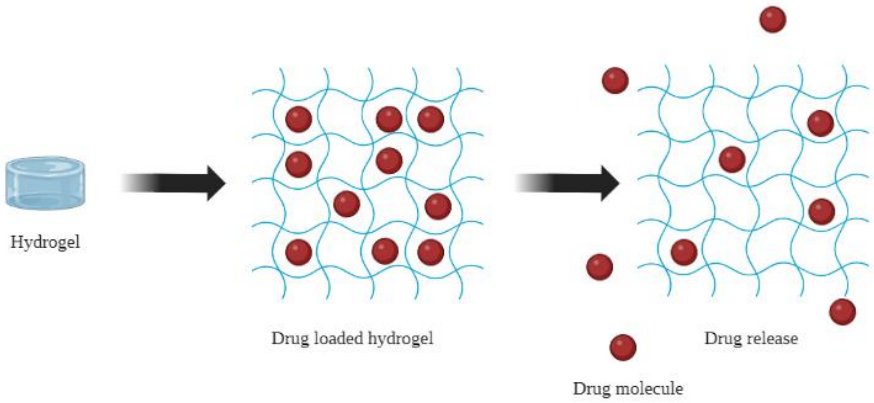

Figure 2: Mechanism of drug release from hydrogel [12].

Hydrogels for DDS serve as a drug depot in the body of an organism. Drugs are stored inside hydrogels at higher concentrations for a longer period. The encapsulation of drugs slows the degradation and prolongs their life. This also provides sustained local release, which may be controlled by diffusion out of the hydrogel pores or remotely controlled release. Remotely controlled release is performed by using external triggers such as UV light, pH, electrical, and magnetic field. Stimuli-sensitive gelling polymers, or *in situ* polymers, are desirable for DDS because of forming an injectable solution that minimalizes invasion during surgery. Hydrogels for DDS reduce the dose frequency of drugs and reduce the side effect by storing them in target tissue [5], [6], [15], [17].

2.3 PLGA-PEG-PLGA copolymer

PLGA-PEG-PLGA or ABA is a triblock thermosensitive copolymer that consists of poly(D,L-lactic acid-*co*-glycolic acid)-*b*-poly(ethylene glycol)-*b*-poly(D,L-lactic acid-*co*-glycolic acid). ABA contains both hydrophobic segments (PLGA) at the edges and hydrophilic segments (PEG) in the middle; therefore it has an amphiphilic character [18]–[20].

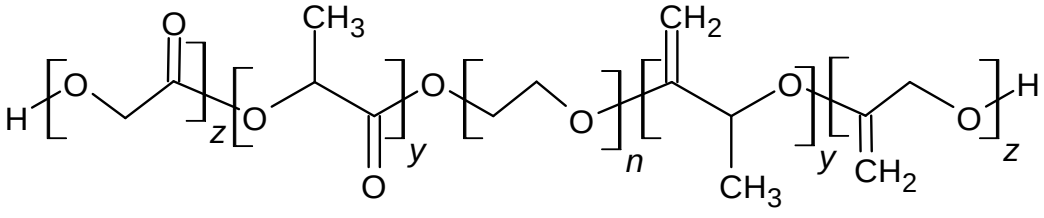

Figure 3: The structure of PLGA-PEG-PLGA [19].

As a result of the amphiphilic character, the copolymer self-assembles into flower-shaped micelles above the critical micellar concentration (CMC). By reaching CMC, ABA becomes an elastic gel in an aqueous environment. Thermosensitive hydrogels that undergo micelles formation in aqueous solution, such as ABA, are important due to their high biodegradation tunability and good properties for encapsulation and drug release [20]–[22].

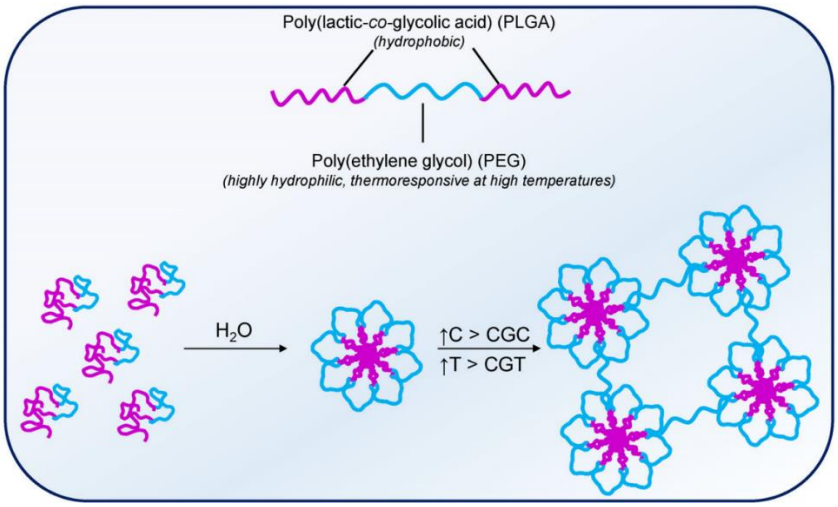

Figure 4:Flower-shaped micelles formation due to the hydrophobic and hydrophilic parts of the PLGA-PEG-PLGA copolymer [23].

The ABA copolymer used in this work is a sol at room temperature and with increasing temperature forms "flower-shaped" micelles turning into gel stage at body temperature (37 ℃). Generally, the sol-gel transition of ABA occurs in the temperature range between 25 ℃ and 37 ℃ depending on the LA/GA ratios. The thermosensitive properties of ABA can be tuned by the LA/GA sequence ratio, different stereochemical structures of copolymers, length, and molecular weight dispersity of PLGA and PEG blocks. Besides all the listed above, thermosensitive properties can be changed by physical modifications [13], [24], [25].

ABA copolymer is nowadays commercially known as ReGel®, which serves for small hydrophobic molecules, peptide or protein delivery, and OncoGel®, which helps in cancer treatment [20], [26].

2.3.1 Poly(lactide acid) (PLA)

PLA belongs to a group of aliphatic polyesters. It is a linear, biogenic, and thermoplastic polymer that is available in three isomeric forms, such as the optically active L-isomer, optically active D-isomer, and optically inactive L,D-isomer. The ratio of these isomers has a major impact on the properties of the polymer, such as the melting temperature and the degree of crystallinity. The L-LA isomer is frequently used for most the biomedical applications because it is primarily metabolised in the human body, and it allows for homogenous dispersion of the drug in its matrix [27]–[30]. In this work, the D,L-LA was used for the PLGA-PEG-PLGA preparation.

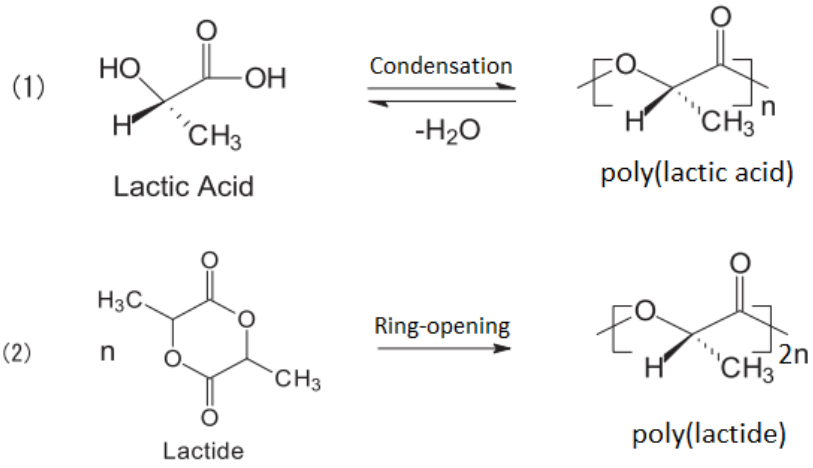

Figure 5: The mechanism of PLA preparation in two ways: (1) preparation by DPC of poly(lactic acid), (2) ring-opening polymerisation (ROP) of lactide to poly(lactide) [31].

It is possible to obtain PLAs in two ways. The first approach is by direct polycondensation (DPC) to PLA; however, this method is difficult because of the complications with generated water removal. Most PLAs are produced by a more efficient ROP of lactide [31].

2.3.2 Poly(glycolic acid) (PGA)

PGA is the simplest of the aliphatic polyesters. It is a rigid thermoplastic polymer with high crystallinity and a chemical structure similar to PLA; however, it shows different characteristics. Because of its high crystallinity, PGA is insoluble in most organic solvents, except solvents such as hexafluoro-isopropanol. It is acquired from either petroleum or renewable resources, such as sugarcane or sugar beet. PGAs can be prepared, similarly to PLAs, by two approaches. The first is obtained by polycondensation of glycolic acid, which is easy but not very efficient. The second approach is by ROP of glycolide, which represents high efficiency [29], [31].

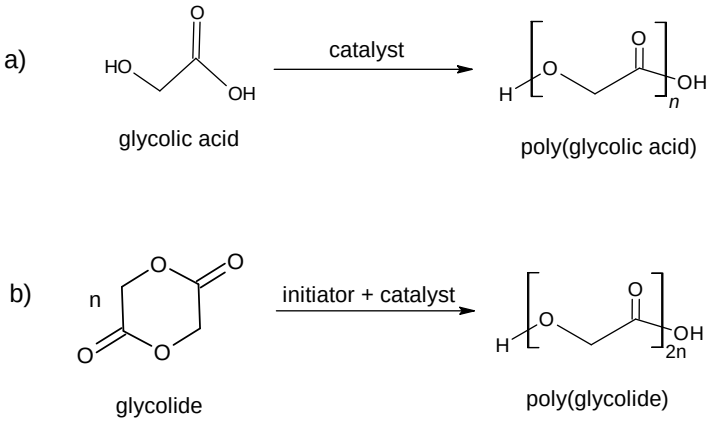

Figure 6: Two methods of approaching PGA: a) preparation by polycondensation of poly(glycolic acid), b) preparation by ROP of poly(glycolide) [32], *inspired by* [31].

2.3.3 Poly(ethylene glycol) (PEG)

PEG belongs to the group of aliphatic polyethers. This synthetic polymer is used extensively in applications where biodegradation is required. It is frequently used for biomedical purposes such as DDS for its special properties. PEG is a biocompatible hydrophilic water-soluble polymer that is rarely biodegradable and performs properties such as the absence of antigenicity and immunogenicity. PEGs and their derivatives are broadly used in cosmetics, soaps, detergents, the pharmaceutical industry (vehicles for DDS, drug binders and veterinary drugs preparations) and textile, paper, leather, wood, food, plastics, metal, rubber and other industries [28], [33]–[35].

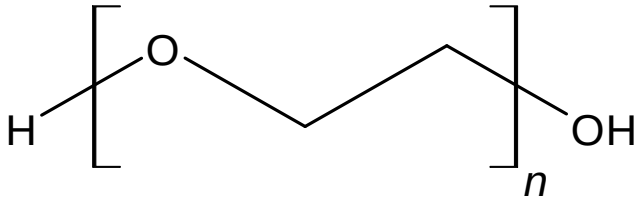

Figure 7: The structure of PEG [32].

2.4 Degradation of polymers

Degradation is a process of changing the properties and weakening of polymers as a result of chemical or physical reactions initiated by the environment. Changes in the polymer may occur in losing original properties, such as cracking, erosion, discoloration, and mechanical, optical, or electrical attributes. Ordinarily, polymer degradation is an undesirable process, but in certain cases, it is highly requested (such as in the controlled release of drugs, tissue engineering applications, or degradation of polymers in the environment). Important factors for the initiation of degradation are heat, light, and air [36], [37].

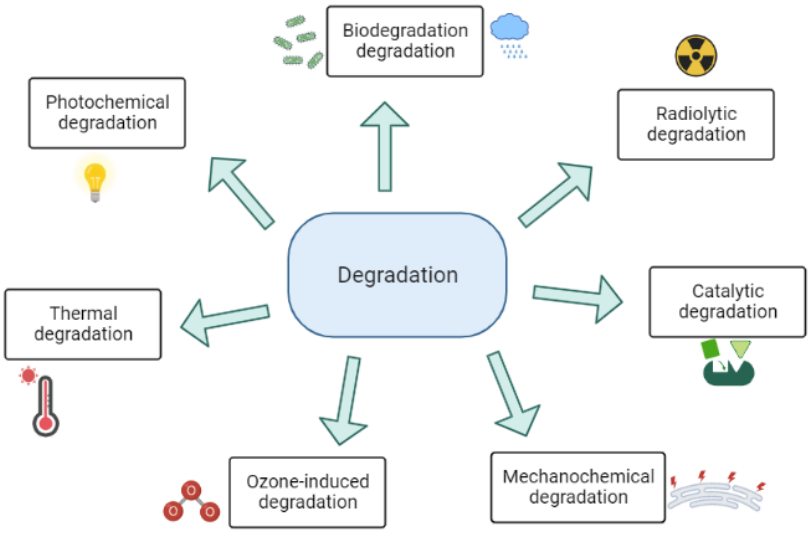

Figure 8: Causing agents of degradation of materials [12].

Polymer degradations are classified according to the origin of the causing agent into several types [36].

2.4.1 Photochemical degradation

Polymers exposed to the presence of protons found especially in sunlight, such as infrared radiation (IR) (760 – 2500 nm), visible light (400 – 760 nm), and ultraviolet light (UV) (10 – 400 nm), may undergo photochemical degradation. Materials undergo degradation due to the absorption of emitted protons from light and create radicals [37], [38].

2.4.2 Thermal degradation

Once exposed to elevated temperatures, polymers undergo chemical changes without other compounds involved. Normally, photochemical and thermal degradations are analogous. The main differences between these two degradations are in the sequence of initial steps and at the place of occurrence. Thermal degradation occurs throughout the entire volume of the polymer, while photochemical degradations occur on the surface. The mechanism of thermal degradation is done either by chain end degradation (depolymerization reaction) or by random degradation route [36], [37], [39].

2.4.3 Ozone-Induced degradation

Ozone-induced degradation is common in polymers when the ageing processes are very slow, and the material maintains its properties for a very long time. This type of degradation is induced by atmospheric ozone and is highly sensitive even to very small amounts of ozone in the air. Ozone-induced degradation was observed in natural rubber for the first time [36], [40].

2.4.4 Mechanochemical degradation

Generally, radicals in materials are formed because of the influence of external mechanical stress. Mechanical forces might be initiated by strong ultrasounds or mastication that forms radicals. Radicals in the absence of oxygen can recombine. On the other hand, when exposed to air, oxygen serves as a radical scavenger and reacts with them causing permanent chain breakage [36], [38], [41].

2.4.5 Catalytic degradation

Catalytic and thermal degradations are widely used in plastic waste recycling. Recycling serves as an alternative method of producing fuels, gases, oils, and chemicals. These outcomes could be processed into valuable raw materials by the petroleum refining industry. The products of catalytic degradation are high-quality hydrocarbons that require low-energy and low temperature operation. For catalytic degradation, different types of catalysts such as zeolites, Pt-CO, and Pt-Mo supported over SiO_2 and transition metal catalysts (Cr, Ni, Mo, Co, Fe) supported over Al_2O_3 or SiO_2 are used. [36], [42].

2.4.6 Radiolytic degradation

Polymers used in the food industry (packaging) such as polyvinyl chloride (PVC) and manufacturing of medical and pharmaceutical devices require the absence of microorganisms, thus sterilisation. The most common method for sterilising such materials is ionizing radiation due to its high efficiency and practicability. Polymers exposed to gamma radiation undergo

radiolytic degradation that changes their molecular structure, and mechanical physical, and chemical properties, hence materials used in the food industry, medicine and pharmacy require certain additives to retard the impacts of radiation [43].

2.4.7 Biodegradation

Polymer materials are an inseparable part of our daily life in a wide variety of applications, whether it is packaging, the automotive industry, clothing, electronics, or medicine. Biodegradable synthetic polymers provide numerous advantages in tissue engineering. Their key ability is the possibility of tuning properties. Biopolymers or biodegradable polymers provide a solution to the problem of plastic pollution [44], [45].

Polysiloxanes, polyurethanes, polyesters, and polyolefins are groups belonging to synthetic polymer families that are nowadays used in bioengineering, such as biodegradable materials [46].

Biodegradable materials are subject to European standards such as the composability standard for packaging EN 1343 and the standard for solid materials biodegradation ASTM D6400. According to these standards, biodegradation occurs when there is CO_2 release. Generally speaking, biodegradation is a process in which complex organic matter is broken down by microorganisms or by enzymatic action into less complex biomass together with CO_2, water, and minerals. These products are not toxic to the environment and organisms, and can easily be eliminated from the body of an organism by metabolic processes. [36], [44], [47].

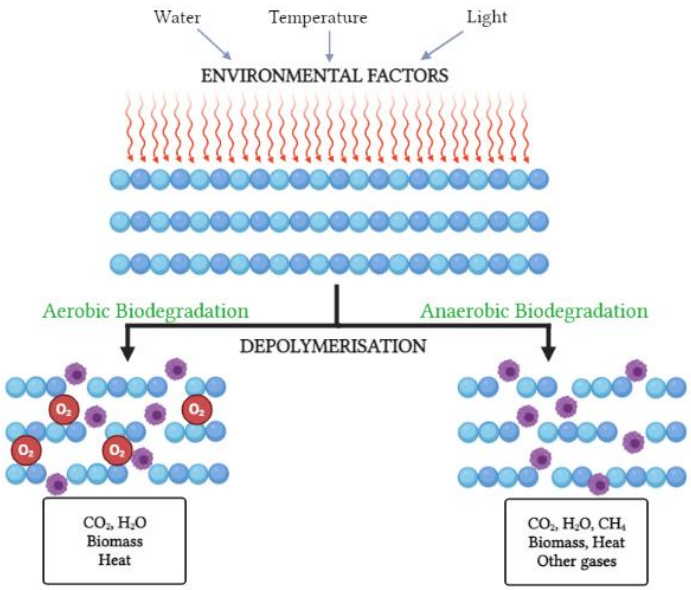

Figure 9: Comparison of the anaerobic and aerobic biodegradation of polymers and their products. Purple symbols represent microorganisms that are part of degradation [12].

If biodegradation occurs in the presence of air and aerobic conditions, then there are degradation products such as CO_2, water, and biomass. In the case of anaerobic conditions, in the absence of air, methane and other gases are produced. The first step in polymer biodegradation is the biodeterioration process. In this process, polymeric materials are broken down into smaller

segments by various microbial communities and organisms with the help of other agents present in the particular environment. The next step includes the cleavage of polymeric backbones by hydrolytic enzymes, catalytic agents, or free radicals that are created by the activity of microorganisms. Whether the polymer is molecular, macromolecular, microscopic, or macroscopic, biodegradation can occur at different structural levels depending on the mechanism. The biodegradation mechanism follows different pathways [36], [47]–[49].

Polymers have a wide variety of functions in biomedical fields, such as drug delivery systems, scaffold development, implantation of medical devices and artificial organs, ophthalmology, dentistry, bone repair, and many others. In all fields, they serve as a drug-binders and a tool for controlled drug delivery; thus, it is of high priority to study and understand the degradation mechanisms and effects of the factors. The degradation study is a key factor in understanding the stability and behaviour of polymers in different environments. Through the degradation study, degradation pathways can be determined, and it also allows to study the degradation of products and vehicles, and propose the prediction of polymer life in living organisms [50]–[53].

2.4.7.1 Hydrolytic degradation

Hydrolytic degradation is a process that combines water uptake and the cleavage of hydrolysable bonds. There are two types of hydrolytic degradation. When the water uptake in the polymer is faster than the breaking of the bonds, *bulk degradation* takes place. The polymer is first completely soaked with water, and after that, degradation occurs in the bulk. On the other hand, when the rate of hydrolysis is faster than that of water uptake, *surface erosion* occurs. This happens after the encounter of the polymer and the aqueous environment. The decrease in thickness and mass loss occurs only in the outer surface area. In reality, both types occur simultaneously, but one is prevalent and usually the most prevalent is bulk degradation. If the polymer contains hydrolytically unstable bonds (hydrophilic bonds), then hydrolysis may occur. Polymers such as polyesters, polyanhydrides, polycarbonates, and polyamides are mainly degraded by hydrolytic degradation. Their primary hydrolysis leads to low molecular weight oligomers with the following microbial aid [36], [54]–[56].

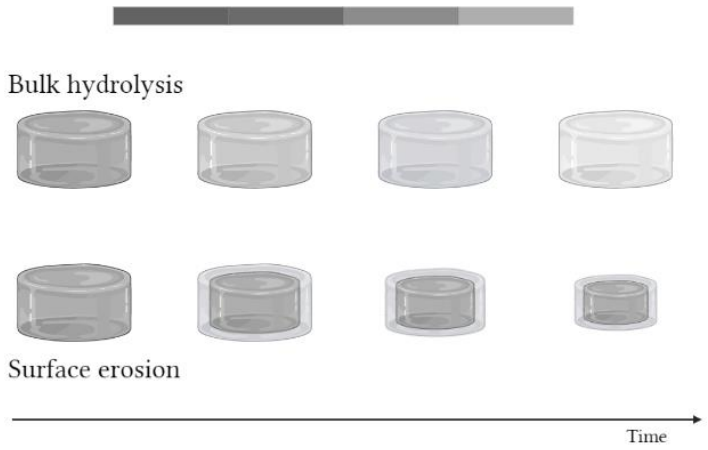

Figure 10: Difference between the bulk hydrolysis mechanism and the surface erosion mechanism over time [12], *inspired by* [54].

2.4.7.2 Enzymatic degradation

Enzymes are biological catalysts that reduce the activation energy of reactions. Hydrogels for biomedical applications may experience enzymatic degradation when encountering enzymes in the body fluids of an organism. The majority of enzymes in enzyme-catalysed degradation are proteins with complex three-dimensional structures. Different enzymes assist in different degradation mechanisms. Polymers for DDS that undergo enzyme-catalysed degradation are highly appealing. They serve as site-specific vehicles that travel to a particular place with a higher concentration of enzymes. Long-chain synthetic polymers, encountering enzymes, are broken down into small chains followed by aerobic or anaerobic degradation until completely mineralized. Certain enzymatic degradation of natural polymers follows the unzipping or chain-end mechanisms. In the case of *enzyme-catalysed hydrolysis* by hydrolytic enzymes (such as lipase, phosphates, esterase, and protease) help with the breakdown of the material and its subsequent metabolic use [36], [55], [57], [58].

2.4.7.3 Lipase

A lot of research on the synthesis and degradation of PLGA-PEG-PLGA copolymer for DDS have been published, but there are only a few articles that study the degradation of PLGA-PEG-PLGA copolymer with lipases. Some articles indicate that naturally occurring esterase enzymes, such as lipase, may accelerate the degradation rate of polymer hydrogels by cleaving ester bonds [59].

Ke Shi et. al. [60] studied the impact of lipase and cutinase (hydrolytic enzymes) on the biodegradation rate of poly(ε-caprolactone) (PCL) films. The research contributed that lipase significantly accelerated the degradation rate of PCL films. On the other hand, after degradation with cutinase, no significant changes were observed [60]. This experiment demonstrated that polymer degradation depends on the type of enzyme and whether the enzyme is compatible with the copolymer enough.

In our experiment, commercially available lipase from Candida rugosa without further purification was used. The impact of lipase on the polymer was studied and compared with degradation without enzyme in different degradation media.

2.5 Factors affecting polymer degradation

Degradation is a complex process that leads to the deterioration of the properties of a polymer. It is affected by many factors. These factors may speed up degradation or inhibit it. The degradation process can be controlled by these factors. These factors can be divided into external factors and polymer characteristics [61], [62].

2.5.1 Physiological factors

Physiological factors are external factors or exposure factors. These factors present properties of the environment in which the polymer occurs [53], [62].

2.5.1.1 Humidity

Humidity is an extremely important external factor. Exposure to the moist and wet environment allows the polymer to undergo hydrolysis and, thus may speed up the degradation process.

Rocca-Smith J. at al. [63] showed an increase in the level of degradation products after an increase in relative humidity. The moist environment also provides suitable conditions for the reproduction of many microbes and their growth. The degradation of polymers by microorganisms in a warm and humid environment increases [36], [53], [62]–[64].

2.5.1.2 pH and temperature

The pH can influence the rate of hydrolysis, and thus degradation. It is believed that strongly acidic or basic degradation media initiate the degradation process. Moreover, degradation products can change the pH conditions of the medium, thus shifting the degradation rate. Increased temperature speeds up polymer degradation. Adhariya J. et al. [65] studied the release kinetics of naltrexone in PLGA microspheres. The degradation increased dramatically at a higher pH (7,4 – 9,0) than in a lower pH lever (4,5 – 6,0). Furthermore, the study showed that an elevated temperature (45 ℃) increased polymer degradation; therefore, drug release [53], [62], [65].

2.5.2 Material characteristics factors

2.5.2.1 Molecular weight

The molecular weight of the polymer is a crucial characteristic that influences not only polymer degradation but also many other properties. Generally, the higher the M_w of the polymer, the slower the degradation [36], [53], [62] .

2.5.3 Composition

The chemical composition has crucially affected degradation. In our case, the most important is the LA/GA ratio. Higher concentrations of GA make the polymer more hydrophilic and more amorphous, and thus it speeds up degradation. On the other hand, higher concentrations of LA may slow down degradation. [36], [53].

2.5.4 Drugs

Drugs loaded inside polymers for DDS also can influence degradation. It depends on their type and whether they are of *hydrophobic* or *hydrophilic character*. The work of Cabezas L. et al. [66] shows the difference between the release of hydrophobic drugs (indomethacin) and hydrophilic drugs. Drugs of hydrophobic character have slower release than hydrophilic. This is caused by the low aqueous solubility in the water of hydrophobic drugs and the slow initial burst. The *size* of drug particles is related to their solubility. The influence of particle size is of great importance for partially soluble or insoluble drugs. For soluble drugs, size is important only if the polymer is highly hydrophobic or the drug molecule is large [53], [66].

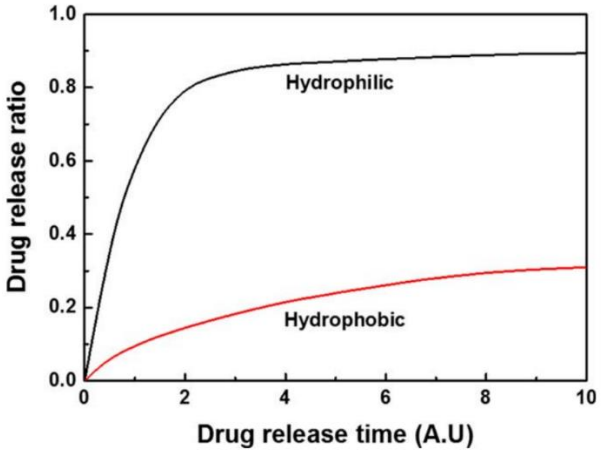

Figure 11: Difference in drug release between the hydrophobic and hydrophilic types of drugs [53].

Interactions between bounded drugs and polymers are another extremely important factor in the degradation rate. Drug particles can be of acidic or basic nature. The degradation of acidic drugs is faster because of the support of the autocatalysis of polymer. On the other hand, the view on basic drugs is conflicted. Some studies point out that basic drugs (such as tertiary amines or nucleophilic drugs) accelerate degradation. On the other hand, Klose et al. [67] studied the potential effects of drugs (ibuprofen and lidocaine) on the kinetics of polymer degradation. In this case, slightly basic lidocaine is released slower than acidic ibuprofen due to the neutralization of hydrolysis and the retardation of autocatalysis [53], [66], [67].

In our case, we assume that degradation with enzyme lipase will exceed degradation because it is a hydrolytic enzyme; thus, it will encourage hydrolysis.

2.6 Degradation of the PLGA-PEG-PLGA copolymer

The degradation of the PLGA copolymer implies random hydrolysis of the ester bonds. All biodegradation products of the PLGA-PEG-PLGA copolymer are biocompatible, nontoxic, and non-inflammatory, and after degradation can be excluded from the body by metabolic pathways [29], [30].

2.6.1 Degradation of PLA

PLA undergoes hydrolytic biodegradation, which is followed by a random cleavage of the ester bonds. The main degradation product is lactic acid, which is regularly found in the human body. The whole reaction is speeded up by the production of acids and H^+ ions. This also causes a reduction in the pH level. Lactic acid enters a tricarboxylic acid cycle, also known as a Krebs cycle, and is later excreted as water and CO_2, mostly by respiration, but also in small amounts in the form of urine and faces. The rate of hydrolysis of PLA is much slower than that of PGA [29], [55], [64], [68].

2.6.2 Degradation of PGA

The degradation of PGA in an organism is introduced, in addition to the random hydrolysis of the ester bond, by numerous enzymes with esterase activity. Glycolic acid is a breakdown

product of PGA degradation and also enters the Krebs cycle. However, it can also be excreted from the body in the urine. The rate of PGA degradation is rapid [29], [30], [55].

2.6.3 Degradation of PEG

The biodegradation of PEG has been investigated since 1962. PEG is non-biodegradable polymer but can be broken down into smaller sections by oxidation of the terminal alcohol group or by specific PEG-assimilated bacteria. The breakdown of PEG and the subsequent excretion from an organism depend on M_w. PEGs with lower M_w are eliminated by the kidney. High M_w PEGs undergo renal filtration and then are excreted into the bile. [55], [69], [70].

3. MAIN GOAL OF THE WORK

The main goal of this thesis is divided into several parts:

- Synthesis and characterisation of the thermosensitive PLGA-PEG-PLGA copolymer via ring-opening polymerisation.
- Preparation samples of the hydrogel of various wt. concentration for degradation with different degradation media.
- Characterisation of a degraded hydrogel via Gel Permeation Chromatography/Size Exclusion Chromatography (GPC/SEC) and degradation products via Ultra performance liquid chromatography in tandem arrangement with electrospray ionisation mass spectrometry (LC-MS).
- Study the impact of lipase in enzymatic degradation on the degradation rate of PLGA-PEG-PLGA copolymer.

4. EXPERIMENTAL PART

4.1 Chemicals

- Acetonitrile for LC-MS Chromasolv® (MeCN, ≥ 99.9%) (VWR Collection, USA)
- Dipotassium hydrogen phosphate (K_2HPO_4, ≥ 98 %) (Sigma-Aldrich, Germany)
- D,L-lactide (≥ 99.5%) (Polysciences, Inc., USA)
- Ethanol (EtOH, ≥ 96%) (Penta, Czech Republic)
- Formic acid (≥98%) (Sigma-Aldrich, Germany)
- Glycolic acid (GA, ≥ 99.9%) (Sigma-Aldrich, USA)
- Glycolide (≥ 99.9%) (Polysciences, Inc., USA)
- L-(+)-Lactic acid (LA, ≥ 98%) (Sigma-Aldrich, Switzerland)
- Lipase from Candida rugosa (Sigma-Aldrich, Germany)
- Liquid nitrogen (99.999%) (Linde Gas a.s., Czech Republic)
- Potassium dihydrogen phosphate (K H_2PO_4, ≥ 98%) (Sigma-Aldrich, Germany)
- Poly(ethylene glycol) (PEG, with an average M_w of 1500 $g \cdot mol^{-1}$) (Sigma-Aldrich, Germany)
- Polystyrene (PS, with M_w 30.000 $g \cdot mol^{-1}$ and M_w/M_n 1.06) (Pressure chemical CO, USA)
- Sodium chloride (NaCl, ≥ 99.5%) (Lach-Ner, s.r.o., Czech Republic)
- Sodium hydroxide (NaOH, ≥ 98%) (mikroCHEM, Slovakia)
- Tetrahydrofuran (THF for HPLC, ≥ 99.9%) (Sigma-Aldrich, Germany)
- Thin octanoate (SnII 2-ethylhexanoate, ≥ 92.5%) (Sigma-Aldrich, USA)
- Ultrapure water (Ultrapure water type I) was prepared on Millipore Direct- Q® 3 UV
- Water HiPerSolv CHROMANORM® for HPLC (VWR Collection, USA)

4.2 Equipment

- Common laboratory equipment and glass such as flasks, reagent bottles, vials, funnels, spatulas and injections, needles, filters
- Analytical scales Adventurer Pro AV264C (OHAUS, Switzerland)
- Incubator CO2cell (MMM group, Germany)
- Automatic pipettes (Hirschmann Laborgeräte, Germany)
- Gel permeation chromatography (HPLC GPC/SEC system) 1260 Infinity (Agilent Technologies, USA) with multiangle light scattering (MALS) photometry DAWN HELEOS II (Wyatt, USA) and differential refractometer T-rEX (Wyatt, USA)
- Cooled incubator VWR INCU-Line (VWR Collection, USA)
- Pocket-size waterproof pH Meter H138 miniLab™ with ISFET sensor (Hach Company, USA)
- Ultra High Performance Liquid Chromatography (UHPLC) Agilent 1290 Infinity (Agilent Technologies, USA) with Luna® Omega Polar C18 column (100 x 2.1 mm; 1.6 μm) (Phenomenex, USA) connected with BRUKER EVOQ LC-TQ Mass spectrometer (Agilent Technologies, USA) and Nitrogen and dry air gas generator Genius 3045 (PEAK Scientific, UK) (at FCH VUT)
- Glass high-vacuum line (hand-made at CEITEC)

- Proton Nuclear Magnetic Resonance Spinsolve 60 benchtop ^{1}H and ^{19}F NMR spectrometer (Margitek, Germany)
- Tissue Culture Plates Inserts 12 wells, PC membrane, 0.4 μm, sterile (VWR Collection, USA)

4.3 Sample preparation

4.3.1 Synthesis of PLGA-PEG-PLGA copolymer

The ABA copolymer with a D,L-LA/GA molar ratio of 3.0 and a PLGA/PEG weight ratio of 2.5 was prepared by ring-opening polymerisation (ROP) in two steps. The first step was the purification of PEG, and the second step was the addition of LA and a GA and the subsequent reaction using stannous octanoate as the catalyst. The entire reaction process was carried out under a nitrogen atmosphere at 130 °C for 3 hours [18], [19].

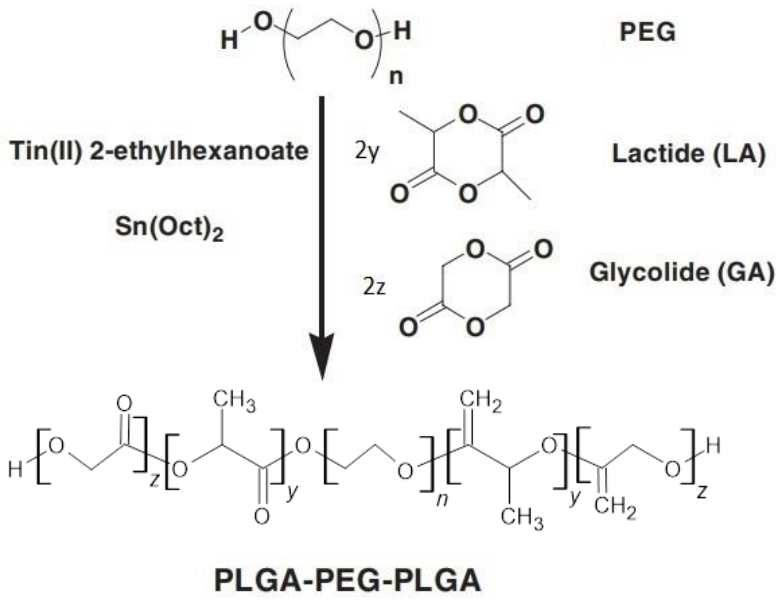

Figure 12: Ring-opening polymerisation of PLGA-PEG-PLGA [19].

The living ring-opening synthesis of the PLGA-PEG-PLGA copolymer was prepared following the guidelines given in an article written by Michlovská L. et. al. (2010) [18].

Figure 13: Vacuum line for copolymer synthesis.

After the synthesis, the copolymer was purified from the unreacted monomers by repeated dissolving in ultrapure water (UPW) in a refrigerator and then subsequent precipitation at 80 °C. The polymer precipitate was separated from the water by decantation. The entire purification process was repeated three times. The purified polymer product was later freeze-dried [71].

4.3.2 Preparation of the copolymer solution

Three different concentrations of copolymer aqueous solutions were prepared, 10, 15 and 20 wt. % solutions. The copolymer was weighed into vials, and the necessary amount of solvent was pipetted into vials. The polymer was dissolved in UPW, saline solution (0.9 % NaCl) or phosphate-buffered saline (PBS) (with a pH of 7.54) in the case of enzymatic degradation. The entire mixture was stirred for 3 – 4 days in the refrigerator at a temperature of 12 °C.

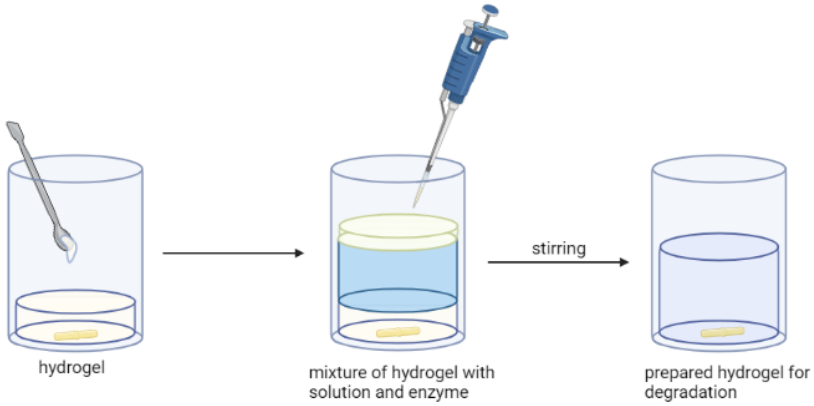

Figure 14: Process of preparation of copolymer solution [12].

In the case of enzymatic degradation, lipase solution was prepared. Then the required volume of lipase solution was added to the already dissolved copolymer solution (after 3 – 4 days) so that the final concentration of lipase in the enzyme - hydrogel mixture was 0.8 mg/ml. This mixture was stirred for 30 min in the refrigerator.

Figure 15: Copolymer solution - dissolved copolymer in the required solution after 4 days of stirring in the refrigerator.

4.3.3 Sample preparation for degradation

After the hydrogel dissolved in the required medium, 0.5 ml of this solution was pipetted into the cultivation plate inserts with a porous membrane allowing hydrogel to diffuse into the degradation medium and vice versa. The prepared samples in inserts were allowed to gel for 45 min at 37 °C in an incubator. After whole samples were gelled, the cultivation plates were inserted into a bottom pan containing a degradation medium and allowed to degrade.

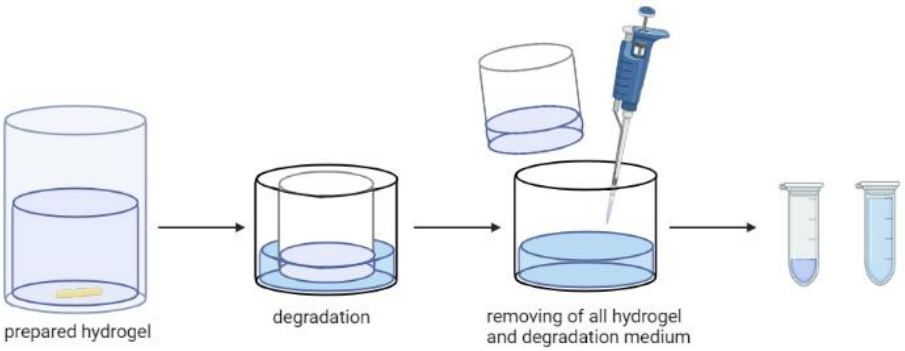

Figure 16: Process of preparing hydrogel samples for degradation [12].

Samples for enzymatic degradation were prepared in the same way. The prepared samples in inserts were allowed to gel for 45 min at 37 °C in an incubator. Gelled samples in the cultivation plates were inserted into a bottom pan containing degradation medium and allowed to degrade.

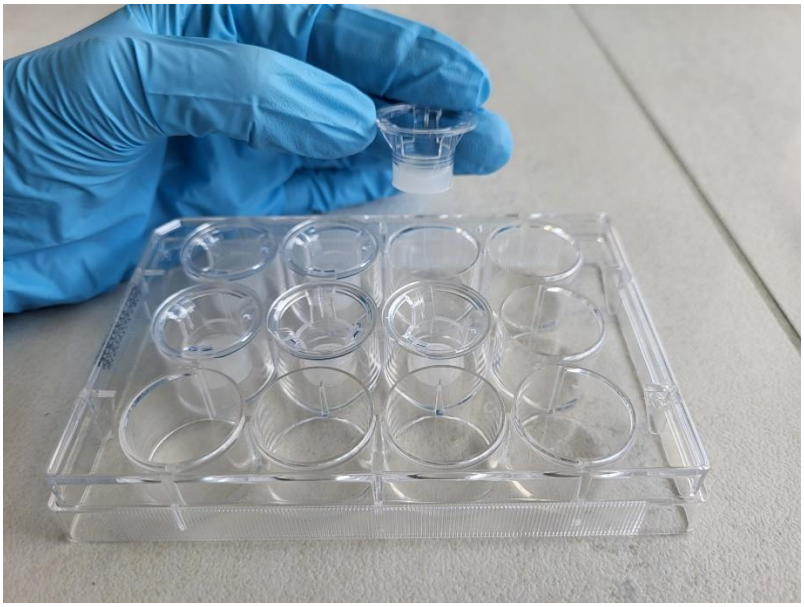

Figure 17: The prepared loaded samples of hydrogel in cultivation plate with membrane and a bottom pan with degradation medium.

4.4 Degradation

4.4.1 Sample degradation

To study the behaviour of the hydrogel in different degradation mediums, the dissolved hydrogels were allowed to degrade in an incubator at 37 ℃. The samples were removed from the degradation medium after 1, 2, 3, 7, 9, 14, 16, 21, 23 and 30 days.

In the case of lipase degradation, the samples were removed after 1,2 and 3 days.

4.4.2 Sample removal

Samples of hydrogel and degradation medium were withdrawn after a certain time. The degraded hydrogel after a certain time was collected into Eppendorf tubes for later GPC/SEC analysis. The pH of the removed degradation medium was measured, and then this liquid fraction was pipetted into Eppendorf tubes for later analysis of degradation products via LC-MS. Usually, after 21 days, there was no degraded hydrogel left to be removed, because most of it already diffused through the porous membrane into the degradation medium. To determine the weight loss percentage, the hydrogel samples in inserts before and after degradation were weighed and mass loss was calculated by the following equation (1):

$$W_{loss}[\%] = \frac{W_{before} - W_{after}}{W_{before}} \cdot 100\% \qquad (1)$$

where W_{before} is the mass of hydrogel loaded into the cultivation plate insert [g] before degradation, and W_{after} is the mass of the removed insert with the hydrogel [g] after a certain time of degradation.

4.5 Methods

4.5.1 Gel permeation chromatography / Size exclusion chromatography (GPC/SEC)

Size Exclusion Chromatography (SEC), also called Gel Permeation Chromatography, uses a chromatographic technique that separates dissolved molecules based on their size in a chromatographic column [72]. GPC/SEC analysis was performed at CEITEC laboratories.

Figure 18: GPC technique for separating dissolved molecules according to their size [12].

GPC was used to determine the number average molecular weight (M_n) and polydispersity index (M_w/M_n or PDI) of polymer samples. The instrument used two PLgel 5 μm Mixed-C columns for separation. It was also equipped with multi-angle light scattering photometer HELEOS-II and with refractive index Optilab T-rEX detectors. Tetrahydrofuran (THF) was used as a mobile phase with a flow rate of 1 ml/min. As a standard, polystyrene (PS) (with M_w 30 kg·mol^{-1} and PDI 1.06) was used. The data were evaluated in ASTRA 6.1 programme.

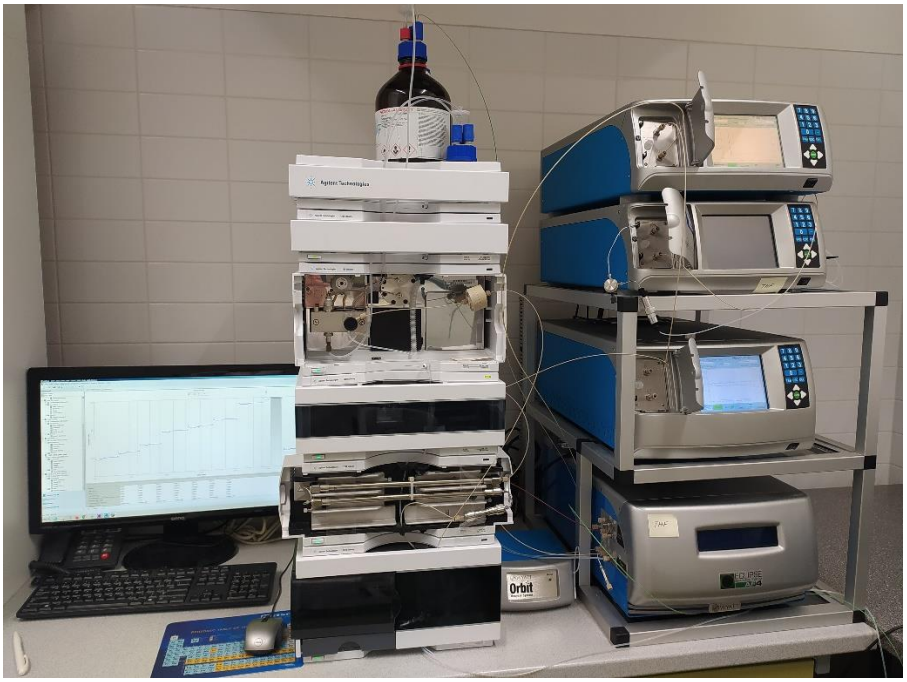

Figure 19: Gel permeation chromatography GPC/SEC system 1260 Infinity with Multi-Angle Light Scattering (MALS) photometry DAWN HELEOS II.

4.5.1.1 Sample preparation for GPC analysis

15 mg of degraded hydrogel and 3 mg of PS as standard were weighed into 4 ml vials. Then 1 ml of THF was added as a solvent to the vials. The prepared samples with solvent were allowed to dissolve. If necessary, the samples were shaken. After the copolymer and PS dissolved, the mixture was filtrated using 2 ml syringes, needles, and 0.2 μm polytetrafluoroethylene (PTFE) syringe filters (Ahlstrom) in small 2 ml screw cap septum vials used for GPC or HPLC. After preparation, samples were placed in an autosampler for analysis.

4.5.2 Proton Nuclear Magnetic Resonance spectroscopy (^{1}H NMR)

Nuclear Magnetic Resonance (NMR) is a spectroscopic technique used for determining the structure of organic compounds or those with protons, their purity and content. NMR provides information about the compound through the chemical shift δ that is characteristic of various nuclei. ^{1}H NMR is used to determine the types and number of hydrogen atoms in the molecule [73]. ^{1}H NMR analysis of copolymer was performed at CEITEC laboratories.

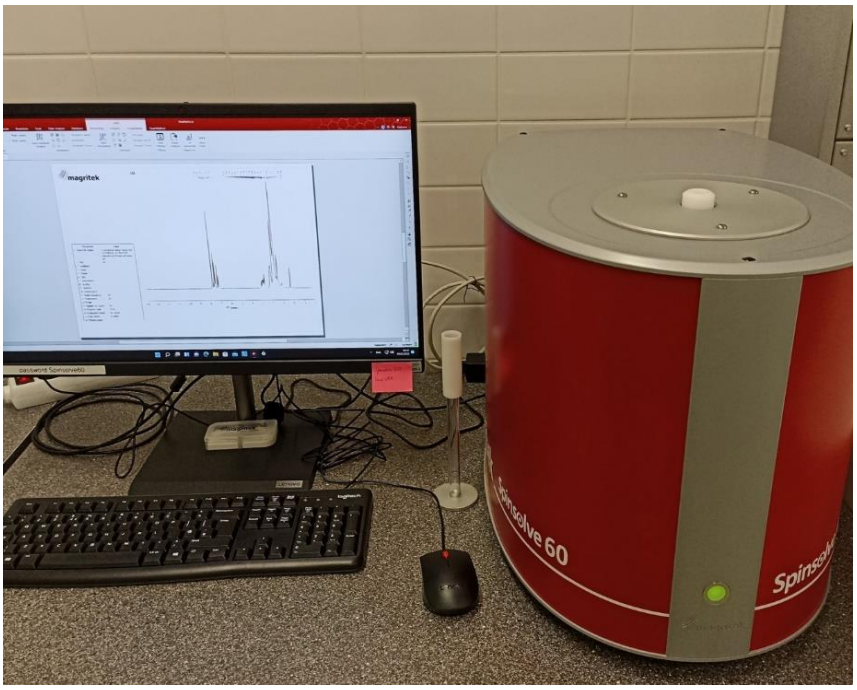

Figure 20: Proton Nuclear Magnetic Resonance Spinsolve 60 benchtop ^{1}H and ^{19}F NMR spectrometer.

Polymer characterization as PLGA/PEG, LA/GA rations and M_w of the synthesized copolymer were acquired using ^{1}H NMR spectrometer Spinsolve with operating frequency of 60 MHz with 1.6 s acquisition time, 10 s repetition time, 90 ° pulse angle, and 128 scans at 25 °C. Deuterated chloroform ($CDCl_3$) was used as a solvent. The ^{1}H NMR spectra were evaluated using an ACD / 1D NMR Processor.

4.5.2.1 Sample preparation for ^{1}H NMR spectroscopy

20 mg of the synthesized copolymer was weighed into 4 ml vials. Then 1 ml of $CDCl_3$ was added as a solvent to the vials. The samples were allowed to dissolve, and if necessary, samples were shaken. When the copolymer was dissolved, the required volume of the mixture was pipetted into NMR tubes. After preparation, the sample was placed in NMR for analysis.

4.5.3 Ultra-High-Performance Liquid Chromatography (UHPLC)

Ultra-high-performance liquid chromatography (UHPLC) is a modern chromatographic technique for the identification and quantification of mixture components. It can separate dissolved ions, organic substances, and macromolecules. The separation process is based on the affinity of substances to the stationary phase (in the chromatographic column) [74]. LC-MC analysis was performed at the Faculty of Chemistry BUT.

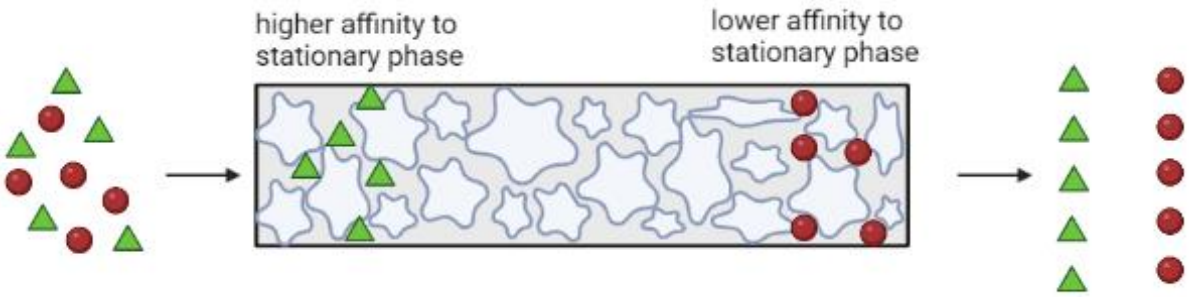

Figure 21: UPLC technique for separating dissolved substances according to their affinity to the stationary phase [12].

UHPLC in tandem arrangement with electrospray ionisation mass spectrometry (LC-MS) was used for the quantitative analysis of degradation products (LA, GA) in the degradation medium. The instrument used a Luna® Omega Polar C18 column for the separation of substances. A mixture of 0.1 % formic acid (HCOOH) in UPW with acetonitrile (MeCN) was used as a mobile phase with a flow rate of 0.5 ml/min. The acquired data were evaluated in the TASQ software.

Figure 22: Ultra-High-Performance Liquid Chromatography (UHPLC) Agilent 1290 Infinity with Luna® Omega Polar C18 column connected with BRUKER EVOQ LC-TQ Mass spectrometer.

4.5.3.1 Preparation of the calibration solutions

Calibration solutions are used in quantitative analysis to determine the unknown concentration of a given sample in solution. First, stock standard solutions were prepared. 10 mg of each standard (GA, LA) was weighed, transferred to a 10 ml volumetric flask, and filled with a mixture of MeCN with UPW for liquid chromatography (in a 50:50 ratio). Such prepared

solution had a concentration of 1 $mg \cdot ml^{-1}$. The other stock standard solution with a concentration of 10 $\mu g \cdot ml^{-1}$ was prepared from a 1 $mg \cdot ml^{-1}$ solution by dilution with UPW for liquid chromatography.

After the preparation of standard stock solutions, calibration solutions with concentrations of 50; 100; 250; 500; 750; 1000; 1500 and 2000 $Ng \cdot ml^{-1}$ were prepared by dilution with UPW for liquid chromatography.

4.5.3.2 Sample preparation for LC-MS analysis

Due to the high concentration of LA and GA, samples of degradation medium were appropriately diluted. After dilution, the samples were filtrated using 2 ml syringes, needles and 0.22 μm Nylon syringe filters (Agilent Technologies) in small 2 ml screw cap septum vials used for GPC or HPLC. After preparation, samples were placed in an autosampler for analysis.

5. RESULTS AND DISCUSSION

5.1 Characterisation of synthesized PLGA-PEG-PLGA polymer

The thermosensitive PLGA-PEG-PLGA copolymer was synthesized on an advanced high vacuum line via ROP according to the article written by Michlovská L. et. al. (2010) [18]. The synthesized copolymer after purification had a slightly yellowish colour, was semi-transparent (see Figure 23) and was sticky and stretchy. Other characteristics of the synthesized polymer were obtained from ^{1}H NMR spectroscopy and GPC/SEC measurements.

Figure 23: Synthesized and purified PLGA-PEG-PLGA copolymer.

5.1.1 Characterisation of polymer via ^{1}H NMR spectroscopy

^{1}H NMR spectroscopy was used to receive the number average molecular weight (M_n) and the LA/GA and PLGA/PEG rations of the synthesized copolymer batches. A typical ^{1}H NMR spectrum of the PLGA-PEG-PLGA copolymer was obtained (see Figure 24). Each peak represents a different proton in the structure of the copolymer. Four different batches were used for the degradation of the hydrogel. ^{1}H NMR characterisation of all the used batches is shown in Table 1.

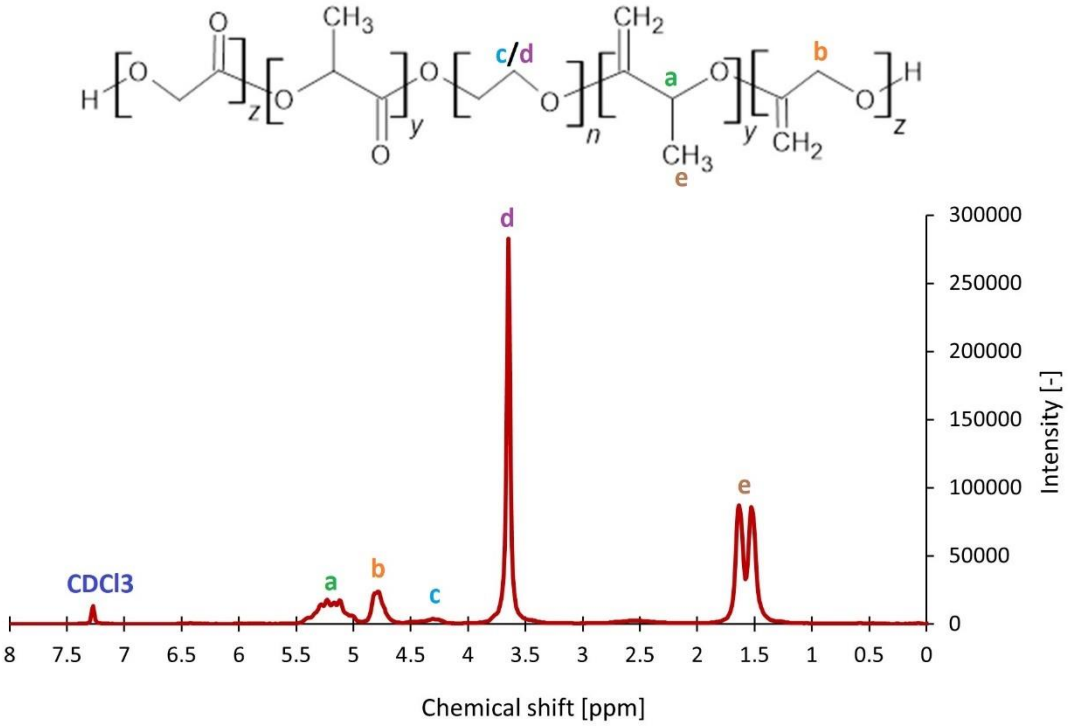

Figure 24: ^{1}H NMR spectrum of the PLGA-PEG-PLGA copolymer with chemical shift in ppm.

Figure 24 shows the ^{1}H NMR spectrum of the copolymer with six peaks. The peak with the largest chemical shift represents deuterated chloroform $CDCl_3$ used as a solvent. The double peak with the lowest chemical shift δ = 1.4 - 1.7 ppm (**a**), represents (O-(C**H**$_3$)CHO) and the peak with chemical shift δ = 5.0 – 5.5 ppm (**e**) represents (O-(CH_3)C**H**O) of the LA. PEG protons (OC**H**$_2$C**H**$_2$O) are characterized by a chemical shift δ = 3.5 – 3.7 ppm (**b**). The GA proton (OC**H**$_2$O) is demonstrated by a peak with chemical shift δ = 4.6 – 4.9 ppm (**d**). The peak with the lowest intensity and chemical shift of δ = 4.2 – 4.35 ppm (**c**) is characteristic of protons in the bond between PEG and PLA (OC**H**$_2$C**H**$_2$O) [18].

Table 1: Table of values obtained for each batch of copolymer from the ^{1}H NMR measurement compared with the theoretical values.

Batch of polymer	Theoretical values	1	2	3	4	Average
M_n [g·mol^{-1}]	5250	5389	5296	5156	5235	**5269 ± 99**
PLGA/PEG [wt./wt.]	2.5	2.59	2.53	2.44	2.49	**2.51 ± 0.07**
LA/GA [mol/mol]	3.00	2.91	2.89	2.91	3.04	**2.94 ± 0.7**

Furthermore, the M_n, PLGA/PEG ratio data and LA/GA molar ratio were obtained from NMR (see Table 1). The results showed that, copolymers were synthesized with approximately PLGA/PEG weight ratio of 2.51 ± 0.07, an LA/GA molar ratio of 2.94 ± 0.7 and an average M_n of 5269 ± 99 g·mol^{-1}.

5.1.2 Characterisation of polymer via GPC/SEC

GPC/SEC analysis was used to determine the number average molecular weight (M_n, curve 2 in Figure 25) and the polydispersity index (PDI). The linearity of the synthesized polymer is represented by the Mark–Houwink plot (curve 1 in Figure 25) in graphs. Samples for GPC/SEC analysis were prepared as mentioned in Chapter 4.5.1.1. Each sample was measured twice for the control.

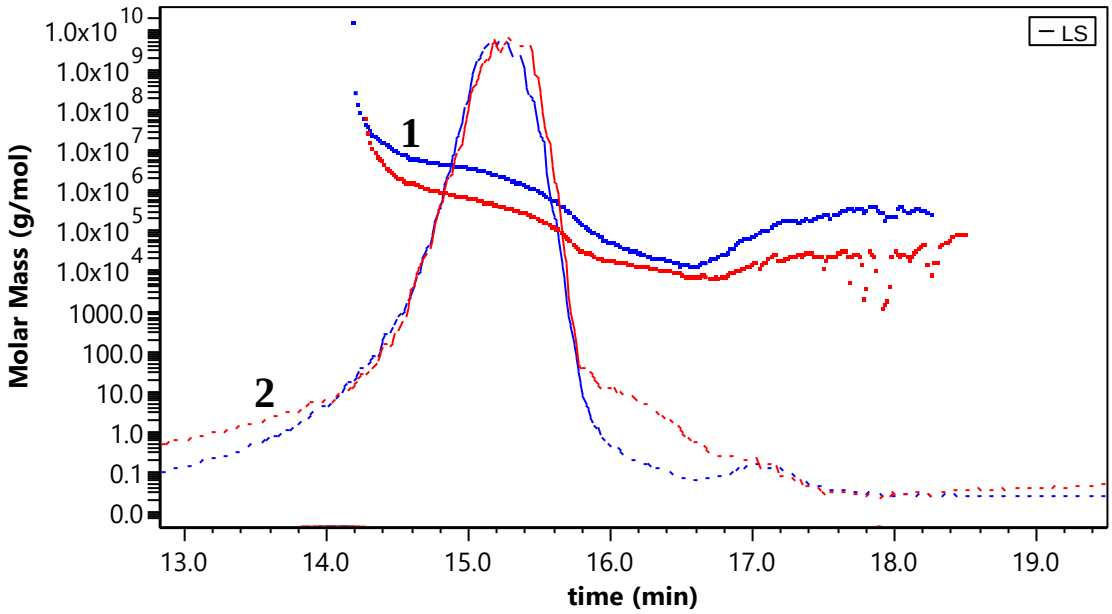

Figure 25: Graph of 1st batch synthesized copolymer ABA, measured two times for control. The curve 1 represents Mark-Houwink plot and the curve 2 represents distribution of molecular weight of polymer.

The Figure 25 shows the chromatogram of synthesized ABA copolymer. The curve 1 shows that polymer is linear. And curve 2 represents molecular weight distribution. Table 2 shows characterisation of the 1st batch of synthesized polymer determined by GPE/SEC analysis. The number average molecular weight of polymer was 5670 g·mol^{-1} with polydispersity index 1.09.

Table 2: Measured values of 1st batch synthesized PLGA-PEG-PLGA copolymer received from GPC/SEC compared to the theoretical values.

	M_n [g·mol^{-1}]	PDI
Theoretical values	5250	1.09
Batch of copolymer	5670 ± 344	1.143 ± 0.01

5.2 Characterisation of degradation products

Hydrogel samples were degraded during the designed period (1, 2, 3, 7, 9, 14,16, 21, 23, 30 days). At particular time, each degraded hydrogel was collected and removed from the cultivation plate inserts, and degradation medium was removed from the bottom pan. However, before the samples were removed, gravimetric analysis was performed. Later, the final samples were sent for further analysis. Degraded hydrogels were analysed via GPC/SEC, and degradation media were analysed via LC-MS. However, some data are not available because the amount of some samples was insufficient for further analysis.

5.2.1 Gravimetric analysis

Gravimetric analysis was used to characterise mass loss percentage of polymer during degradation in various medium in bottom pan. The pH of degradation media was also measured. After preparing the samples for degradation, were pipetted into the cultivation plate inserts and let to gel for 45 minutes at 37 °C. After the hydrogels were gelled, every single insert plate with sample was weighed on analytical scales. At a particular time after degradation, insert plates with degraded hydrogel were removed from the bottom pan. Exes surface liquid was carefully dried with cellulose fibre from samples and inserts were weighed. Later, the weight loss percentage was determined.

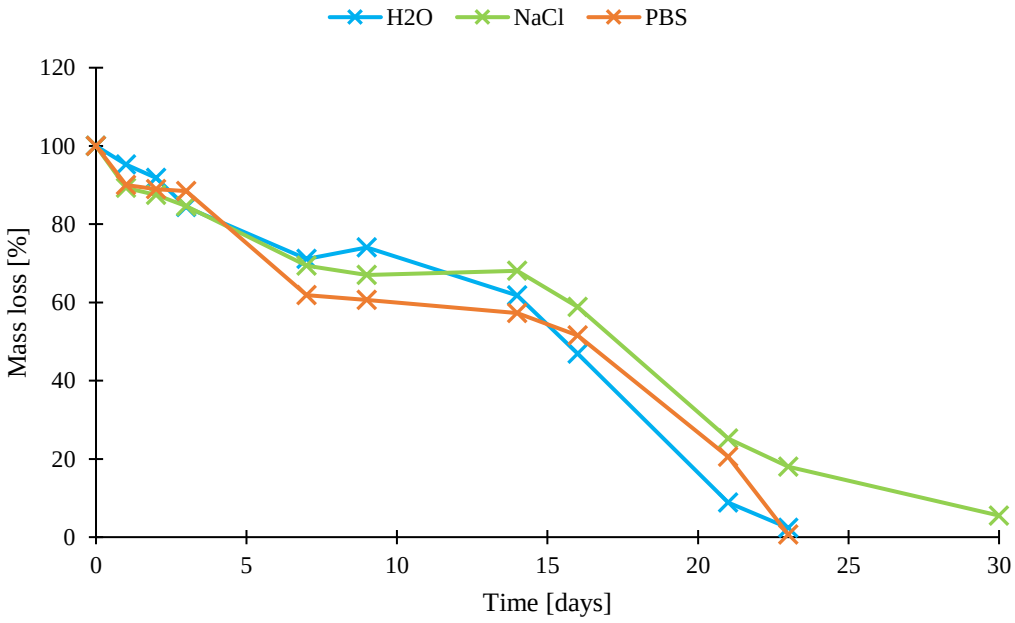

Figure 26: Graph of 10 wt. % hydrogel mass loss progress during the designed period in various degradation media.

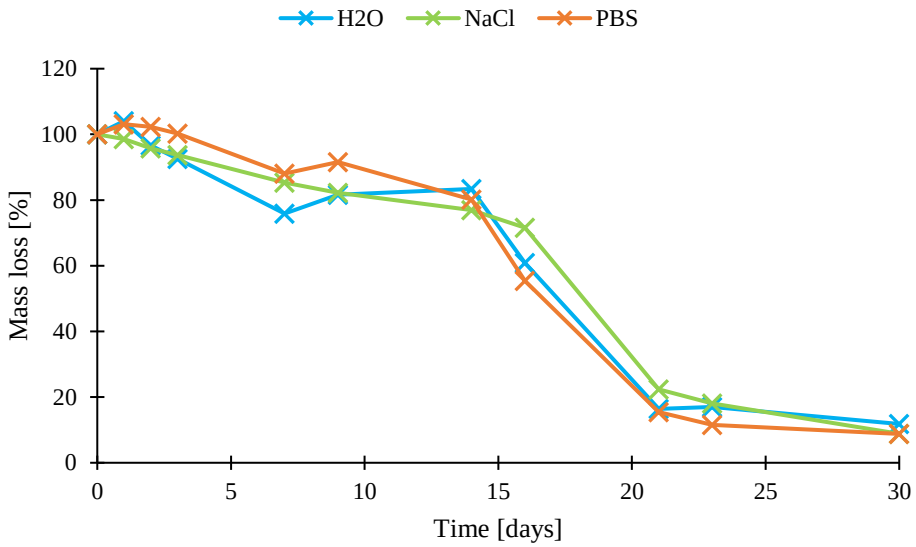

Figure 27: Graph of 15 wt. % hydrogel mass loss progress during the designed period of time in various degradation media.

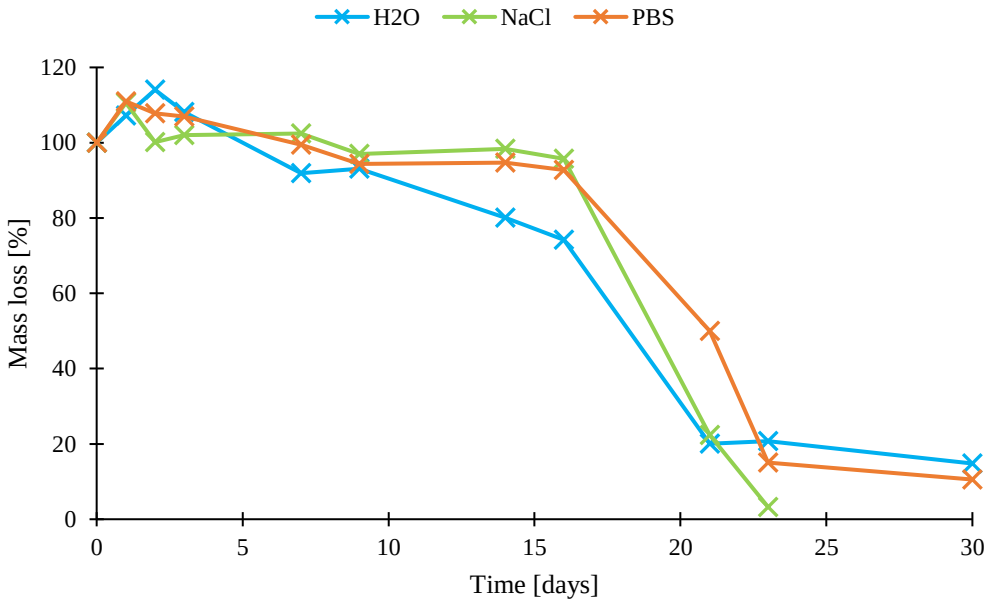

Figure 28: Graph of 20 wt. % hydrogel mass loss progress during the designed period of time in various degradation media.

From graphs above, it can be seen that the degradation curves are similar for each hydrogel concentration. In general, we can divide curves into 4 phases. The first phase is the water intake phase or swelling. The hydrogel samples in Figure 27 and Figure 28 underwent a swelling phase for approximately the first three days, except for 15 wt. % hydrogel in NaCl (Figure 27). The most significant swelling was observed in 20 wt. % hydrogel degraded in UPW. However, none of the 10 wt. % hydrogel samples in Figure 26 swelled. We can generally say that, with

increasing concentration of hydrogel, the percentage of swelling increases [75]. The second phase is a moderate drop that includes a small increase in mass. The next phase is a section of significant drop, and the last one is a static phase. The largest mass loss is visible in Figure 26 in case of 10 wt. % hydrogel degraded in PBS. After 23 days 99.35 % of the hydrogel mass degraded. Half of the degraded mass occurred in the case of 10 % hydrogels around 15 days, and with increasing concentration of hydrogel, this time was shifting closer to approximately 20 days.

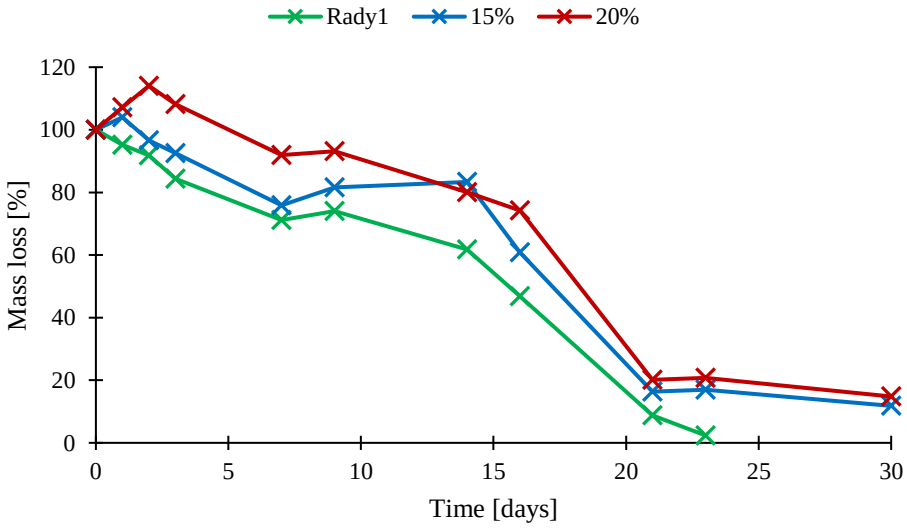

Figure 29: Graph of various concentrations of hydrogel degraded in H_2O.

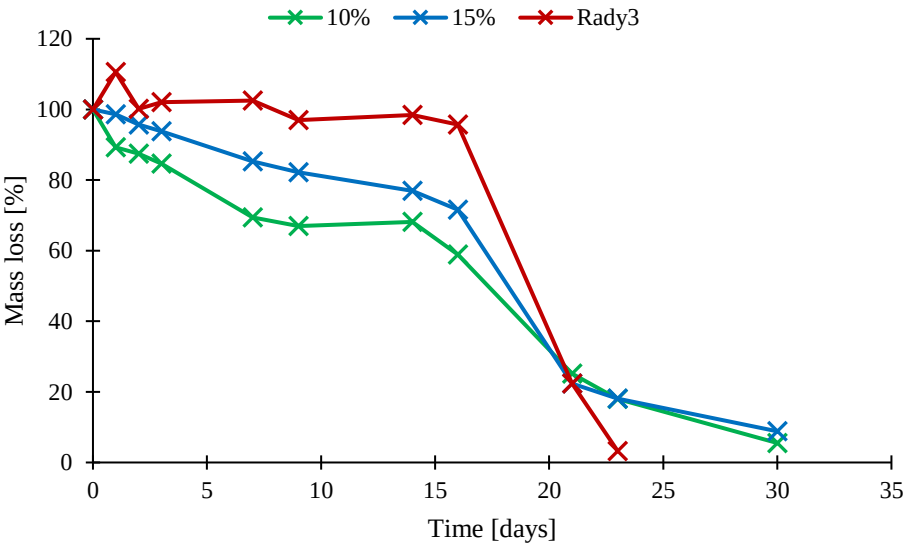

Figure 30: Graph of various concentrations of hydrogel degraded in NaCl.

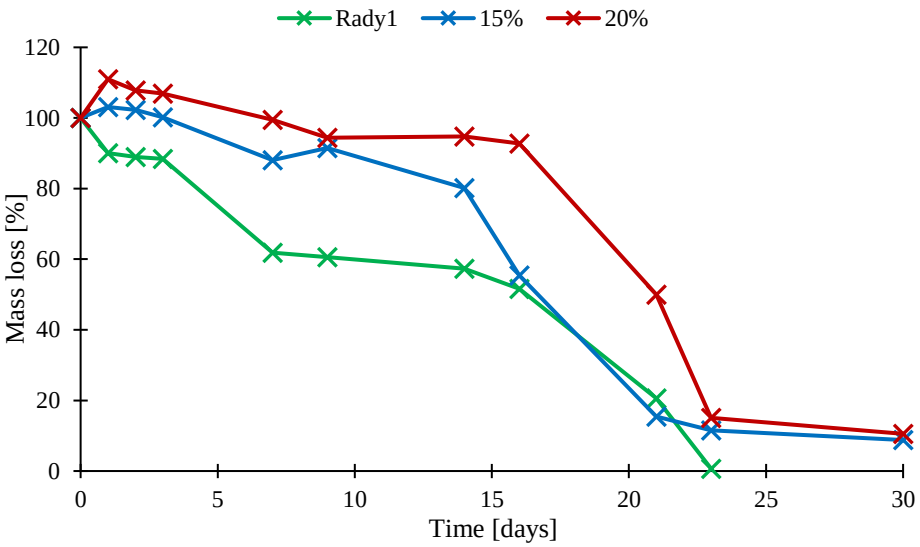

Figure 31: Graph of various concentrations of hydrogel degraded in PBS medium.

Figure 29, Figure 30, and Figure 31 show the degradation progress of various hydrogel concentrations in particular media.

Our hydrogel is degraded through the hydrolytic degradation, which generates LA and GA as degradation products. As degradation proceeds, more degradation products (LA, GA) started to occur in the bottom pan liquid. Due to the higher concentration of acids in the degradation medium, the pH decreased gradually, as shown in graphs below (Figure 32, Figure 33 and Figure 34). From the graphs below, it is visible that the pH progress in time depends on the type of a degradation medium [76]. For each medium, the curve is specific apart from hydrogel concentration (see Figure 35, Figure 36 and Figure 37).

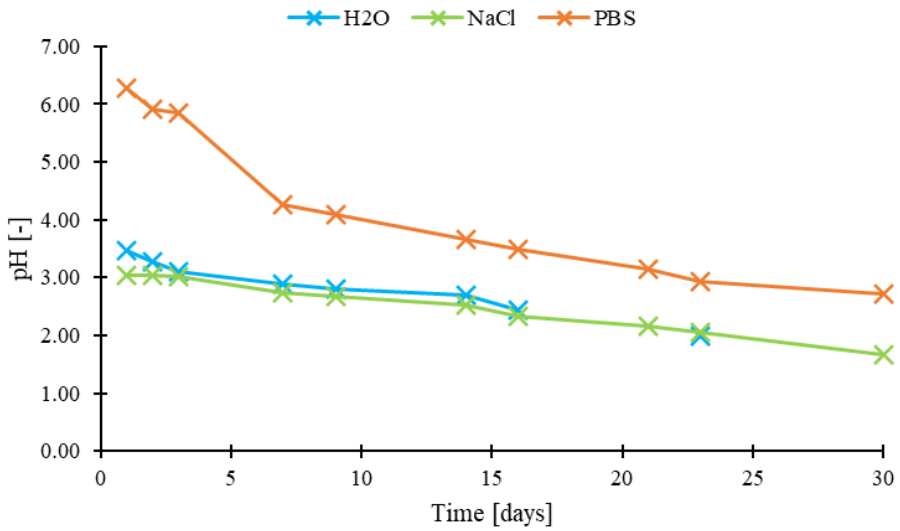

Figure 32: Graph of pH medium development used for 10 wt. % hydrogels.

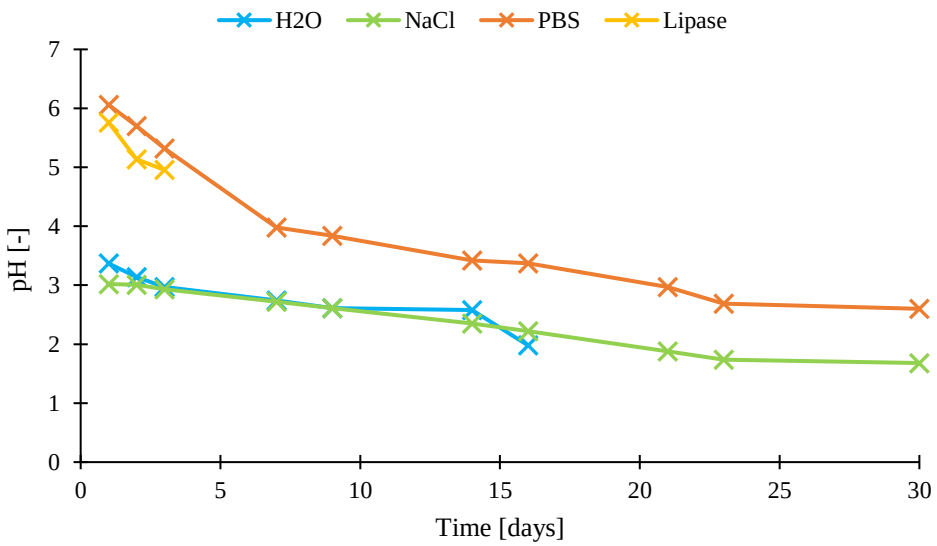

Figure 33: Graph of pH medium development used for 15 wt. % hydrogels.

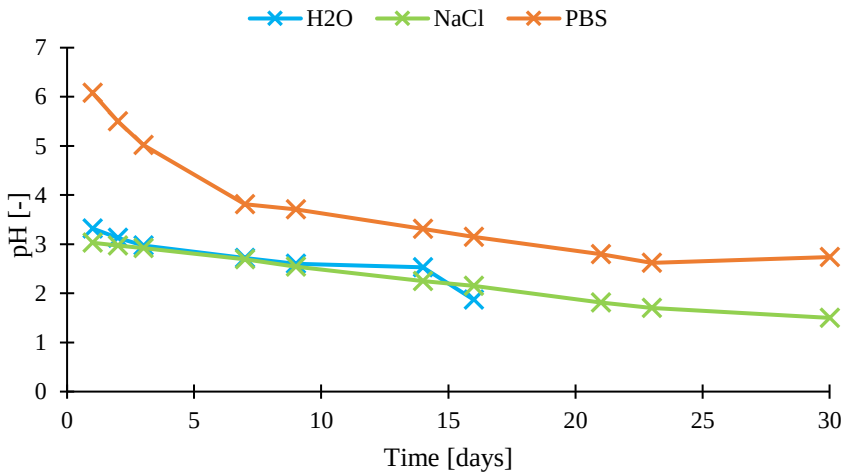

Figure 34: Graph of pH medium development used for 20 wt. % hydrogels.

The lowest pH value, 1.50 was achieved with 20 wt. % hydrogel in NaCl solution after 23 days of degradation (see Figure 34). Altogether we can say that the biggest decrease in pH values is during first three days of degradation. Figure 35, Figure 36 and Figure 37 show the pH progress of different hydrogel concentrations in particular solutions.

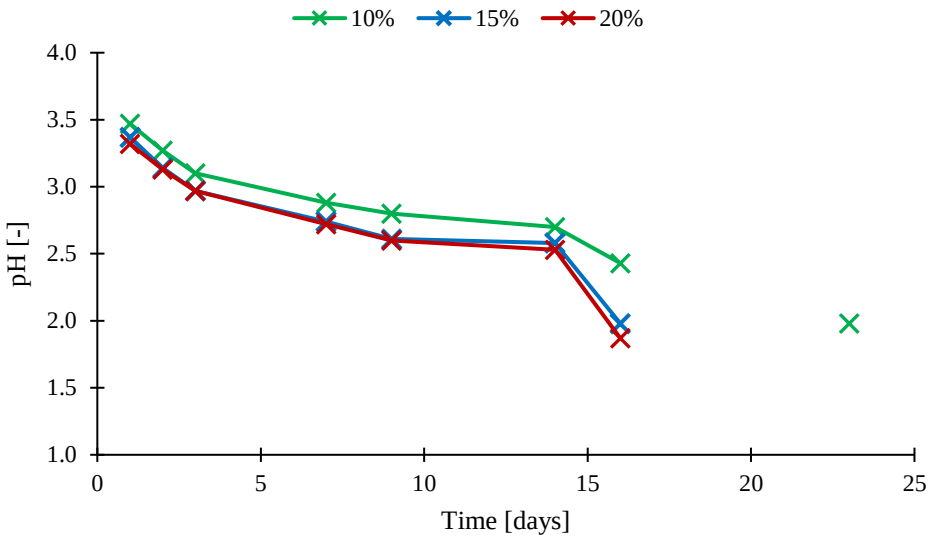

Figure 35: Graph of pH progress in time of various concentrations of hydrogel in H_2O.

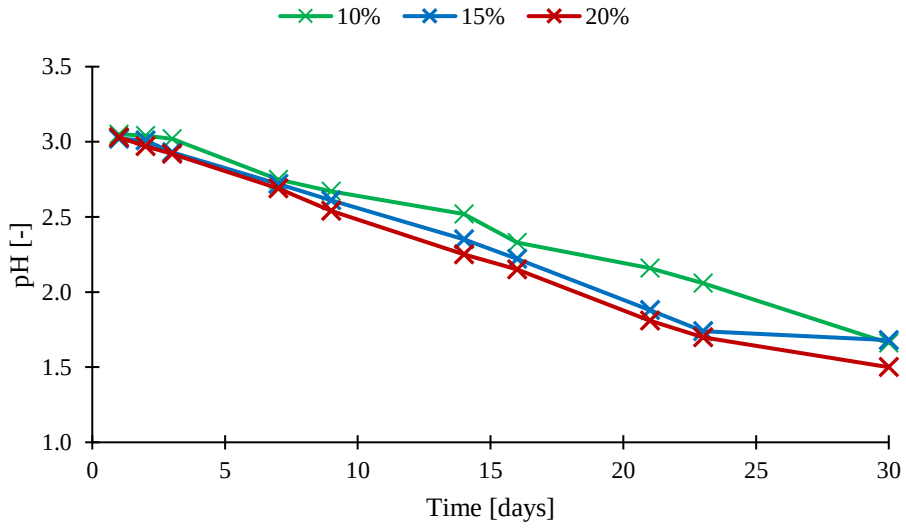

Figure 36: Graph of pH progress in time of various concentrations of hydrogel in NaCl.

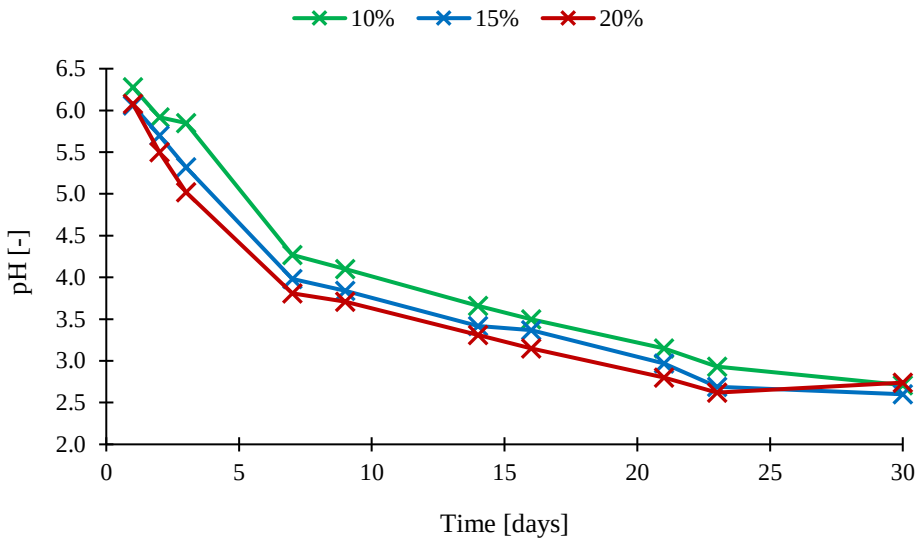

Figure 37: Graph of pH progress in time of various concentrations of hydrogel in PBS.

15 wt. % hydrogel samples were let to degrade with lipase in PBS. In this work we tried to simulate environment for sample degradation to the environment in human body, thus the degradation of hydrogels in human body will be different. Human body contain numerous enzymes, proteins and substances that may have an impact on the degradation. We chose to use lipase as enzyme effecting degradation because its abundance in human body and ability to accelerate biodegradation of aliphatic polyesters. The degradation time was designed for three days, though we expected a rapid degradation rate, as was suggested in the article by Ke Shi et. al. [60].

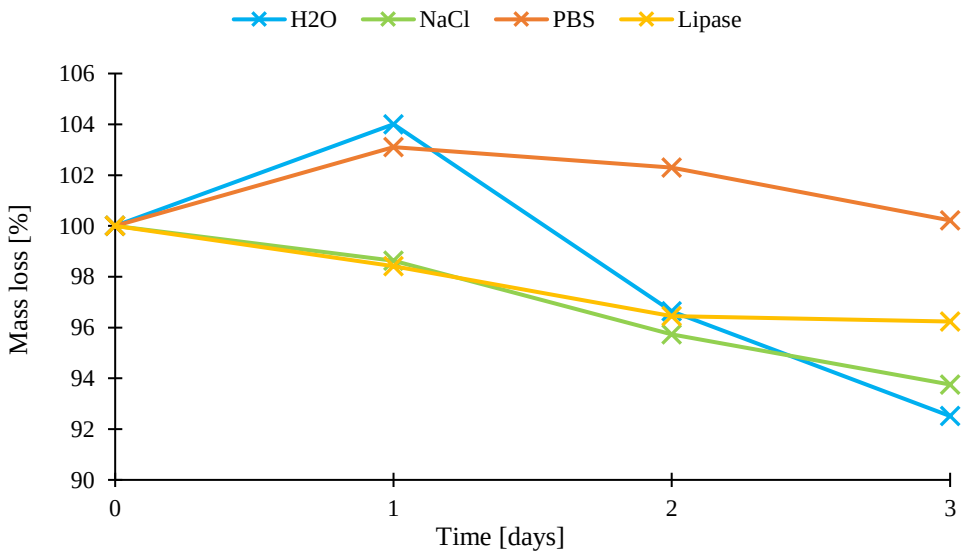

Figure 38: Comparison graph of the first three days degradation of 15 wt. % hydrogel in multiple degradation media, H_2O; NaCl; PBS, and PBS with lipase.

Degradation of 15 wt. % hydrogel with lipase was not swelling, rather degrading from the beginning (see Figure 38). That is the first observable difference if we compare it with other hydrogels (except for the 15 wt. % hydrogel in NaCl). After three days, the hydrogel mass dropped by 3.77 %. Similar behaviour was observed in the case of NaCl, where the mass loss was after three days 6.24 %.

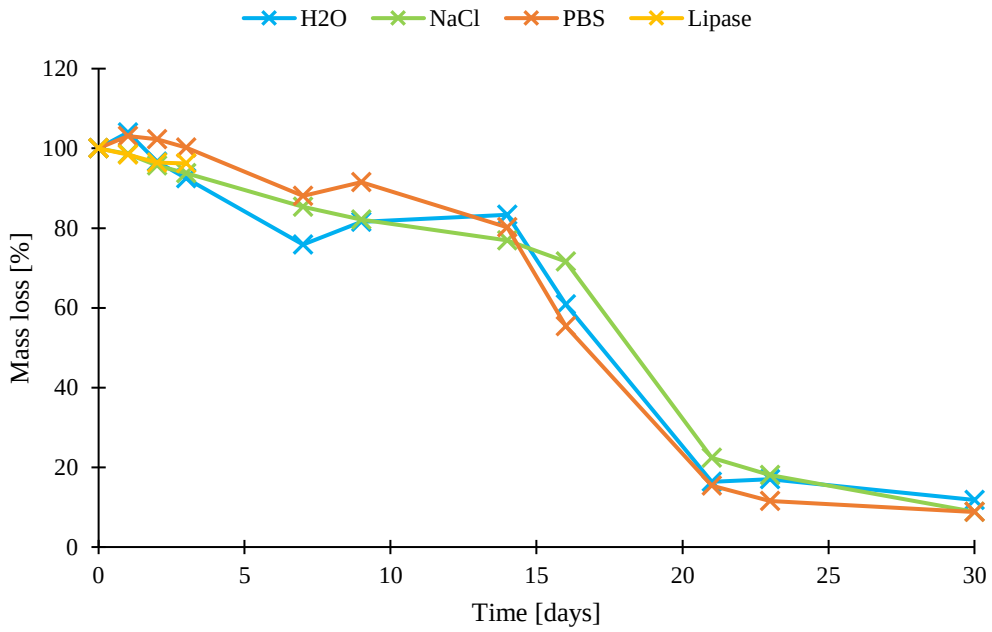

Figure 39: Graph of 15 wt. % hydrogel mass loss progress during the designed period of time in various degradation media plus enzymatic degradation.

Figure 39 shows that the hydrogel with lipase did not undergo the swelling phase and as mentioned above, the degradation curves are similar for each hydrogel concentration; thus, we can predict, that after three days the hydrogel with lipase may be in the moderate drop phase. It seems like progress in enzymatic degradation with lipase will have a sharper decline in the next phases.

5.2.2 Characterisation of hydrogel degradation via GPC

The change in the average molecular weight and PDI during the time of hydrogel degradation was studied via GPC/SEC analysis. After degradation of each sample, the hydrogel was removed from the cultivation plate inserts. Degraded samples for GPC/SEC analysis were prepared as mentioned in Chapter 4.5.1.1. Each sample was measured twice for control.

The degradation was confirmed by a gradual decrease in the molecular mass of the polymer. Figure 40 shows us the change of the molecular weight distribution curve in the case of 15 wt. % hydrogel in NaCl solution after 1, 7 and 16 days. The peak is gradually getting smaller, which means that a significant decrease in the molecular weight of the polymer was observed.

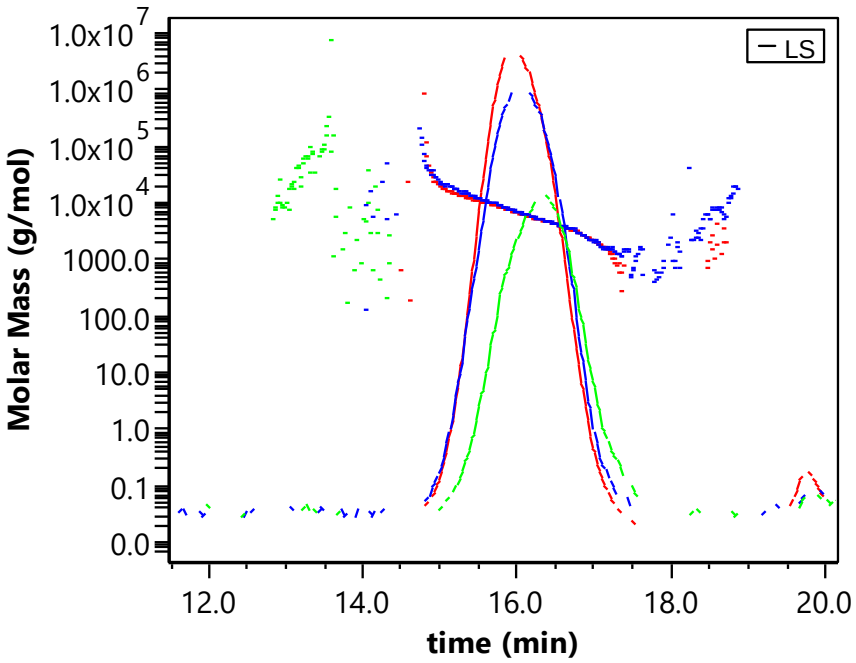

Figure 40: Graph of the molecular weight distribution of 15 wt. % hydrogel in NaCl. The red curve represents the hydrogel after 1 day, the blue curve represents the hydrogel degraded for 7 days, and the blue green represents the hydrogel after 16 days.

Figure 41 shows the difference in degradation with lipase. 15 wt. % hydrogels degraded in NaCl, UPW and PBS have longer degradation time decreasing trends. On the other hand, the trend of lipase degradation is not yet visible. GPC/SEC analysis showed that lipase has a minor impact on hydrogel degradation, though gravimetric analysis and LC-MS analysis of degradation products showed different results. The next graphs below (Figure 42 and Figure 43) show decreasing trends of molecular weight for 10 and 20 wt. % hydrogels.

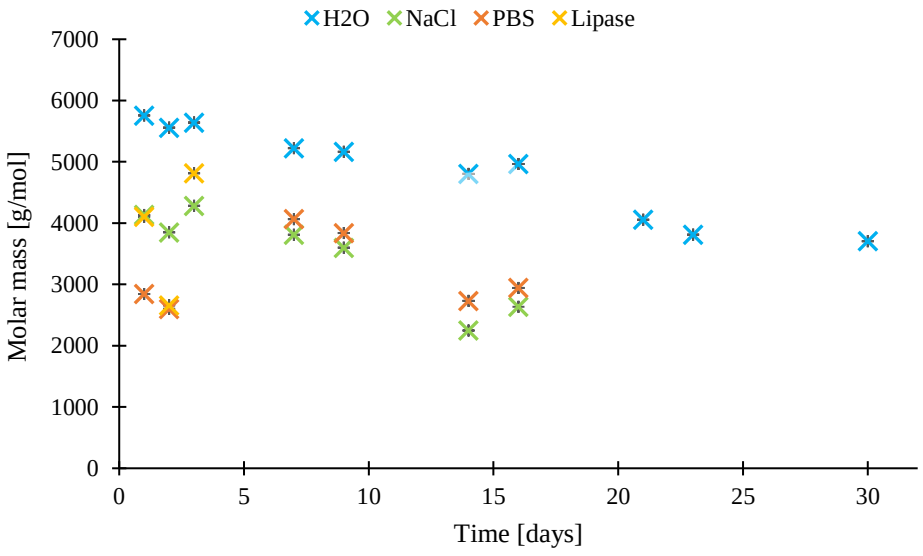

Figure 41: Graph of molar mass decrease during time of various 15 wt. % hydrogels. Data were obtained from GPC/SEC measurements.

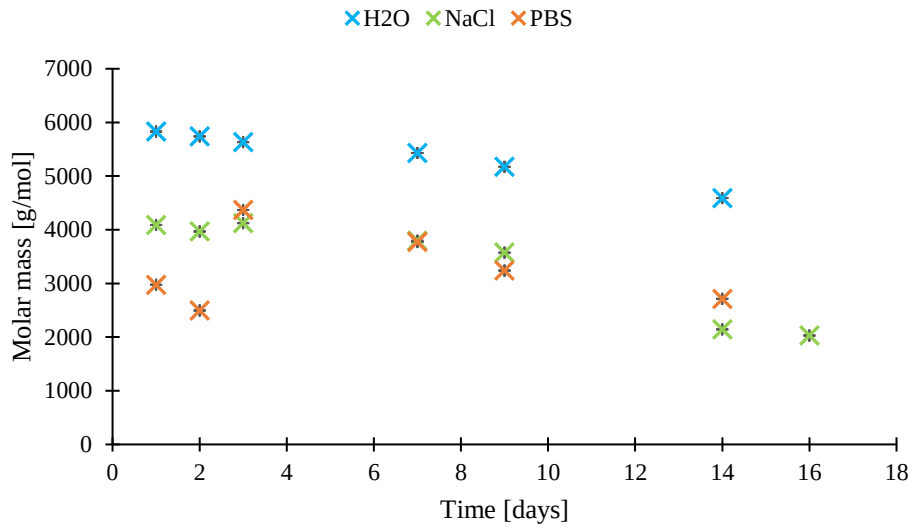

Figure 42: Graph of molar mass degrease during time of various 10 wt. % hydrogels. Data were obtained from GPC/SEC measurements.

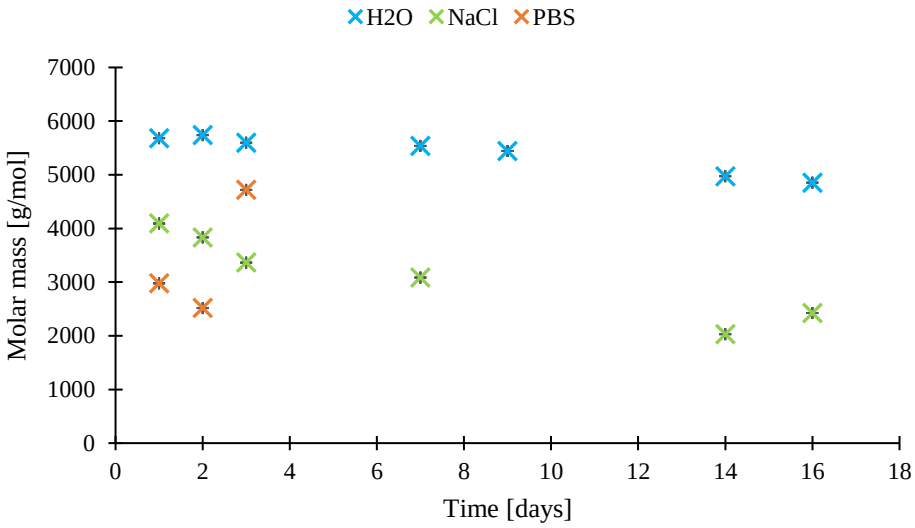

Figure 43: Graph of molar mass degrease during time of various 20 wt. % hydrogels. Data were obtained from GPC/SEC measurements.

The behaviour of PDI during the degradation of 15 wt. % hydrogels is visible in Figure 44. Figure 45 and Figure 46 show PDI trends for 10 and 15 wt. % hydrogels. The PDI of the lipase samples has a decreasing character, although the average value of 8.4 is extremely high. The appropriate PDI value should be around 1.09 (as visible in other samples). This may be caused by the enzyme with the hydrogel creating agglomerates. The degraded samples with lipase were not specially treated to remove lipase before GPC/SEC analysis. The degradation of the

hydrogel is performed not by the break down of a material into fragments but via chipping the chain ends. This is proved by a gradual decrease in the PDI values.

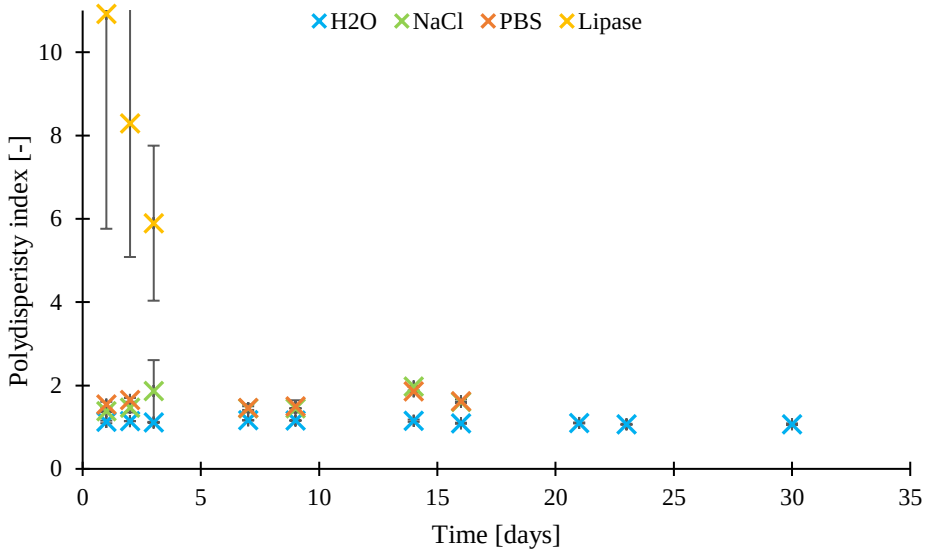

Figure 44: Graph of polydispersity index change during the time of degradation in case of 15 wt. % hydrogels degraded in various fluids.

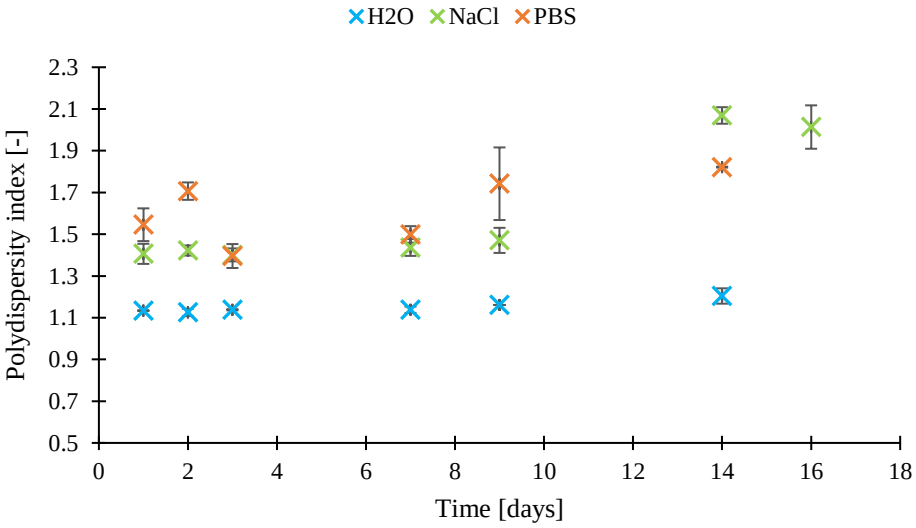

Figure 45: Graph of polydispersity index change during time of 10 wt. % hydrogels degradation in various fluids.

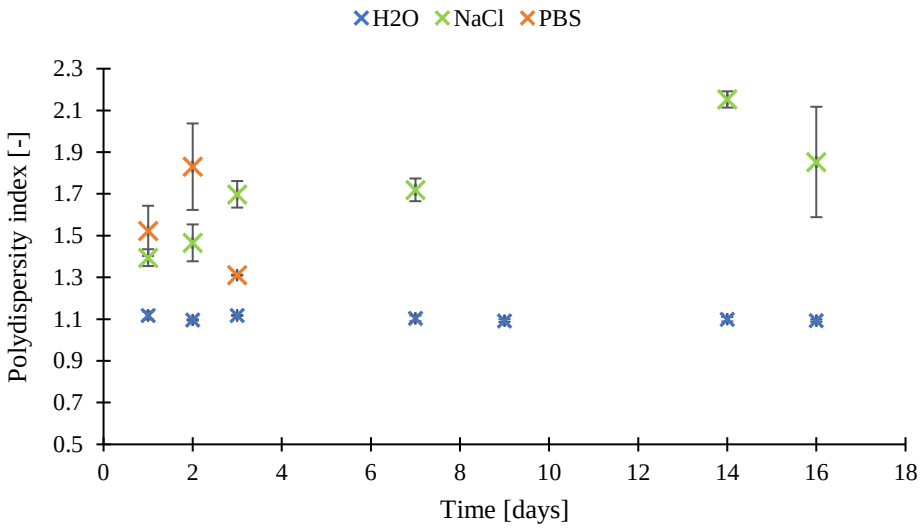

Figure 46: Graph of polydispersity index change during time of 20 wt. % hydrogel degradation in various fluids.

5.2.3 Characterisation of degradation products via LC-MS

The degradation products of the PLGA-PEG-PLGA copolymer are LA and GA. The concentration of these degradation products increases with a longer degradation time. Degradation products in the degradation medium were characterised via LC-MS analysis.

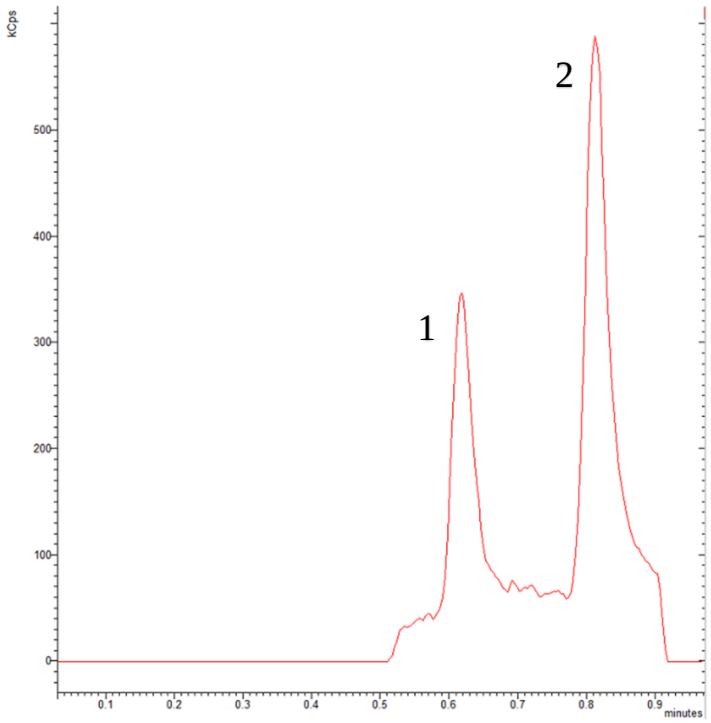

Figure 47: Chromatogram of 20 wt. % hydrogel after 30 days of degradation in NaCl. The 1st peak represents GA, and the 2nd peak represents LA.

Figure 47 represents a chromatogram from LC-MS analysis of a degradation medium of 20 wt. % hydrogel after 30 days.

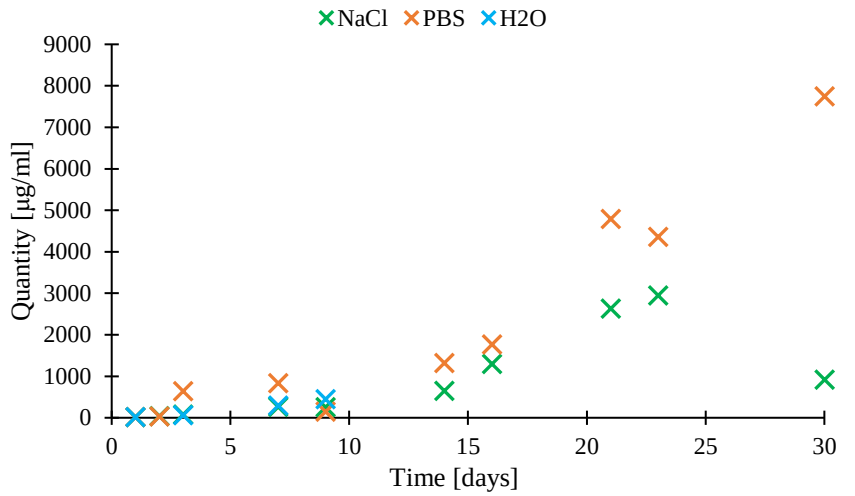

Figure 48: Graph of an increase in the concentration of GA during the degradation of 15 wt. % hydrogel in various degradation media.

From Figure 48 and Figure 49, and other graphs below, it is visible that LA occurred in degradation media after degradation in greater concentrations than GA due to the initial concentrations [77]. We can see in Figure 49 a massive increase in LA concentration after 15 days in the case of degradation in NaCl. In the case of 20 wt. % hydrogel samples, GA concentration was highest in the case of PBS and LA was highest in NaCl samples. A similar scenario but reversed can be observed in 10 wt. % hydrogel samples. The highest quantity of LA occurred in PBS and the highest concentration of GA occurs in NaCl.

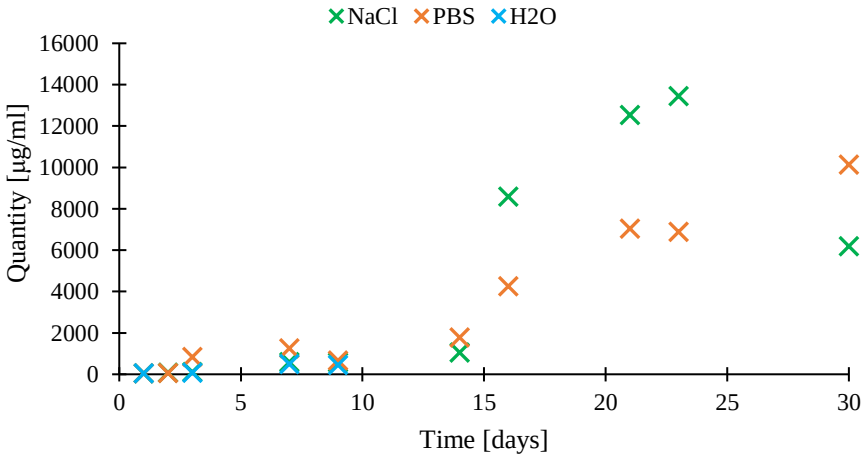

Figure 49: Graph of an increase in the concentration of LA during the degradation of 15 wt. % hydrogel in various degradation media.

The following graphs (Figure 50-53) show the progress of LA and GA increase in 10 and 20 wt. % hydrogels in time.

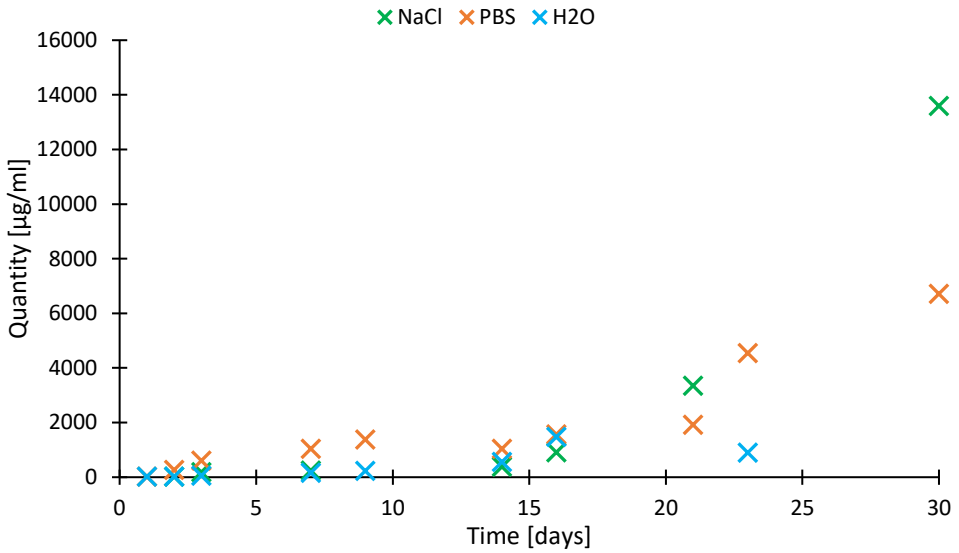

Figure 50: Graph of an increase in the concentration of GA during the 10 wt. % hydrogel degradation in various degradation media.

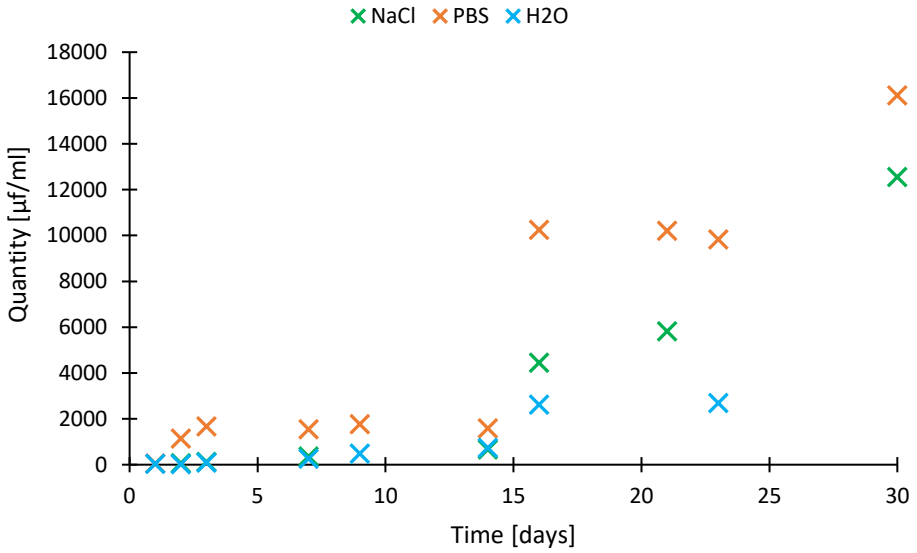

Figure 51: Graph of an increase in the concentration of LA during the 10 wt. % hydrogel degradation in various degradation media.

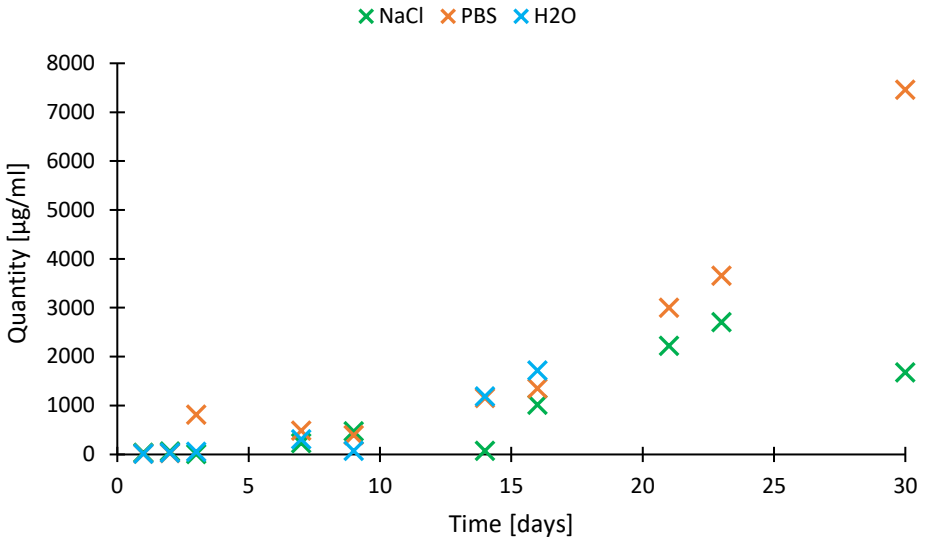

Figure 52: Graph of an increase in the GA concentration during the 20 wt. % hydrogel degradation in various degradation media.

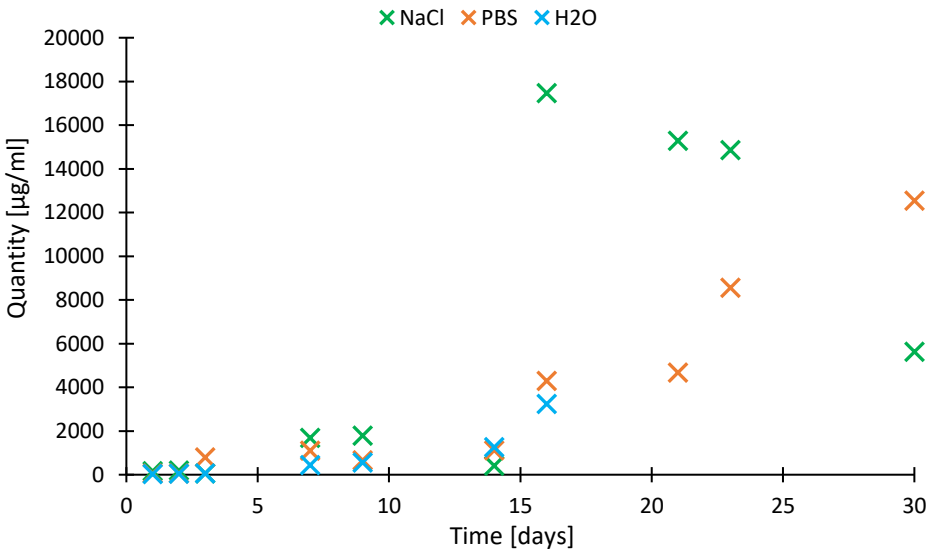

Figure 53: Graph of an increase in the concentration of LA during the 20 wt. % hydrogel degradation in various degradation media.

Figures 54 and Figure 55 have similar trends. The amount of LA and GA in $\mu g \cdot ml^{-1}$ is increasing. In both graphs, samples with lipase contain a larger amount of degradation products than other samples. On the other hand, after three days, samples with PBS have a higher concentration of both acids.

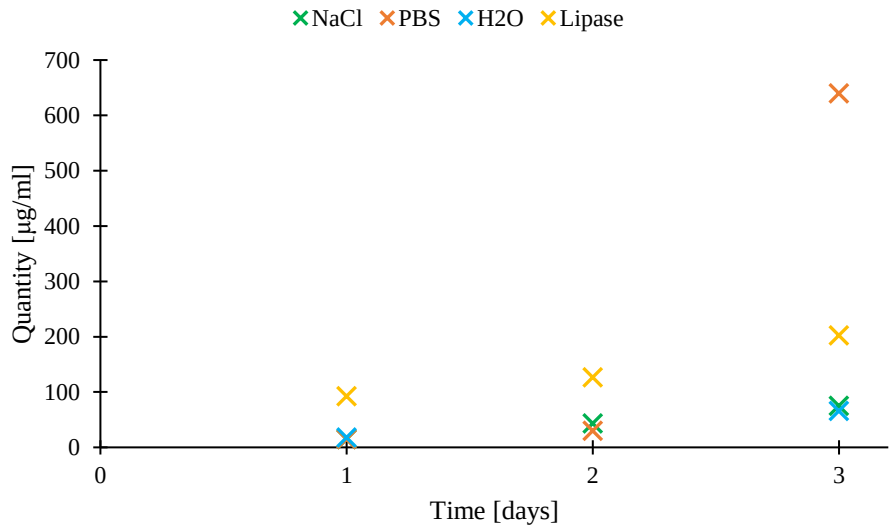

Figure 54: Graph of an increase in the concentration of GA during the 3 days of degradation of 15 wt. % hydrogel in various degradation media and with lipase.

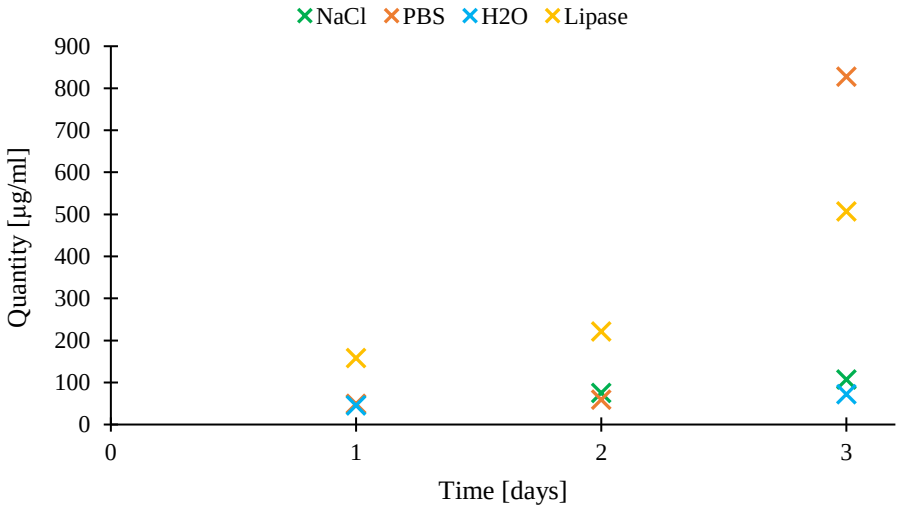

Figure 55: Graph of an increase in LA concentration for 3 days degradation of 15 wt. % hydrogel in various degradation media and with lipase.

The data obtained from LC-MS analysis were used to calculate the degradation rate constants. The calculations were performed at CEITEC BUT.

Table 3: Table of calculated degradation rate constant for hydrogels in degradation media.

Hydrogel concentration	Medium	Degradation product	*k*
10 %	H_2O	LA	154.13
10 %	H_2O	GA	39.82
15 %	H_2O	LA	47.05
15 %	H_2O	GA	28.52
20 %	H_2O	LA	927.73
20 %	H_2O	GA	261.01
10 %	NaCl	LA	1097.42
10 %	NaCl	GA	664.50
15 %	NaCl	LA	1210.52
15 %	NaCl	GA	239.25
20 %	NaCl	LA	1030.70
20 %	NaCl	GA	83.53
10 %	PBS	LA	703.85
10 %	PBS	GA	468.33
15 %	PBS	LA	459.86
15 %	PBS	GA	404.90
20 %	PBS	LA	672.17
20 %	PBS	GA	438.11
15 %	Lipase	LA	-
15 %	Lipase	GA	337.52

Data from LC-MS analysis propose that lipase may accelerate the degradation process; however, acquired data from the calculated degradation rate constant visible in Table 3 show different results. The greater the *k* constant, the faster the degradation. Knowing this fact, it is visible from Table 3 that the fastest degradation overall and also for the 15 wt. % hydrogel was in the case of NaCl solution, with the highest constant (in the case of LA as a degradation product). From available data, the constant for the PBS solution is higher than that for lipase, but it is not such a big difference.

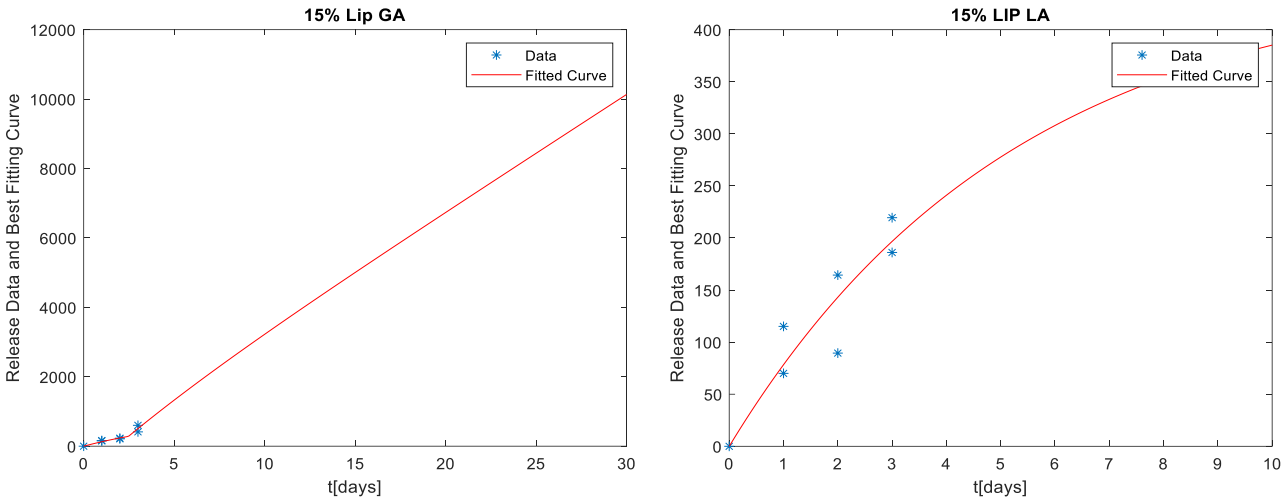

Figure 56: Model of the LA, GA degradation products behaviour of 15 wt. % hydrogel in PBS solution with lipase. From the left to the right, the left graph is for GA as degradation product and the right graph is for LA as degradation product.

The lipase behaviour in Figure 56 suggests that lipase does not significantly affect hydrogel degradation. This may mean that lipase is not suitable for the degradation of hydrogels made from PLGA-PEG-PLGA copolymer. The graphs below show models for other degraded hydrogels.

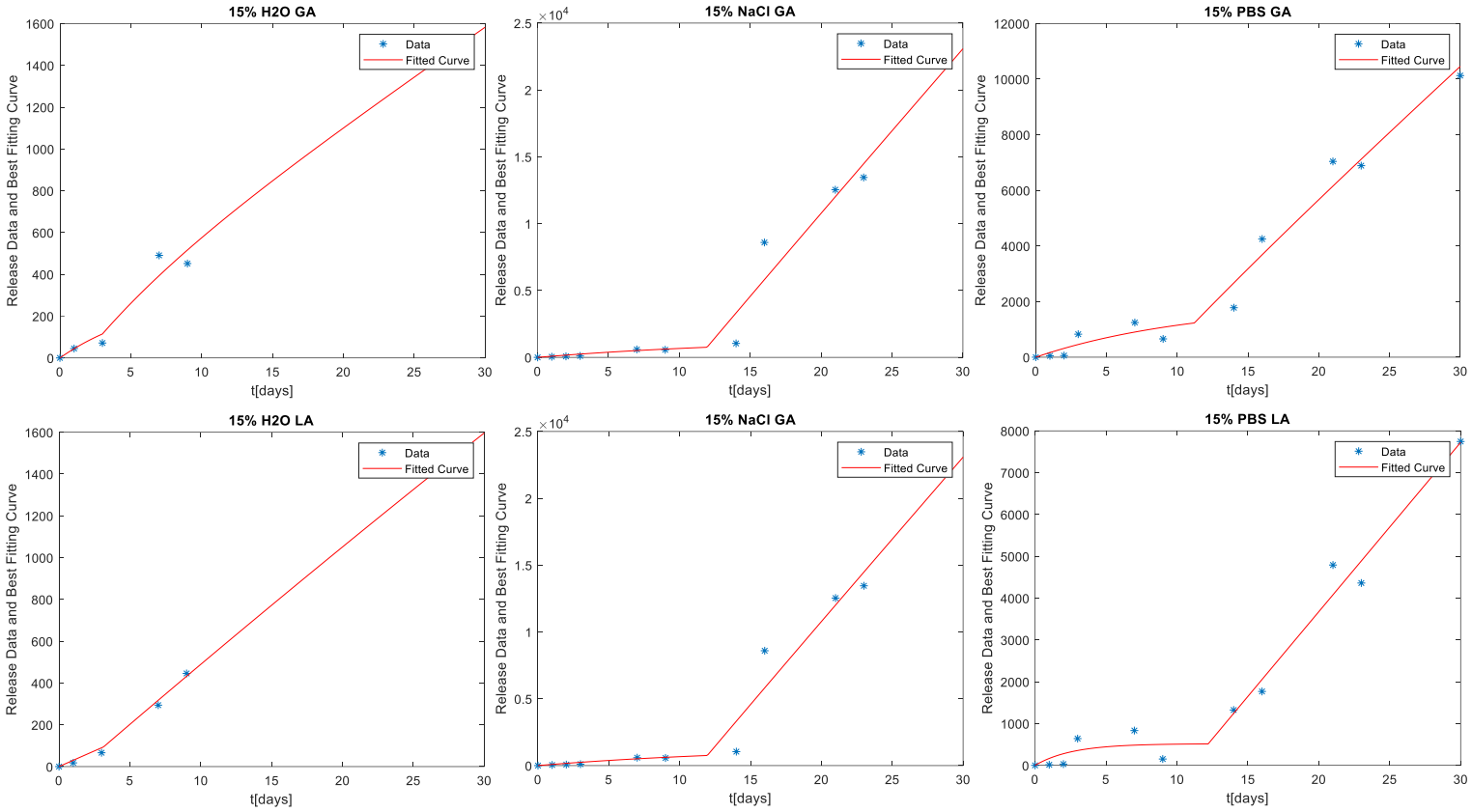

Figure 57: Model of the LA, GA degradation products behaviour in 15 wt. % hydrogel in various solutions. From left to right, the left graphs are for H_2O, the middle graphs are for NaCl, and the right graphs are for PBS. The graphs in the upper part are for GA as the degradation product, and the graphs in the lower part are for LA as the degradation product.

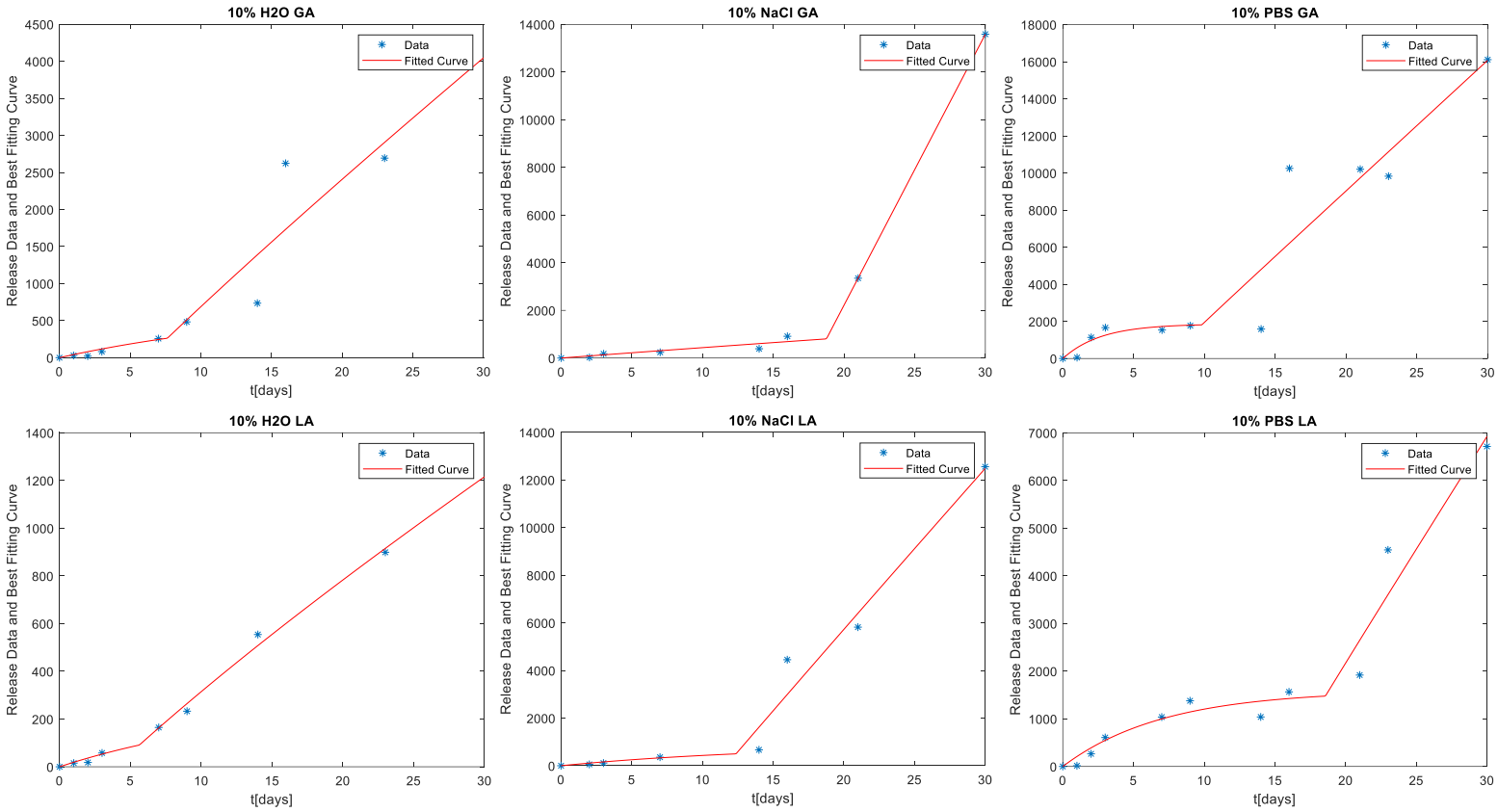

Figure 57: Model of the LA, GA degradation products behaviour in 10 wt. % hydrogel in various solutions. From left to right, the left graphs are for H_2O, the middle graphs are for NaCl, and the right graphs are for PBS. The graphs in the upper part are for GA as the degradation product, and the graphs in the lower part are for LA as the degradation product.

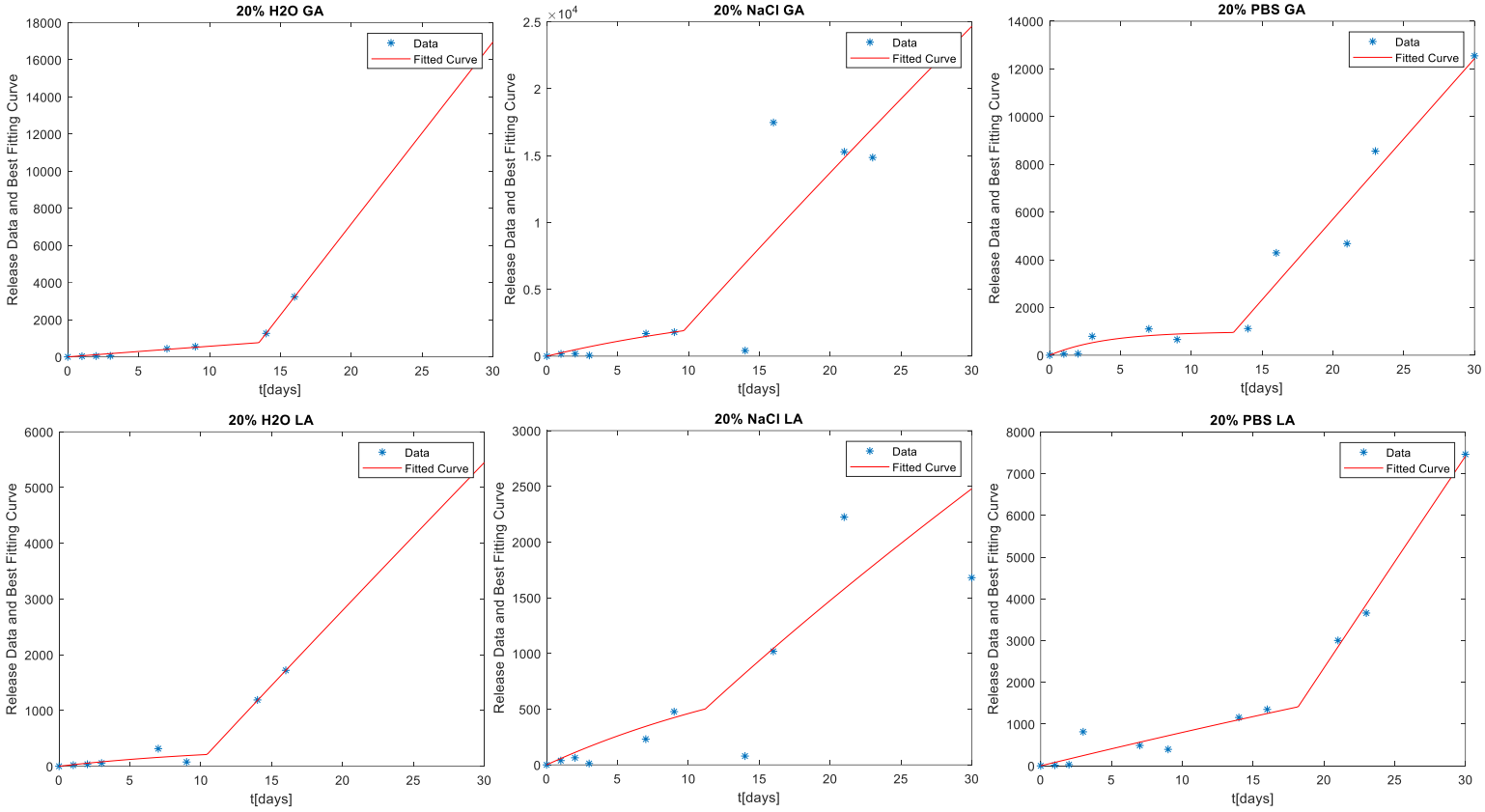

Figure 58: Model of the LA, GA degradation products behaviour in 20 wt. % hydrogel in various solutions. From left to right, the left graphs are for H_2O, the middle graphs are for NaCl, and the right graphs are for PBS. The graphs in the upper part are for GA as the degradation product, and the graphs in the lower part are for LA as the degradation product.

6. CONCLUSION

The main aim of this work was to study the degradation process of hydrogels and their degradation products. One of the goals was to synthesize a thermosensitive PLGA-PEG-PLGA copolymer. From such prepared copolymer, hydrogel solutions of 10, 15 and 20 wt. % were prepared. Later, hydrogel solutions were left to degrade in various media. After degradation, degraded hydrogel samples were examined together with degradation products in fluids used for degradation.

Four batches of the ABA copolymer were synthesized, with an average number molecular weight (M_n) of 5.25 kg·mol^{-1}, PLGA/PEG ratio of 2.5 and LA/GA ratio of 2.9 confirmed by ^{1}H NMR analysis. GPC/SEC analysis was used to determine PDI and M_n. PDI of 1st batch was 1.14 and M_n of 5.6 kg·mol^{-1}. M_n of copolymer characterized via GPC/SEC was a bit higher than the one obtained from ^{1}H NMR but results from ^{1}H NMR were close to the theoretical value (5.25 kg·mol^{-1}). After the evaluation of all data, we can say the synthesis of PLGA-PEG-PLGA thermosensitive copolymer was successful.

Gravimetric analysis was performed to observe the mass loss of 10, 15 and 20 wt. % hydrogels during the degradation time in UPW, 0.9 % NaCl solution, PBS and enzyme lipase in PBS solution. It was observed that the progress of mass loss was similar in the same hydrogel concentrations regardless of the degradation medium. Additionally, with a higher concentration of hydrogel, the mass loss over degradation time was smaller than in a lower concentration of hydrogels. We expected the biggest mass loss in the case of lipase, but gravimetry showed that it was similar to in the case of NaCl, though the results were not so different. Degradation with lipase was evaluated only for 3 days and for 15 wt. % hydrogel, so we can just predict what will be the progress. However, this data will serve as a great foundation for further studies of lipase´s impact on hydrogel degradation, since there are not many articles about it. Also, the change in pH was studied over time. It was observed that the biggest pH drop occurred during the first three days in every sample. After those three days, the pH decrease remained constant. Generally, the lowest pH values occurred during degradation in UPW and NaCl solutions.

The degradations of hydrogels were analysed via GPC/SEC. Some samples could not be analysed because most of the hydrogel had already diffused through the porous membrane into the bottom pan medium, and there was an insufficient amount of hydrogel required for GPC/SEC analysis. The majority of samples showed a visible trend in the decrease in M_n and PDI over time. The decrease in M_n tells us that hydrogel degradation occurred, and the decrease in PDI shows that degradation was performed by the chipping of end chains. Although, samples with lipase were not treated to remove lipase from gel before analysis, thus they show higher PDI values.

The liquid in the bottom pan was after sample removal prepared for LC-MS analysis of degradation products – LA and GA. Generally speaking, the concentration of LA was higher than that of GA in every sample. This was caused by the initial concentrations of PLA and PGA during the synthesis of copolymer. LC-MS showed nice trends of increasing concentrations of degradation products in solutions over time. Lipase samples showed on average much higher concentrations during the first three days than the other samples. This fact should indicate the acceleration of degradation by lipase; however, results from degradation rate constant calculations show that lipase does not significantly accelerate the degradation process.

Gravimetry and LC-MS analysis proposed a potential acceleration of degradation by lipase; however, a degradation rate constant calculation and modelling of the degradation product behaviour showed that the degradation with lipase does not significantly accelerate the process. This may mean that lipase may not be suitable for the degradation of hydrogels made from the PLGA-PEG-PLGA copolymer. Although a more complex experiment about this problem should be carried out in the future.

7. REFERENCES

[1] A. Ahsan *et al.*, "An overview of hydrogels and their role in transdermal drug delivery," *International Journal of Polymeric Materials and Polymeric Biomaterials*, vol. 70, no. 8, pp. 574–584, 2021, doi: 10.1080/00914037.2020.1740989.

[2] R. Narayanaswamy and V. P. Torchilin, "Hydrogels and Their Applications in Targeted Drug Delivery," *Molecules*, vol. 24, no. 3, p. 603, Feb. 2019, doi: 10.3390/molecules24030603.

[3] C. Li *et al.*, "Recent progress in drug delivery," *Acta Pharm Sin B*, vol. 9, no. 6, pp. 1145–1162, Nov. 2019, doi: 10.1016/j.apsb.2019.08.003.

[4] F. Doberenz, K. Zeng, C. Willems, K. Zhang, and T. Groth, "Thermoresponsive polymers and their biomedical application in tissue engineering – a review," *J Mater Chem B*, vol. 8, no. 4, pp. 607–628, 2020, doi: 10.1039/C9TB02052G.

[5] C. A. Dreiss, "Hydrogel design strategies for drug delivery," *Curr Opin Colloid Interface Sci*, vol. 48, pp. 1–17, Aug. 2020, doi: 10.1016/j.cocis.2020.02.001.

[6] T. R. Hoare and D. S. Kohane, "Hydrogels in drug delivery: Progress and challenges," *Polymer (Guildf)*, vol. 49, no. 8, pp. 1993–2007, Apr. 2008, doi: 10.1016/j.polymer.2008.01.027.

[7] J. A. Hunt, R. Chen, T. van Veen, and N. Bryan, "Hydrogels for tissue engineering and regenerative medicine," *J. Mater. Chem. B*, vol. 2, no. 33, pp. 5319–5338, 2014, doi: 10.1039/C4TB00775A.

[8] J. Maitra, V. K. Shukla, and V. Kumar Shukla, "Cross-linking in hydrogels-a review," *American Journal of Polymer Science*, vol. 2014, no. 2, pp. 25–31, 2014, doi: 10.5923/j.ajps.20140402.01.

[9] M. Kitagawa, T. Maeda, and A. Hotta, "PEG-based nanocomposite hydrogel: Thermo-responsive sol-gel transition and degradation behavior controlled by the LA/GA ratio of PLGA-PEG-PLGA," *Polym Degrad Stab*, vol. 147, pp. 222–228, Jan. 2018, doi: 10.1016/j.polymdegradstab.2017.11.024.

[10] H. Kamali, E. Khodaverdi, and F. Hadizadeh, "Ring-opening polymerization of PLGA-PEG-PLGA triblock copolymer in supercritical carbon dioxide," *J Supercrit Fluids*, vol. 137, pp. 9–15, Jul. 2018, doi: 10.1016/J.SUPFLU.2018.03.001.

[11] M. C. Jones and J. C. Leroux, "Polymeric micelles – a new generation of colloidal drug carriers," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 48, no. 2, pp. 101–111, Sep. 1999, doi: 10.1016/S0939-6411(99)00039-9.

[12] "BioRender."

[13] P. Wang *et al.*, "Modified PLGA–PEG–PLGA thermosensitive hydrogels with suitable thermosensitivity and properties for use in a drug delivery system," *J Mater Chem B*, vol. 5, no. 8, pp. 1551–1565, 2017, doi: 10.1039/C6TB02158A.

[14] J. A. Hunt, R. Chen, T. Van Veen, and N. Bryan, "Hydrogels for tissue engineering and regenerative medicine," 2014, doi: 10.1039/c4tb00775a.

[15] A. Bordbar-Khiabani and M. Gasik, “Smart Hydrogels for Advanced Drug Delivery Systems,” *Int J Mol Sci*, vol. 23, no. 7, p. 3665, Mar. 2022, doi: 10.3390/ijms23073665.

[16] K. J. Hogan and A. G. Mikos, “Biodegradable thermoresponsive polymers: Applications in drug delivery and tissue engineering,” *Polymer (Guildf)*, vol. 211, p. 123063, Dec. 2020, doi: 10.1016/j.polymer.2020.123063.

[17] P. S. Chan, J. W. Xian, Q. Li, C. W. Chan, S. S. Y. Leung, and K. K. W. To, “Biodegradable Thermosensitive PLGA-PEG-PLGA Polymer for Non-irritating and Sustained Ophthalmic Drug Delivery,” *AAPS J*, vol. 21, no. 4, p. 59, Jul. 2019, doi: 10.1208/s12248-019-0326-x.

[18] L. Michlovská, L. Vojtová, L. Mravcová, S. Hermanová, J. Kučerík, and J. Jančář, “Functionalization Conditions of PLGA-PEG-PLGA Copolymer with Itaconic Anhydride,” *Macromol Symp*, vol. 295, no. 1, pp. 119–124, Sep. 2010, doi: 10.1002/masy.200900071.

[19] N. Khameh Khorshid *et al.*, “Novel Structural Changes during Temperature-Induced Self-Assembling and Gelation of PLGA-PEG-PLGA Triblock Copolymer in Aqueous Solutions,” *Macromol Biosci*, vol. 16, no. 12, pp. 1838–1852, Dec. 2016, doi: 10.1002/MABI.201600277.

[20] K. Lysáková *et al.*, “Novel approach in control release monitoring of protein-based bioactive substances from injectable PLGA-PEG-PLGA hydrogel,” *Express Polym Lett*, vol. 16, no. 8, pp. 798–811, 2022, doi: 10.3144/expresspolymlett.2022.59.

[21] Z. Song *et al.*, “Curcumin-loaded PLGA-PEG-PLGA triblock copolymeric micelles: Preparation, pharmacokinetics and distribution in vivo,” *J Colloid Interface Sci*, vol. 354, no. 1, pp. 116–123, Feb. 2011, doi: 10.1016/J.JCIS.2010.10.024.

[22] L. Yu, Z. Zhang, H. Zhang, and J. Ding, “Biodegradability and Biocompatibility of Thermoreversible Hydrogels Formed from Mixing a Sol and a Precipitate of Block Copolymers in Water,” *Biomacromolecules*, vol. 11, no. 8, pp. 2169–2178, Aug. 2010, doi: 10.1021/bm100549q.

[23] A. P. Constantinou and T. K. Georgiou, “Pre-clinical and clinical applications of thermoreversible hydrogels in biomedical engineering: a review,” *Polym Int*, vol. 70, no. 10, pp. 1433–1448, Oct. 2021, doi: 10.1002/pi.6266.

[24] M. Kitagawa, T. Maeda, and A. Hotta, “PEG-based nanocomposite hydrogel: Thermo-responsive sol-gel transition and degradation behavior controlled by the LA/GA ratio of PLGA-PEG-PLGA,” *Polym Degrad Stab*, vol. 147, pp. 222–228, Jan. 2018, doi: 10.1016/j.polymdegradstab.2017.11.024.

[25] L. Vojtova *et al.*, “The Effect of the Thermosensitive Biodegradable PLGA–PEG–PLGA Copolymer on the Rheological, Structural and Mechanical Properties of Thixotropic Self-Hardening Tricalcium Phosphate Cement,” *Int J Mol Sci*, vol. 20, no. 2, p. 391, Jan. 2019, doi: 10.3390/ijms20020391.

[26] N. L. Elstad and K. D. Fowers, “OncoGel (ReGel/paclitaxel) — Clinical applications for a novel paclitaxel delivery system,” *Adv Drug Deliv Rev*, vol. 61, no. 10, pp. 785–794, Aug. 2009, doi: 10.1016/j.addr.2009.04.010.

[27] L. McKeen, “Renewable Resource and Biodegradable Polymers,” *The Effect of Sterilization on Plastics and Elastomers*, pp. 305–317, 2012, doi: 10.1016/B978-1-4557-2598-4.00012-5.

[28] S. Kliem, M. Kreutzbruck, and C. Bonten, “Review on the Biological Degradation of Polymers in Various Environments,” *Materials*, vol. 13, no. 20, p. 4586, Oct. 2020, doi: 10.3390/ma13204586.

[29] P. Gunatillake, “Biodegradable synthetic polymers for tissue engineering,” *Eur Cell Mater*, vol. 5, pp. 1–16, Oct. 2003, doi: 10.22203/eCM.v005a01.

[30] R. A. Jain, “The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices,” *Biomaterials*, vol. 21, no. 23, pp. 2475–2490, Dec. 2000, doi: 10.1016/S0142-9612(00)00115-0.

[31] K. J. Jem and B. Tan, “The development and challenges of poly (lactic acid) and poly (glycolic acid),” *Advanced Industrial and Engineering Polymer Research*, vol. 3, no. 2, pp. 60–70, Apr. 2020, doi: 10.1016/j.aiepr.2020.01.002.

[32] “SCD/ChemSketch.”

[33] A. Dal Pozzo, L. Vanini, M. Fagnoni, M. Guerrini, A. de Benedittis, and R. A. A. Muzzarelli, “Preparation and characterization of poly(ethylene glycol)-crosslinked reacetylated chitosans,” *Carbohydr Polym*, vol. 42, no. 2, pp. 201–206, Jun. 2000, doi: 10.1016/S0144-8617(99)00134-4.

[34] J. P. Eubeler, M. Bernhard, and T. P. Knepper, “Environmental biodegradation of synthetic polymers II. Biodegradation of different polymer groups,” *TrAC Trends in Analytical Chemistry*, vol. 29, no. 1, pp. 84–100, Jan. 2010, doi: 10.1016/j.trac.2009.09.005.

[35] H.-J. Jang, C. Y. Shin, and K.-B. Kim, “Safety Evaluation of Polyethylene Glycol (PEG) Compounds for Cosmetic Use,” *Toxicol Res*, vol. 31, no. 2, pp. 105–136, Jun. 2015, doi: 10.5487/TR.2015.31.2.105.

[36] B. Singh and N. Sharma, “Mechanistic implications of plastic degradation,” *Polymer Degradation and Stability*, vol. 93, no. 3. pp. 561–584, Mar. 2008. doi: 10.1016/j.polymdegradstab.2007.11.008.

[37] J. Vohlídal, “Polymer degradation: a short review,” *Chemistry Teacher International*, vol. 3, no. 2, pp. 213–220, Jun. 2021, doi: 10.1515/cti-2020-0015.

[38] E. Yousif and R. Haddad, “Photodegradation and photostabilization of polymers, especially polystyrene: review,” *Springerplus*, vol. 2, no. 1, p. 398, Dec. 2013, doi: 10.1186/2193-1801-2-398.

[39] K. Pielichowski and J. Njuguna, “Thermal Degradation of Polymeric Materials.” [Online]. Available: http://www.rapra.net

[40] W. L. Hawkins, “Stabilization Against Degradation by Ozone,” pp. 91–97, 1984, doi: 10.1007/978-3-642-69376-2_6.

[41] J. Li, C. Nagamani, and J. S. Moore, “Polymer Mechanochemistry: From Destructive to Productive,” 2015, doi: 10.1021/acs.accounts.5b00184.

[42] K. H. Lee and D. H. Shin, “Catalytic degradation of waste polyolefinic polymers using spent FCC catalyst with various experimental variables,” *Korean Journal of Chemical Engineering*, vol. 20, no. 1, pp. 89–92, 2003, doi: 10.1007/BF02697190/METRICS.

[43] G. M. Vinhas, R. M. S. Maior, and Y. M. B. De Almeida, “Radiolytic degradation and stabilization of poly(vinyl chloride),” *Polym Degrad Stab*, vol. 83, no. 3, pp. 429–433, Mar. 2004, doi: 10.1016/J.POLYMDEGRADSTAB.2003.08.005.

[44] S. Kliem, M. Kreutzbruck, and C. Bonten, “Review on the Biological Degradation of Polymers in Various Environments,” *Materials*, vol. 13, no. 20, p. 4586, Oct. 2020, doi: 10.3390/ma13204586.

[45] P. Gunatillake, “Biodegradable synthetic polymers for tissue engineering,” *Eur Cell Mater*, vol. 5, pp. 1–16, Oct. 2003, doi: 10.22203/eCM.v005a01.

[46] P. Gunatillake, R. Mayadunne, and R. Adhikari, “Recent developments in biodegradable synthetic polymers,” *Biotechnol Annu Rev*, vol. 12, pp. 301–347, 2006, doi: 10.1016/S1387-2656(06)12009-8.

[47] Nasrollahzadeh Mahmoud, *Biopolymer-Based Metal Nanoparticle Chemistry for Sustainable Applications*, vol. 2. Elsevier, 2021. doi: 10.1016/C2020-0-03104-X.

[48] Katarzyna Leja and Lewandowicz Grażyna, “Polymer Biodegradation and Biodegradable Polymers – a Review,” *Pol J Environ Stud*, vol. 19, no. 2, pp. 255–266, Accessed: Feb. 21, 2023. [Online]. Available: http://www.pjoes.com/Polymer-Biodegradation-and-Biodegradable-Polymers-a-Review,88379,0,2.html

[49] A. Banerjee, K. Chatterjee, and G. Madras, “Enzymatic degradation of polymers: a brief review,” *Materials Science and Technology*, vol. 30, no. 5, pp. 567–573, May 2014, doi: 10.1179/1743284713Y.0000000503.

[50] S. S. Liow, A. A. Karim, and X. J. Loh, “Biodegradable thermogelling polymers for biomedical applications,” *MRS Bull*, vol. 41, no. 7, pp. 557–566, Jul. 2016, doi: 10.1557/MRS.2016.139.

[51] M. Blessy, R. D. Patel, P. N. Prajapati, and Y. K. Agrawal, “Development of forced degradation and stability indicating studies of drugs—A review,” *J Pharm Anal*, vol. 4, no. 3, p. 159, 2014, doi: 10.1016/J.JPHA.2013.09.003.

[52] G. E. Zaikov, “QUANTITATIVE ASPECTS OF POLYMER DEGRADATION IN THE LIVING BODY,” *Journal of Macromolecular Science, Part C*, vol. 25, no. 4, pp. 551–597, Nov. 1985, doi: 10.1080/07366578508081964.

[53] Y. Xu, C.-S. Kim, D. M. Saylor, and D. Koo, “Polymer degradation and drug delivery in PLGA-based drug-polymer applications: A review of experiments and theories,” *J Biomed Mater Res B Appl Biomater*, vol. 105, no. 6, pp. 1692–1716, Aug. 2017, doi: 10.1002/jbm.b.33648.

[54] D. Hofmann, M. Entrialgo-Castaño, K. Kratz, and A. Lendlein, “Knowledge-based approach towards hydrolytic degradation of polymer-based biomaterials,” *Advanced*

Materials, vol. 21, no. 32–33, pp. 3237–3245, Sep. 2009, doi: 10.1002/ADMA.200802213.

[55] R. Smith, *Biodegradable polymers for industrial applications*. Taylor & Francis, 2005.

[56] B. Laycock *et al.*, "Lifetime prediction of biodegradable polymers," *Prog Polym Sci*, vol. 71, pp. 144–189, Aug. 2017, doi: 10.1016/j.progpolymsci.2017.02.004.

[57] A. Banerjee, K. Chatterjee, and G. Madras, "Enzymatic degradation of polymers: a brief review," *Materials Science and Technology*, vol. 30, no. 5, pp. 567–573, May 2014, doi: 10.1179/1743284713Y.0000000503.

[58] J. M. Knipe, F. Chen, and N. A. Peppas, "Enzymatic Biodegradation of Hydrogels for Protein Delivery Targeted to the Small Intestine," *Biomacromolecules*, vol. 16, no. 3, pp. 962–972, Mar. 2015, doi: 10.1021/bm501871a.

[59] M. K. Gupta, J. R. Martin, B. R. Dollinger, M. E. Hattaway, and C. L. Duvall, "Thermogelling, ABC Triblock Copolymer Platform for Resorbable Hydrogels with Tunable, Degradation-Mediated Drug Release," *Adv Funct Mater*, vol. 27, no. 47, p. 1704107, Dec. 2017, doi: 10.1002/adfm.201704107.

[60] K. Shi, J. Jing, L. Song, T. Su, and Z. Wang, "Enzymatic hydrolysis of polyester: Degradation of poly(ε-caprolactone) by Candida antarctica lipase and Fusarium solani cutinase," *Int J Biol Macromol*, vol. 144, pp. 183–189, Feb. 2020, doi: 10.1016/j.ijbiomac.2019.12.105.

[61] B. Singh and N. Sharma, "Mechanistic implications of plastic degradation," *Polymer Degradation and Stability*, vol. 93, no. 3. pp. 561–584, Mar. 2008. doi: 10.1016/j.polymdegradstab.2007.11.008.

[62] T. Ahmed *et al.*, "Biodegradation of plastics: current scenario and future prospects for environmental safety," *Environmental Science and Pollution Research*, vol. 25, no. 8, pp. 7287–7298, Mar. 2018, doi: 10.1007/s11356-018-1234-9.

[63] J. R. Rocca-Smith *et al.*, "Beyond Biodegradability of Poly(lactic acid): Physical and Chemical Stability in Humid Environments," 2017, doi: 10.1021/acssuschemeng.6b03088.

[64] N. F. Zaaba and M. Jaafar, "A review on degradation mechanisms of polylactic acid: Hydrolytic, photodegradative, microbial, and enzymatic degradation," *Polym Eng Sci*, vol. 60, no. 9, pp. 2061–2075, Sep. 2020, doi: 10.1002/pen.25511.

[65] J. V. Andhariya, S. Choi, Y. Wang, Y. Zou, D. J. Burgess, and J. Shen, "Accelerated in vitro release testing method for naltrexone loaded PLGA microspheres," *Int J Pharm*, vol. 520, no. 1–2, pp. 79–85, Mar. 2017, doi: 10.1016/J.IJPHARM.2017.01.050.

[66] L. I. Cabezas, I. Gracia, A. De Lucas, and J. F. Rodríguez, "Validation of a Mathematical Model for the Description of Hydrophilic and Hydrophobic Drug Delivery from Biodegradable Foams: Experimental and Comparison Using Indomethacin as Released Drug," 2014, doi: 10.1021/ie500760m.

[67] D. Klose, F. Siepmann, K. Elkharraz, and J. Siepmann, “PLGA-based drug delivery systems: Importance of the type of drug and device geometry,” *Int J Pharm*, vol. 354, no. 1–2, pp. 95–103, Apr. 2008, doi: 10.1016/J.IJPHARM.2007.10.030.

[68] M. A. Elsawy, K.-H. Kim, J.-W. Park, and A. Deep, “Hydrolytic degradation of polylactic acid (PLA) and its composites,” *Renewable and Sustainable Energy Reviews*, vol. 79, pp. 1346–1352, Nov. 2017, doi: 10.1016/j.rser.2017.05.143.

[69] J. Ulbricht, R. Jordan, and R. Luxenhofer, “On the biodegradability of polyethylene glycol, polypeptoids and poly(2-oxazoline)s,” *Biomaterials*, vol. 35, no. 17, pp. 4848–4861, 2014, doi: 10.1016/J.BIOMATERIALS.2014.02.029.

[70] C. Su, Y. Liu, R. Li, W. Wu, J. P. Fawcett, and J. Gu, “Absorption, distribution, metabolism and excretion of the biomaterials used in Nanocarrier drug delivery systems,” *Adv Drug Deliv Rev*, vol. 143, pp. 97–114, Mar. 2019, doi: 10.1016/J.ADDR.2019.06.008.

[71] K. Lysakova *et al.*, “Novel approach in control release monitoring of protein-based bioactive substances from injectable PLGA-PEG-PLGA hydrogel,” *Express Polym Lett*, vol. 16, no. 8, pp. 798–811, 2022, doi: 10.3144/expresspolymlett.2022.59.

[72] G. Giridhar, R. K. N. R. Manepalli, and G. Apparao, “Size-Exclusion Chromatography,” in *Thermal and Rheological Measurement Techniques for Nanomaterials Characterization*, Elsevier, 2017, pp. 51–65. doi: 10.1016/B978-0-323-46139-9.00003-7.

[73] Madhusha, “Difference Between 1H NMR and 13C NMR,” *https://pediaa.com/difference-between-1h-nmr-and-13c-nmr/*, Jan. 26, 2018.

[74] L. Nahar, A. Onder, and S. D. Sarker, “A review on the recent advances in HPLC, UHPLC and UPLC analyses of naturally occurring cannabinoids (2010–2019),” *Phytochemical Analysis*, vol. 31, no. 4, pp. 413–457, Jul. 2020, doi: 10.1002/pca.2906.

[75] V. Dhote, S. Skaalure, U. Akalp, J. Roberts, S. J. Bryant, and F. J. Vernerey, “On the role of hydrogel structure and degradation in controlling the transport of cell-secreted matrix molecules for engineered cartilage,” *J Mech Behav Biomed Mater*, vol. 19, pp. 61–74, Mar. 2013, doi: 10.1016/j.jmbbm.2012.10.016.

[76] K. Jelonek, S. Li, J. Kasperczyk, X. Wu, and A. Orchel, “Effect of polymer degradation on prolonged release of paclitaxel from filomicelles of polylactide/poly(ethylene glycol) block copolymers,” *Materials Science and Engineering: C*, vol. 75, pp. 918–925, Jun. 2017, doi: 10.1016/j.msec.2017.03.006.

[77] H. K. Makadia and S. J. Siegel, “Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier,” *Polymers (Basel)*, vol. 3, no. 3, pp. 1377–1397, Aug. 2011, doi: 10.3390/polym3031377.

8. LIST OF ABREVIATIONS AND SYMBOLS

ABA	PLGA-PEG-PLGA copolymer
ASTM D6400	European standard for solid materials biodegradation
$CDCl_3$	Deuterated chloroform
CGT	Critical gelation temperature
CMC	Critical micellar concentration
CO_2	Carbon dioxide
DDS	Drug delivery system
DPC	Direct polycondensation
EN 1343	European composability standard for packaging
GA	Glycolic acid
GPC	Gel permeation chromatography
HCOOH	Formic acid
IR	Infrared radiation
LA	Lactic acid
LC	Liquid chromatography
MALS	Multi-Angle Light Scattering photometry
MeCN	Acetonitrile
M_n	Number average molecular weight
M_w	Weight average molecular weight
MS	Mass spectrometry
NaCl	Saline solution – physiological saline
NMR	Nuclear magnetic resonance spectroscopy
PTFE	Polytetrafluoroethylene
PBS	Phosphate-buffer saline
PCL	Poly(ε-caprolactone)
PDI	Polydispersity index
PEG	Poly(ethylene glycol)
PGA	Poly(glycolic acid)
PLA	Poly(lactide acid)
PS	Polystyrene

PVC	Polyvinyl chloride
ROP	Ring-opening polymerisation
SEC	Size exclusion chromatography
TERM	Tissue engineering and regenerative medicine
THF	Tetrahydrofuran
UHPLC	Ultra-High-Performance Liquid Chromatography
UPW	Ultra-pure water
UV	Ultraviolet radiation
wt. %	Weight percentage