TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

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DRUG RELEASING POLY (LACTIDE-*CO*-GLYCOLIDE) MICROPARTICLES

Master of Science Thesis

Examiner: Professor Minna Kellomäki
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ABSTRACT

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The aim of this thesis was to improve already developed drug release system for model drug dexamethasone (DEX), in which biodegradable, poly (lactide-co-glycolide) (PLGA) – polyvinyl alcohol (PVA) microparticles for drug release was designed, using combination of two preparation methods. Gamma irradiation as a sterilization method for the microparticles was also studied. There were also attempt to sustain the release rate even more and minimize the burst release of DEX by designing hydrogel-microparticle composite using aldehyde-modified hyaluronic acid (HA) crosslinked by hydrazide-modified PVA. Also mechanical properties of the composite were determined with compression test. In addition to DEX, encapsulation of a second drug, muraglitazar was investigated. Until now, muraglitazar has not been used in any drug delivery device.

Improvement of the existing PLGA-PVA-DEX microparticles succeeded. Yield of the spray dried microparticles increased approximately 60 %. Despite of smaller size of the microparticles, slower drug release rate, during four weeks, was achieved. However, prolonged drug delivery tests revealed unexpected problem, degradation of the DEX in aqueous solution, and analytical problems. It resulted in a need to design the new method for analyzing DEX with high performance liquid chromatography (HPLC). Gamma irradiation was proved to have adverse effects to the microparticle morphology and also drug release profile. Hydrogel-microparticle composite showed very similar mechanical properties with control hydrogel. Unfortunately, modification of the drug release profile of DEX failed. Still, analysis of drug release samples from composite gel denoted possible stabilization of the DEX in presence of hydrogel or one of its components. Hydrophobic drug, muraglitazar was successfully encapsulated in PLGA-PVA microparticles using the same method. Drug was released from the microparticle within one week.

As a conclusion, slightly modified preparation method combining emulsion method and spray drying is suitable for producing drug loaded microparticles with improved properties. Gamma sterilization has adverse effects to the microparticles and is therefore not recommended to use for sterilization. *In vitro* drug release from the particles is relatively fast, so further investigation is needed to modify drug release profiles. Especially modifying with hydrogel is recommended, as hydrogel-microparticle composite with good properties and larger amount of microparticles was developed in this study.

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Tämän diplomityön tarkoituksena oli kehittää paremmaksi jo olemassa olevaa lääkeluovutussysteemiä, joka perustuu kahden valmistusmenetelmän yhdistelmää käyttäen kehitettyihin, deksametasonia (DEX) sisältäviin, biohajoaviin poly (laktidi-*co*-glykolidi) (PLGA) mikropartikkeleihin. Myös gammasäteilytystä mahdollisena mikropertikkeleiden sterilointimenetelmänä tutkittiin. Työssä oli myös tarkoituksena hidastaa lääkeluovutusta entisestään ja minimoida alun nopea lääkeaineen vapautuminen kehittämällä hydrogeeli-mikropartikkelikomposiitti käyttäen aldehydi-modifioitua hyaluronihappoa (HA), ja ristisilloittajana hydrazidi-modifioitua PVA:a. Lisäksi komposiittigeelin mekaanisia ominaisuuksia tutkittiin puristuskokeilla. Deksametasonin lisäksi, myös uuden, tähän mennessä lääkeluovutussysteemeissä käyttämättömän lääkeaineen, muraglitazarin, kapselointia mikropartikkeleihin tutkittiin.

PLGA-PVA-DEX mikropartikelisysteemia onnistuttiin parantamaan. Prosessin saantoprosentti nousi noin 60 %, ja mikropartikkeleiden pienestä koosta huolimatta hitaampi, neljän viikon aikana tapahtuva lääkeluovutus saavutettiin. Pidennetyt lääkeluovutuskokeet paljastivat odottamattoman ongelman, deksametasonin hajoamisen, joka johti uuden analysointimenetelmän kehittämisen korkean erotuskyvyn nestekromatografialle (HPLC). Gammasteriloinnilla oli haitallinen vaikutus mikropartikkeleiden morfologiaan ja lääkeluovutukseen. Hydrogeeli-mikropartikkelikomposiitti käyttäytyi puristuskokeessa samalla tavalla kuin kontrolligeeli. Lääkeluovutusprofiilin muuttaminen kuitenkin epäonnistui. Sen sijaan mahdollinen hydrogeelin deksametasonia stabiloiva vaikutus paljastui. Myös muraglitazarin kapselointi PLGA-PVA mikropartikkeleihin onnistui täysin samaa valmistusmenetelmää käyttäen. Lääkeluovutus mikropartikkeleista tapahtui viikon kuluessa.

Johtopäätöksenä voidaan todeta, että hieman muunneltu, emulsio/liuottimen haihdutus- ja suihkukuivausmenetelmän yhdistelmä on sopiva lääkeainetta sisältävien mikropartikkeleiden valmistukseen. Gammasterilointia ei suositella sen haitallisten vaikutusten vuoksi. *In vitro* lääkeluovutus mikropartikkeleista on suhteellisen nopea, joten lisätutkimuksia lääkeluovutusprofiilin hidastamiseksi tarvitaan. Erityisesti hydrogeelin käyttöä lääkeluovutusprofiilin muuttamiseen suositellaan, sillä hydrogeeli-mikropartikkelikomposiittimateriaali hyvillä ominaisuuksilla ja suuremmalla mikropartikkelipitoisuudella saatiin kehitettyä tässä tutkimuksessa.

PREFACE

This thesis was performed at the Department of Electronics and Communications Engineering, Tampere University of Technology. This thesis project was part of the Human Spare Part project at the Institute of Biosciences and Medical Technology, BioMediTech. It is also collabortion projects with the Immunopharmacy research group at Tampere University.

First, I want to thank my supervisors, Professor Minna Kellomäki and PhD Jenny Párraga for the opportunity to take part of this project, and make myself familiar with the reseach among the biomaterials. I am very thankful also to the rest of the hydrogel group for advices, support and great team spirit. Especially, thank you Jennika Karvinen and Janne Koivisto for the help with hydrogels.

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TERMS AND DEFINITIONS

DCM	Dichloromethane
DDD	Drug delivery device
DEX	Dexamethasone
EtOH	Ethanol
H_3PO_4	Phosphoric acid
HA	Hyaluronic acid
m-HA	Aldehyde-modified hyaluronic acid
HPLC	High performance liquid chromatography
O/W	Oil-in-water solvent evaporation method
PBS	Phosphate buffer saline solution
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Poly(lactic-*co*-glycolic) acid
PPAR-agonist	Peroxisome proliferator-activated reseptor agonist
PVA	Poly(vinyl alcohol)
m-PVA	Hydrazide-modified polyvinyl alcohol
RPM	Revolutions per minute
RT	Room temperature
T_g	Glass transition temperature
TFA	Trifluoroacetic acid

1 INTRODUCTION

Controlled drug delivery has been under intensive investigation for decades, as it has many advantages compared to conventional administration routes, peroral delivery and injections. With controlled drug delivery devices improved efficacy, reduced toxicity, and improved patient contentment of drug therapy can be achieved. (Uhrich et al. 1999) Controlled drug delivery can be achieved by using polymeric drug delivery systems. All drug release systems aim to the same goal: improvement of drug therapy. This improvement is needed especially in case of hydrophobic drugs with poor solubility and permeability, which leads to low oral bioavailability in the body (Wischke & Schwendeman 2008).

Many forms of drug delivery devices exist, but one of the most popular forms is microparticles (Raman et al. 2005). They offer more efficient drug therapy by overcoming challenges, such as controlling drug concentration in the body, prolonging time that drug affects in the body, targeting of the drug to specific site (Langer & Peppas 2003), as well as protecting drug from too short life span in the body (Eniola et al. 2002). Polymeric microparticles have endless possibilities in the drug encapsulation. Investigation of the drug releasing microparticles continues with never ending development of new therapeutic molecules and polymeric materials. For example, by combining microparticles with hydrogels even more wide range of possibilities and advantages are achieved.

The aim of this study was to improve previously developed microparticle drug release system concerning hydrophobic, anti-inflammatory drug dexamethasone (DEX) (Ugur 2014). Microparticles were made of biodegradable polymer poly (lactide-co-glycolide) and polyvinyl alcohol. Gamma irradiation as possible sterilization method and its effect to drug release from the microparticles was investigated. In addition, novel hydrogel-microparticle composite material was designed in order to modify drug release profile of the DEX. Hydrogel was based on aldehyde-modified hyaluronic acid and crosslinked with hydrazide-modified polyvinyl alcohol. Finally, encapsulation using the same preparation method, and drug release of new, hydrophobic drug muraglitazar was examined.

This thesis starts with the theoretical background, in which the main topics, such as the drug delivery, the microparticles and the hydrogels are introduced through literature review. It is followed by experimental part, where materials, methods, and results with discussion are described to each separate group of interest: DEX loaded microparticles, hydrogel-microparticle composite and muraglitazar loaded microparticles. Finally, there will be conclusions and future propositions.

THEORETICAL BACKROUND

2 MICROPARTICLES IN DRUG DELIVERY

2.1 General

The term microparticle means small, sperical system with diameter smaller than 250 μm. Micro- and also nanoparticles are composed of solid matrix of polymer where therapeutic agent is distributed through the whole particle (Lassalle & Ferreira 2007). They have gained lots of interest among researchers, since they offer many possibilities to the drug delivery. (Park et al. 2005).

Drug delivery can be defined as delivering drug to the human body with the system called drug delivery device (DDD). Controlled drug delivery takes place when drug or some other therapeutic agent is incorporated in the polymeric material, either natural of synthetic, and released from the material in a desired and/or predictable way. (Peppas & Brannon-Peppas 2001) Targets of the drug delivery is to control, usually prolong, time that drug dose affects in the body, to control the amount of drug in the body, to target drug to specific places or cells, and to overcome tissue or cellular barriers. (Langer & Peppas 2003)

Wide range of drugs is suitable for drug delivery applications. These include protein-based drugs like insulin or growth factors, and conventional drugs like antibiotics (Künzler 2002). Drug release from the DDD can be constant or cyclic over a time period, or it can be triggered by some stimuli from external environment. Controlled drug delivery offers many benefits for the patients, as constant plasma drug concentration with conventional ways of drug administration, oral delivery or injection is difficult. It reduces risk of over and under dosing when drug level can be maintained longer in the therapeutic level. (Park 1997; Künzler 2002) Therapeutic level indicates range of drug concentration, in which desired effects occurs without harmful side effects. When concentration decreases below it, only little effect occurs, and concentrations above it are toxic. (Uhrich et al. 1999) So, higher local drug concentrations are achieved while minimizing the systemic drug concentration (Langer 1998). It also reduces need of frequent administration of the drug, and so on increases patient compliance and convenient. (Park 1997)

Controlled drug delivery via DDD offers also benefits to the drug. For example matrix material protects the drug from too short life span in the body caused by degradation or digestion. (Eniola et al. 2002) If traditional oral or injectable drugs cannot be used, it may be only option for the drug therapy.

Controlled drug delivery can be divided into two categories: temporal control and distribution control. In temporal control, the drug is released from the DDD the over an extended period of time or at the certain time during therapy. This kind of control is

important to the drug that is metabolized and eliminated from the body very fast after administration. Otherwise, concentration of the drug in the body may vary extensively, and stay in the therapeutic level relatively short period of time. It demands regular administration, for example injection. In distribution control, the drug is targeted to the specific site of activity in the body. This may be needed when drug molecules encounter tissues when distributed conventional way causing serious side effects, or when drug molecules does not reach their site of action via conventional distribution, for example through blood-brain-barrier. (Uhrich et al. 1999)

2.2 Preparation methods

Many different methods to obtain drug loaded microparticles are reported. Choosing the right method for the certain application is important, because it determines the characteristics, including size and drug interactions, of resulting particles. Thus, experimental parameters involved in particular method such as solvents, stirring rate and stabilizers have decisive importance. Furthermore, it may be even more important to know, how the change of these parameters affects the characteristics of the microparticles. (Lassalle & Ferreira 2007) In addition to the experimental parameters, also nature of the polymer, incorporated drug, intended use and duration of the therapy have an influence to the choice of the method (Jain 2000).

These factors set many requirements to the preparation method. Chemical and biological activity of the encapsulated drug must maintain during the process. Some drugs, especially polypeptides are denatured or decomposed when exposed to high temperatures, light or organic solvents. Yield and encapsulation efficiency of the obtained particles has to be high enough, so that process can be converted to mass production. Microparticles obtained from the chosen method should have certain size range that is small enough to administration through the syringe needle. Only that way microparticles can be administrated parenterally, in other words through other pathways than digestive track. Release profile of the encapsulated drug has to be reproducible in order to get predictable therapeutic response. And last, obtained microparticles must be able to re-suspend easily. (Park et al. 2005)

Microparticles have been prepared using many different methods. However, many of them are modifications of three basic methods, which are solvent evaporation/extraction, phase separation and spray drying. (Freitas et al. 2005)

2.2.1 Emulsion/solvent evaporation

Emulsion based methods include solvent evaporation and solvent extraction. The solvent, in which polymer is dissolvent is eliminated by evaporation or extraction. Briefly, polymer and possible therapeutic agent is dissolved in an organic phase and emulsifier in an aqueous phase. Combining these two phases forms emulsion. Emulsion drops form microparticles when solvent is eliminated. (Lassalle & Ferreira 2007) It is

especially used to prepare drug-loaded microparticles from polymers that are water-insoluble (Campos et al. 2013).

Single emulsion process, also called oil-in-water (o/w) emulsification, is the simplest of these methods. That is why other methods are derived from it. It is usually used to encapsulate hydrophobic or poorly water-soluble drugs into the polymer microparticles (Li et al. 2008). First, polymer is dissolved in volatile, organic solvent, which has to be water-immiscible (Figure 1). The most commonly used solvent is dichloromethane (DCM). Then, the drug is dissolved into this polymer solution. (Jain 2000) This co-dissolution may need also another solvent, called co-solvent, for dissolving the drug completely to the polymer solution (Freitas et al. 2005). The organic phase is then added with proper stirring conditions to a larger volume of an aqueous phase with the emulsifier to form an emulsion. Polyvinyl alcohol (PVA) is very often used emulsifier. In other words, polymer solution is broken up into the microdroplets using shear stress caused by stirring, homogenizer or sonicator. Size range of the microparticles is mainly defined by this emulsifying step. (Rosca et al. 2004)

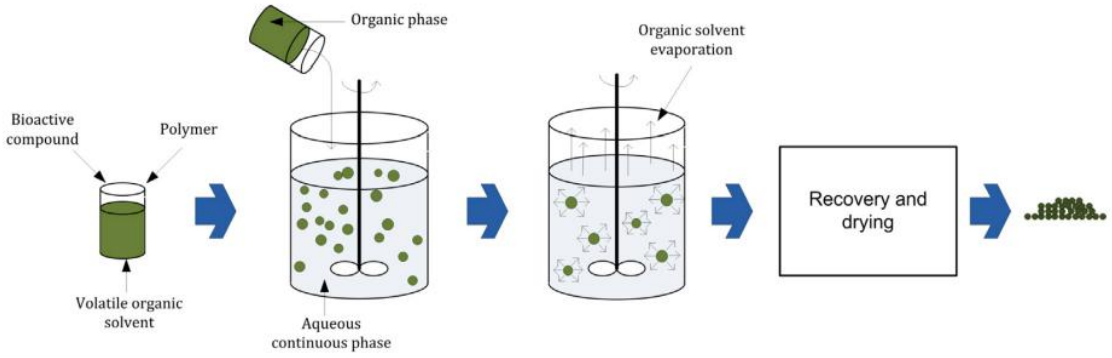

Figure 1. *Steps of the single emulsion process. (Modified from the source Campos et al. 2013)*

Then, solvent is removed by evaporation to the gas phase or extraction into the internal aqueous phase. (Li et al. 2008; Jain 2000) This leads to hardening of the oily droplets into solid particles. As polymer concentration, and so on viscosity of the droplets increases, the phase separation occurs. (Rawat & Burgess 2010) Solvent removal rate affects to the characteristics of the forming microparticles. Appropriate rate depends on many different factors, such as matrix polymer, drug, solvent and desired drug release profile. In case of solvent evaporation, temperature of the medium, pressure and stirring rate controls the removal (evaporation) of the solvent. The faster the rate, the faster microparticles solidify. Usually, fast solidifying results in more porous structure of the microparticles, whereas slower rate favours denser structure. In the last step particles are harvested from the solution and dried. Harvesting is usually performed by filtration or centrifugation. In this stage particles may be washed to eliminate emulsifier and non-encapsulated materials from the surface of the particles. (Freitas et al. 2005)

In addition to solvent evaporation rate, also many other process parameters have an effect on the characteristics of the microparticles. Solvent/co-solvent system has been investigated with both hydrophilic and hydrophobic drugs. In case of hydrophilic drugs, water miscible co-solvent increases the encapsulation efficiency. The oil phase partitions to the aqueous phase faster causing faster phase separation. Thus, hydrophilic drug has less time to move from oil phase to the aqueous phase and it is capsulated to the microparticles more efficiently. (Rawat & Burgess 2010)

In case of hydrophobic drug, it makes the whole system more complicated. Encapsulation efficiency may decrease, and the drug crystals on the surface of the device increase. That is why amount of co-solvent should be kept at minimum level in order to encapsulate hydrophobic drug successfully. (Wischke & Schwendeman 2008) Al-Maaieh & Flanagan (2001) examined effect of ethanol as co-solvent on the properties of hydrophobic drug dexamethasone (DEX) loaded PLGA particles. Increasing ethanol concentration (ethanol % (v/v) of dichloromethane) resulted decrease in drug loading and particle size, and changes in morphology and *in vitro* release profiles. (Vysloužil et al. 2013)

Double emulsion process is also called a water-in-oil-in-water (w/o/w) technique, and it is derived from single emulsion technique. It is more suitable encapsulation method for hydrophilic drugs. When using single emulsion method for hydrophilic drugs or proteins, the drug may diffuse out, or partition from hydrophobic oil phase into the aqueous phase, which decreases the encapsulation efficiency. (Park et al. 2005) In this case, therapeutic agent is dissolved in aqueous phase, either buffered solution or plain water, and polymer is dissolved to the organic phase. Aqueous phase is then added to the organic phase in order to form first o/w emulsion. Then, this first emulsion is added to the larger volume of aqueous phase including the emulsifier to form w/o/w emulsion. (Lassalle & Ferreira 2007) Solvent evaporation or extraction, particle harvesting and drying steps are similar to the single emulsion method.

2.2.2 Spray drying

The main idea of the spray drying is to transform liquid solution into the dried particles by spraying it into the hot, gaseous drying medium. It is very old method. First time it is mentioned as early as 1860, but after World War II the applications started to be in the pharmaceutical industry. Since then, not only the spray drying machines were developed but also concentration turned into the characteristics of the products, and processing parameters affecting to them. (Cal & Sollohub 2010) Nowadays, it is widely used drying method of the aqueous and organic solutions, emulsions and dispersions to produce chemical, pharmaceutical and food industry products (Büchi 2002).

In spray drying method polymer is usually dissolved into the organic, volatile solvent and then drug is dissolved or dispersed into this solution. Also here dichloromethane is very commonly used solvent. This solution is then sprayed into the heated air stream in order to form solid microparticles. Spray drying method has many advantages. It has good reproducibility, ability to scale to the mass production, it offers

mild processing conditions, it is one step process, particle size can be controlled via atomizing conditions (nozzle), and it is not as dependent on the solubility of the drug or the polymer as in the solvent based methods (Arpagaus & Schafroth 2007).

It has also disadvantages, which include the loss of relatively large amount of the particles and formation of the large aggregates. The microparticles are lost because they adhere to the walls of the glass parts of the spray dryer. It is impossible to collect them all. Microparticles are often sticky before the solvent has evaporated completely and this leads to aggregation of the particles. (Park et al. 2005) Another, significant deficiency is the non-uniform particle size (Lassalle & Ferreira 2007). Spray drying method is used especially in large-scale production of the microparticles, and it needs usually big batch sizes, which set economical challenge for microparticle formation. (Wischke & Schwendeman 2008)

The onset of the spray drying process is considered as complicated, as many adjustable processing parameters, such as inlet temperature, drying air flow rate, feeding rate, pressure and amount of the atomizing air, has influence on other parameters. These secondary prameters include outlet temperature, droplet size, drying efficiency and properties of the dried product. Adjusting of these parameters is difficult, and obtained results are hard to predict. So, parameters are often optimized using “trial and error” method, but some estimation can be made. Mutual relationships of the spray drying parameters are presented in Table 1. (Büchi 2002)

Table 1: *Mutual relationships of the spray drying parameters (Büchi 2002).*

parameter / dependence	aspirator rate ↑	air humidity ↑	inlet temperature ↑	spray air flow ↑	feed rate ↑	solvent instead of water	concentration ↑
outlet temperature	↑↑ less heat losses based on total inlet of energy	↑ more energy stored in humidity	↑↑↑ direct proportion	↓ more cool air to be heated up	↓↓ more solvent to be evaporated	↑↑↑ less heat of energy of solvent	↑↑ less water to be evaporated
particle size	-	-	-	↓↓↓ more energy for fluid dispersion	(↑) more fluid to disperse	(↓) less surface tension	↑↑↑ more remaining product
final humidity of product	↑↑lower partial pressure of evaporated water	↑↑ higher partial pressure of drying air	↓↓ lower relative humidity in air	-	↑↑ more water leads to higher particel pressure	↓↓↓ no water in feed leads to very dry product	↓ less water evaporated, lower partial pressure
yield	↑↑ better separation rate in cyclone	(↓) more humidity can lead to sticking product	(↑) eventually dryer product prevent sticking	-	(↓↑) depends on application	↑↑ no hygroscopic behaviour leads to easier dying	↑ bigger particles lead to higher separation

Spray drying method has been used for encapsulation of drugs in order to prepare drug loaded microparticles from many different polymers, hydrophilic and hydrophobic (Mu & Feng 2001). For example, polylactide (PLA), its copolymer poly (lactide-co-glycolide) (PLGA), and chitosan are probably the most used. Also different kinds of drugs have been incorporated into the microparticles, including heat sensitive and heat resistant, water soluble and insoluble, and conventional drugs and proteins. (He et al. 1999; Arpagaus & Schafroth 2007) Advantages of the method are listed in the

beginning of this chapter, but when drug encapsulation is in concern, high encapsulation efficiency in spray drying has to be mentioned (Jain 2000).

As well as in emulsion/solvent evaporation method, designing of the spray dried polymeric DDD may be challenging due to numerous adjustable parameters that have effect on the properties of the drug loaded microparticles microparticles. Spray flow rate (compressed air) needed to disperse the feed solution affects to the size of the microparticles. Higher flow rate results smaller particle size. Concentration of the feed solution affects also to the particle size. The higher solid content in the feed solution, the larger the particles and also the more porous particle structure. Residual moisture content and separation efficiency in the cyclone may be altered through aspirator speed. (Büchi 2002)

Inlet temperature is important factor in the drying process. It has to be high enough to ensure solvent evaporation, but still low enough to avoid destruction of the polymer. Inlet temperature together with aspirator flow rate and pump speed determines outlet temperature. In case of biodegradable polymers, outlet temperature is important to keep below glass transition temperature of the polymer. (Büchi 2002; Arpagaus & Schafroth 2007)

In addition to parameters of the spray dryer, also properties of the polymer, drug and used solvent affect to the final result. As a matter of fact, interaction between all the components in the system plays the most critical role (Gander et al. 1996). In addition, additives such as stabilizers and surface active substances (helper polymers, lipids or other emulsifiers) has an effect on drug delivery properties of the microparticles, bulk properties, and mucoadhesive properties for example. (Mu & Feng 2001; Ungaro et al. 2012)

The spray drying process consists of three phases: atomization, drying of the droplets, particle separation, and collection from the drying gas. Each of these phases, condition in which they are carried out, and used hardware used has own affect to the properties of the product particles. (Cal & Sollohub 2010)

Atomization means the formation of the liquid-gas suspension and it is done in order to reduce the size of the particles. This leads to increase in surface area into which drying gas can influence. Heat from the heated drying gas transfers to the small droplets and owing to this, solvent from the droplets evaporates quickly and solid particles are formed. Drying particles do not reach the temperature of the heated gas (inlet temperature) and that is why spray drying is also suitable drying method for the heat sensitive materials. Furthermore, it offers possibility to get desired physicochemical and morphological properties to the particles. The feed solution is transferred to the atomization device using, for example peristaltic pump. (Cal & Sollohub 2010)

Several different atomization devices exist; these include rotary atomizers, hydraulic nozzles, pneumatic nozzles and ultrasonic nozzles. The selection of atomization device is made based on the properties of the feed solution, desired properties of particles and desired size of particles. (Büchi 2002) Rotary atomizer forms suspension mechanically. It is the most effective atomizer, but it also causes the greatest deposit of the product to

the walls of drying chamber. That is why it is not suitable for the pharmaceutical formulations of high price or small solid content. (Cal & Sollohub 2010)

Hydraulic nozzle uses high pressure to form small droplets. Problem of this atomizer is that it may wear out quickly, especially when feeding solution includes any hard particles. The risk of nozzle clotting is also the highest, possibility to control properties of the particles is limited, and efficiency is low. (Cal & Sollohub 2010)

Pneumatic nozzle, also called multi-fluid nozzles, uses high frictional forces caused by stream of compressed carrier gas to atomize the feed solution into droplets. The atomization is depended on the surface tension, density and viscosity of the feed solution and properties of the carrier gas. The most simple of these nozzles is two-fluid nozzle in which atomizing gas and feed solution intersect immediately after exit from. It is the most popular atomization device when preparing pharmaceutical products. With special pneumatic nozzles it is possible to use two different carrier gases that are independent to each other. These two gases can, in addition, atomize only one feed (three-fluid nozzle) or two independent feeds (four-fluid nozzle). With four-fluid nozzle it is possible to obtain particles less than 1µm with narrower size range and it is used to prepare polymeric particles for sustained or controlled drug release. Problem with pneumatic nozzles, as well as hydraulic nozzles, is clogging of the nozzle. To prevent the clogging some spray dryers have needle, which is driven by compressed air inside the nozzle. (Cal & Sollohub 2010)

The nozzles mentioned above are the most commonly used, but they have limitations. For this reason, specialized nozzles have been developed. For example, ultrasonic nozzles are designed for feeds with high viscosity, and low-voltage hydrodynamic nozzles are for drying very labile particles. (Cal & Sollohub 2010)

After atomization, droplets are exposed to the drying gas in the drying chamber. Drying gas is usually air, and it is drawn through the filters to the system and preheated. Also nitrogen and other inert gases have been used as drying gas, but they demand use of closed circulating systems. It also adds to the costs of the process. Nevertheless, in a case of instable substances it is necessary to use inert gases as drying gas. (Cal & Sollohub 2010)

Air flow in the drying chamber is not laminar. Instead, there are several air streams. That is why droplets are exposed to local air temperature and humidity conditions.So, they lose their solvent under different conditions. Inlet temperature is closely related to the solvent removal from the dispersion droplets. Temperature is adjusted to achieve the best thermal efficiency, but low enough to prevent harmful effects to the material. However, product is never exposed the temperature as high as inlet temperature. In fact, outlet temperature, which is derivative of the inlet temperature is the highest temperature that product is exposed to. Inlet temperature and air humidity both affect to the residual solvent content in the product, and to the whole drying process. Still, only temperature can be adjusted, since air moisture changes according to the season. (Cal & Sollohub 2010)

Drying chambers are usually vertical, thay have shape of cylinder, and they are divided into tall and small chamber according to the height. Temperature profile, in other words temperature distribution along the axis of the chamber and droplet pathway can be derived from the shape of the chamber. Shape and size should be the kind that even the biggest droplets have enough time to dry before collision to the wall. Otherwise product may deposit to the walls of the chamber. It is undesired phenomenon especially in case of expensive pharmaceuticals. (Cal & Sollohub 2010)

Term air-droplet contact is used to describe reciprocal orientation of drying gas stream and the atomizing device. In other words it is the way drying gas contacts the sprayed dispersion droplets. There are three different ways to mix droplets and drying gas, and so on three different types of dryers: co-current dryers, counter-current dryers, and combined flow dryers. (Büchi 2002)

In co-current dryer inlet of the drying gas and sprayed dispersion are both situated in the upper part of the drying chamber. Dispersion is sprayed in a same direction as drying gas flow. Droplets contact the drying gas in the moistest condition and moves along the gas stream to the bottom of the chamber simultaneously losing their solvent. Dried particles leave the chamber through the outlet that is situated in the lowest part of the chamber. This method is gentle to the forming powder, because evaporation of the solvent is so fast even though it is sprayed straight to the hot gas. The co-current dryer is the most popular type, and the drying kinetics and particle behaviour inside this type of chamber are the best known. (Cal & Sollohub 2010)

In counter-current dryers, the drying gas inlet is situated opposite to the inlet of the feed flow. Thus, feed is sprayed in the opposite direction than drying gas. The hot drying gas comes from the bottom of the chamber upwards and loses most of its heat before hitting to the droplets. The droplets, on the other hand are sprayed downwards and when descending to the product uptake, they go through increasingly hot gas. So, dry powder reaches higher temperature than outlet temperature and because of this, counter-current dryer are suitable only for thermally stable materials. (Cal & Sollohub 2010; Büchi 2002)

Third type of dryer is combined flow dryer. According to the name, the drying process is combination of the last two methods. The atomized feed is going upwards from the atomizing device confronting the drying air coming downwards from the upper part of the dryer. The problem is mixing of the moist droplets and already dry product. This is the most economical process for thermally stable substances. (Cal & Sollohub 2010)

Drying of the products is followed by its separation from the gas to the collection vessel. Collection may be carried out using two ways. Descended product can be collected from the bottom of the drying chamber, or product leaves the chamber with outgoing air and is separated from the air in the cyclone, bag filters, electrostatic precipitators or wet collector. The most common way of separation is use of cyclone. There, air is set to the fast rotational motion, which creates centrifugal force. It forces solid particles toward the walls of the cyclone and separates them from the air cone.

(Büchi 2002) Some cyclones have special coating in the inner wall in order to avoid accumulation of the particles. (Cal & Sollohub 2010) The final product is then collected to the sealed container.

Aggregation of the particles during the process can be avoided by coating microparticles with polysaccharide, for example mannitol. Polysaccharide coating has reported to prevent aggregation of the particles and it also increases the yield of the process. This coating can be carried out using double-nozzle technique in which polymer or drug solution is sprayed from one nozzle, and aqueous polysaccharide solution is sprayed from the other nozzle to the surface of the particles at the same time. (Takada et al. 1995)

Another application of spray drying is cryogenic, non-aqueous method in which polymer/drug solution is sprayed into the frozen ethanol that is in the bath of liquid nitrogen. Then, these frozen droplets are transferred into the -80ºC where ethanol causes the extraction of the solvent, and so on the hardening of the microparticles. This method is useful especially in encapsulation of proteins, since it maintains better their biological activity. (Johnson et al. 1997) Spray freeze drying uses the same idea. There, feed is atomized to the freezing medium (liquid nitrogen). Then frozen droplets are removed from the chamber and dried using freeze dryer. It is used in small-scaled pharmaceutical applications. (Yu et al. 2004; Mujumdar 2006)

2.3 Drug loaded poly (lactide-*co*-glycolide) microparticles

Biomaterials are defined as synthetic, natural or modified natural materials intended to be contact and interact with the biological systems in order to evaluate, treat, augment or replace any tissue, organ or function of the body (Williams 1999). Simply, biomaterials may become part of the body temporarily or permanently. They are used in prosthetic, diagnostic and therapeutic applications. (Park et al. 2011) Biomaterials include metals, ceramics, glass, polymers and their combinations. The most essential property of the biomaterial is biocompatibility whereupon material can perform in a host tissue without evoking toxic or injurious effects. In this work we are interested in polymeric materials as carriers for drug delivery.

Biodegradable materials occur naturally or they are made synthetically. They degrade *in vivo* (within the living organism) via either chemical or enzyme-catalysed hydrolysis into biocompatible, non-toxic by-products, which are then eliminated from the body by metabolic pathways. Biodegradability is important characteristic of the material used in DDDs because device does not require the removal by surgical operation. (Park et al. 2005) Through the metabolic and excretion systems in the body, biodegradable materials are completely removed. Degradative enzymes in the body enhance the rate of degradation by facilitating the degradation mechanism. (Uhrich et al. 1999) Biodegradable materials are mainly polymers. Examples of biodegradable polymers are polyesters, poly (ortho esters), polyanhydrides and natural polymers.

Especially aliphatic polyesters have been studied a lot as drug carrier material due to their good biocompatibility. (Park et al. 2005)

2.3.1 Poly (lactide-*co*-glycolide)

Poly (lactide-*co*-glycolide) (PLGA) is a random copolymer of lactide and glycolide. It belongs to the groups of aliphatic polyesters. PLGA, polylactide (PLA), and polyglycolide (PGA) are the most widely studied synthetic, biodegradable polymers in the field of biomedicine. This high interest is not only due to their excellent properties, but the fact that they have been proofed to be safe, non-toxic and biocompatible. In addition, they have already been used successfully in many, approved, injectable medical applications like sutures, bone pins and drug including products for parenteral use. They have also been studied as a material of vascular and urological stents, artificial skin and scaffold for tissue engineering. (Ratner et al. 2004, pp. 121)

Disadvantage in use of PLA, PGA or PLGA is acidic degradation products. They may accumulate at the site of implantation, and cause delayed inflammatory response from few months to years after implantation. Degradation products are discussed in more detailed in next chapter. Another drawback is poor cell attachment and proliferation on the surface of PLA and PGA. (Ratner et al. 2004, pp. 121)

PGA is the oldest biodegradable polymer used in biomedical application. It has simpler structure and is more hydrophilic than PLA (Figure 2). It is highly crystalline polymer, has high melting point and poor solubility in organic solvents. Because of hydrophilic nature and high crystallinity, water uptake into the molecule is fast which leads to the fast degradation rates. (Ratner et al. 2004, pp. 120)

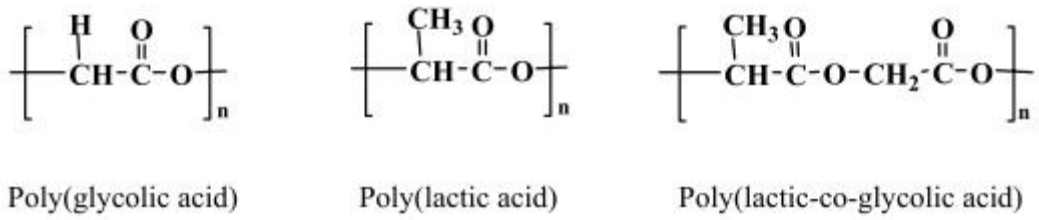

Figure 2: *Chemical structure of polylactides. (Modified from the source Park et al. 2005)*

PLA is more hydrophobic due to methyl group in its structure. It has larger molecular weight than PGA, and it degrades more slowly. (Zhang et al. 2014) Contrary to PGA, PLA molecule has an asymmetric α-carbon and is a chiral molecule. Therefore, it has two optical isomers: D-lactide and L-lactide. PLA containing only one of these isomers (poly (L-lactide) and poly (D-lactide)) is semi-crystalline polymer, but PLA containing both of the isomers poly (DL-lactide) is amorphous polymer due to random division of D-lactide and L-lactide units. (Nair & Laurencin 2007) Amorphous poly (D,L-lactide) is used for example in drug delivery applications where homogenous distribution of active agent is important. Semi-crystalline poly (L-lactide) gives

mechanical strength and stiffness, and is therefore used for example in sutures. In addition, poly (L-lactide) is generally more used compared to poly (D-lactide) since its hydrolysis gives naturally existing stereoisomer of lactic acid. (Ratner et al. 2004, pp. 121)

Polymerization of the PLGA occurs via ring-opening reaction of lactide and glycolide dimer. They degrade through gradual hydrolytic scission of the ester bonds in the backbone. The mechanism of the degradation is bulk erosion (Uhrich et al. 1999). Degradation rate can be adjusted by ratio of lactide to glycolide. (Ratner et al. 2004, pp. 79) Resulting byproduct, also naturally occurring lactic acid, is further eliminated from the body via tricarboxylic acid cycle as carbon dioxide and water (Crotts & Park 1998). In case of biodegradable polymers, often degradation products define the compatibility of the polymer, not always polymer itself. (Uhrich et al. 1999)

Properties of the solid PLGA are considered as invariable, but they change in time when exposed to the humidity. These parameters include glass transition temperature (T_g), moisture content and molecular weight. Also physical properties of the PLGA themselves are dependent on many factors, such as initial molecular weight, ratio of lactide to glycolide, size of the device, surface area of the device (exposure to water) and storage temperature. (Houchin & Topp 2009) The ability of the polymer to form DDD is affected by these properties, since they may have influence to the degradation rate of the device (see Chapter 2.1.2). However, also type of the drug has been shown to control the release rate. (Siegel et al. 2006) T_g of PLGA in general is above physiological temperature (37 °C). Characterization of commercial PLGAs is usually expressed by intrinsic viscosity, which is directly related to the molecular weight. (Makadia & Siegel 2011)

It is important to notice that there is no linear relation in properties of PLGA and corresponding ratio of glycolide and lactide. For example, copolymer consisting of 50:50 lactide to glycolide degrades more rapidly than PGA or PLA alone. (Ratner et al. 2004, pp.121) Nevertheless, PLGA rich in PLA content is less hydrophilic and therefore uptake less water and degrade more slowly than PLGA with higher PGA content. (Makadia & Siegel 2011)

PLGA can be processed into many different sizes and shapes. Different forms include for example scaffolds, rods, discs, films, cylinders, foams microparticles and nanoparticles (Raman et al. 2005; Fredenberg et al. 2011). Microparticles are the most popular form, as they offer many advantages such as ease of fabrication, simple administration (Raman et al. 2005), possibility to control drug release rate, complete biodegradability, good compatibility (Klose et al. 2010), and overall versatility. PLGA can also encapsulate molecules, like drugs or proteins, of almost any size. Examples of encapsulated drugs and macromolecules are proteins, peptides, siRNA, analgesics, anti-cancer drugs, anesthetics, antibiotics, hormones and glucocorticoids (Wischke & Schwendeman 2008; Fredenberg et al. 2011). It is soluble in wide range of organic solvents, for example in chlorinated solvents, tetrahydrofuran, acetone and ethyl acetate. (Wu & Wang 2001) In addition, there are many PLGAs available on the market with

different physico-chemical properties (molecular weight, lactide to glycolide ratio, capped/uncapped carboxylic end groups) (Fredenberg et al. 2011).

2.3.2 Drug release behaviour of the poly (lactide-*co*-glycolide)

Knowing and understanding possible release mechanisms and physico-chemical processes that affect to these mechanisms and so on release rate is crucial in designing controlled drug release device. Two main drug release mechanisms from PLGA drug release devices are diffusion and degradation/erosion. In the beginning it is mostly diffusion controlled and degradation/erosion controlled at the end of the release period. Drug release is often a result of the chain reaction that starts with water absorption and is followed by hydrolysis and erosion. (Fredenberg et al. 2011)

There are four ways for drug release from PLGA-based device. All of these are based on two of main mechanisms mentioned above. These are: 1) diffusion through water-filled pores, 2) diffusion through the polymer, 3) osmotic pumping, and 4) erosion (Figure 3). The transport via pores occurs usually via diffusion, but may be also result of convection driven by for example osmotic pressure. However, osmotic pumping is more common in DDDs based on non-swelling polymers. PLGA absorbs large amounts of water and have tendency to swell. Diffusion through pores is the most common when released drug molecule is large and hydrophilic, for example protein or peptide. (Fredenberg et al. 2011) Diffusion through the polymer is main way of release in case of small, hydrophobic drugs (Raman et al. 2005). Nevertheless, drug has to enter water phase at the surface of the device or in water-filled pores before being released. (Fredenberg et al. 2011)

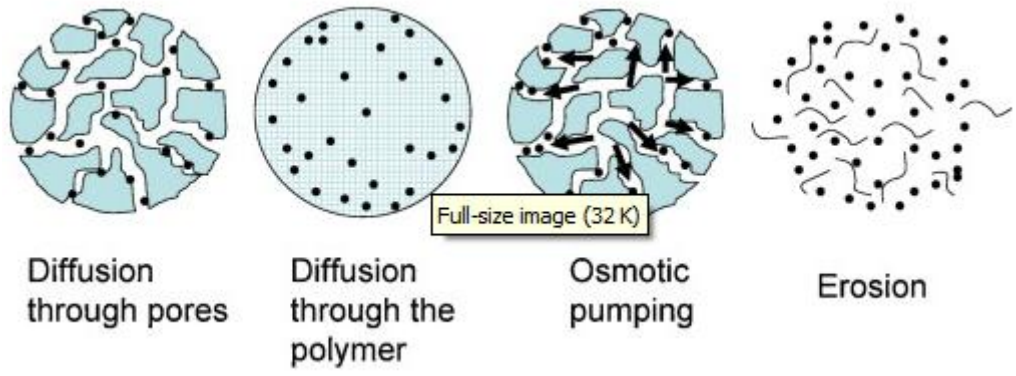

Figure 3: *Drug release mechanisms from PLGA-based devices. (Fredenberg et al. 2011)*

Drug may also be released without any transport via erosion of the matrix polymer. Erosion may also lead to formation of pores, which accelerates drug release via diffusion. Drug release is complicate event and these ways of drug release must be separated from rate-controlling release mechanisms. Rate-controlling mechanisms include water absorption (swelling), dissolution of the drug, hydrolysis, heterogeneous

degradation, changes in polymer chain mobility and density, crystallization of the oligomers, erosion, pore formation, pore closure, polymer-drug interactions, drug-drug interactions, formation of cracks and collapse of the device. (Fredenberg et al. 2011)

The release profile of certain device is hard to predict, as many different processes influence the rate of diffusion and degradation. Here, degradation of the PLGA is discussed in more detail. However, some events affect to both, diffusion and degradation. As an example, water absorption starts immediately after exposing device to the aqueous environment and is also called hydration of the polymer. Hydration leads to hydrolysis of the polymer chains, but also pore formation, as pore can be determined as any volume occupied with water inside the polymer matrix. First, pores are too small for drug transport, but when pore formation proceeds, interconnected network starts to form and it allow drug transport. (Mochizuki et al. 2008) Pore closure may occur. It is related to the mobility of the polymer chains and their ability to rearrange. Mobility of the polymer chains depends on the T_g, which decreases with decreasing molecular weight during degradation of the polymer. (Fredenberg et al. 2011)

As mentioned in previous chapter, PLGA degrades mainly via hydrolytic bulk erosion. Bulk erosion means that water uptake into the polymer matrix is higher than degradation rate of the polymer. Thus, degradation occurs throughout the device. (Uhrich et al. 1999; Klose et al. 2006) PLGA itself is said to autocatalyze the degradation, because hydrolysis produces free carboxylic end groups. Bases in the surrounding media neutralize acids, but since diffusional mass transport in the polymer-based devices is rather slow and acid generation rate may be higher than neutralization rate, this may lead to acidic environment inside the device. Because protons catalyze the cleavage of the ester bond this decreased pH may accelerate the degradation of the matrix polymer. Therefore, degradation inside the device becomes heterogeneous. (Grizzi et al. 1995; Brunner et al. 1999; Lu et al. 1999; Li & Schwendeman 2005) Auto-catalyzing effect is highlighted when dimensions of the devices are increased (Dunne et al. 2000), but it has been noticed also in films and microparticles (Lu et al. 1999; Park et al. 1995). It is reported that pH inside the PLGA microparticles may decrease as low as 1.5 (Fu et al. 2000). Degradation products also plasticize the polymer and increase osmolality inside polymer matrix, and they may crystallize (Fredenberg et al. 2011).

The release profile is hard to predict as real degradation of the PLGA is combination of bulk diffusion, surface diffusion, bulk erosion and surface erosion. (Makadia & Siegel 2011) In addition, many other factors affect to the degradation of PLGA and therefore the release of incorporated drug. Some of these factors are collected to the Table 2.

Table 2: *Factors affecting degradation and drug release of PLGA.*

Factor	Effect	Reference
Composition (The most important factor)	Glycolide proportion ↑ End-capping ↓ Hydrophilicity ↑ Degradation rate ↑	(Park 1995; Lu et al. 1999; Wu & Wang 2001; Wischke & Schwendeman 2008)
Crystallinity	Contradictory results: Crystallinity of PLLA ↑ Sample crystallinity ↓* Degradation rate ↑	(Schliecker et al. 2003*; Alexis 2005)
Molecular weight	Molecular weight ↑ Degradation rate ↓	(Park 1994; Alexis 2005)
pH of the media	Alkalinity ↑ Strong acidity ↑ Degradation rate ↑	(Holy et al. 1999; Zolnik & Burgess 2007)
Size and shape of device	Surface area ↑ Device dimensions ↓ Degradation rate ↑ For large device degradation faster inside than at the surface (autocatalysis)	(Grizzi et al. 1995; Witt & Kissel 2001; Schliecker et al. 2003; Alexis 2005; Klose et al. 2006)
Drug type and additives	Degradation rate, water absorption into the matrix polymer and parameters of the drug release rate varies as function of the drug type (drug chemistry, hydrophilicity) → Not clear relationship	(Frank et al. 2005; Siegel et al. 2006; Wischke & Schwendeman 2008)
Drug loading	Drug loading ↑ (up to certain level, depends on the drug) Burst release ↑	(Eniola et al. 2002)
Temperature	Temperature ↑ Degradation rate ↑	(Zolnik et al. 2006)
Sterilization	Degradation rate ↑	(Rothen-Weinhold et al. 1997; Carrascosa et al. 2003)
Fluid flow	Degradation rate ↓ (Compared to static conditions)	(Agrawal et al. 2000)
Porosity	Porosity ↓ or ↑** Degradation rate ↑	(Agrawal et al. 2000; Klose et al. 2006**)

Degradation of PLGA-based device gives a tri-phasic drug release profile. Sometimes profile is bi-phasic, but only rarely mono-phasic which refers to zero-order release kinetics. Usually large particles give tri-phasic profile because of heterogeneous degradation (autocatalysis) inside the particles whereas smaller particles and particles coated with PLGA gives bi-phasic release profile. Hence, using different sizes of particles makes it possible to alter the release profile. (Berkland et al. 2003; Berchane et al. 2007; Fredenberg et al. 2011) There are three different phases in typical drug release profile of PLGA device.

First phase is burst release. In this phase free, non-encapsulated drug from the surface of the particles and/or drug near the surface is released to the medium. Also crack formation to the device or disintegration of the device may cause burst release. This causes faster drug release immediately after exposing the device in the release medium. It also decreases the effective lifetime of the device. Usually it is regarded as negative phenomenon, but sometimes it may be desirable. The main problem with the burst release is that it is not predictable and the amount of drug released in the burst cannot be controlled. (Huang & Brazel 2001) Burst release may be avoided, for example, by coating device (Wischke & Schwendeman 2008), using block copolymers, variation of process parameters and conjugation of drug to the polymer (Berkland et al. 2002).

Second phase is slow release phase, also called lag-phase. In this phase polymer hydration proceeds and the drug diffuses slowly through the matrix polymer or the already existing pores. Simultaneously, polymer matrix proceeds degrading and hydrating. This phase may be caused by dense polymer matrix with low porosity, pore closure, drug-polymer interactions or drug-drug interactions. (Fredenberg et al. 2011) Lag-phase may be avoided by blending high molecular weight polymer with small amount of low molecular weight polymer, or using only low molecular weight polymer (Wischke & Schwendeman 2008).

Third phase is fast release, also called second burst. This point is deemed as beginning of the erosion. Now polymer is hydrolyzed to oligomers and monomers, which allow the drug to be released, by diffusion and erosion. In this phase different types of drugs attract water into the polymer matrix. (Makadia & Siegel 2011) This phase can be also caused by formed cracks and pores, or complete disintegration of the particles (Matsumoto et al. 2006).

Obviously, all drug release profiles do not follow the traditional tri-phasic release profile. Sometimes, if second phase is rapid, short or lack completely, release may slow down at the end of release. There are huge amounts of factors affecting to the drug release profile and they make prediction of the release profile very difficult.

Mathematical modeling is used to predict drug release from PLGA DDDs. Mathematical models can be divided into two categories: empirical/semi-empirical models and mechanistic empirical models. Empirical/semi-empirical models are only mathematical descriptions and are poor to predict the real drug release, whereas mechanistic models are based on real chemical, physical or biological phenomenon such

as diffusion or erosion. They offer useful tool to understand release process acting as simplification of real drug release system. (Siepmann & Siepmann 2008)

2.3.3 Dexamethasone

Dexamethasone (DEX) belongs to the family of glucocorticoids that are steroid hormones (Figure 4). In a body they bind to the glucocorticoid reseptors affecting this way to immunological and metabolic functions. Their main function is to suppress inflammatory reactions by inhibiting the production of the factors that are responsible for inducing inflammatory response. These factors include vasoactive and chemoattractive factors as well as lipolytic and proteolytic enzymes (Hickey et al. 2002). They also decrease extravasation of the leukocytes to the site of injury. DEX has also demonstrated to promote differentiation of chondrocytes (Na et al. 2007) and inhibiting the formation of blood vessels (Patil et al. 2007). In addition to preventing and suppressing inflammation, it has been used in many biomedical applications, for example cell cultures, treatment of eye diseases, asthma and leukemia (Urbańska et al. 2014)

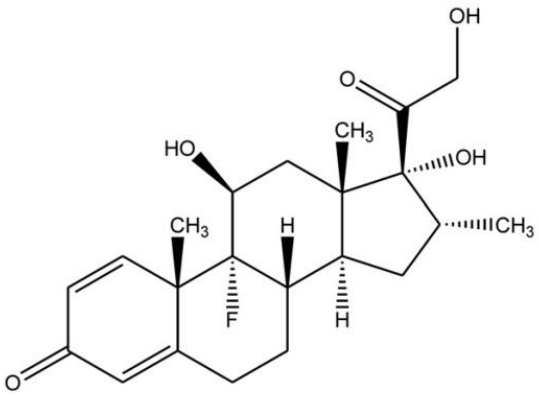

Figure 4. *Structure of the dexamethasone. (Urbańska et al. 2014)*

DEX has also relatively long list of side effects because of its high hydrophobicity and need of high doses for reaching therapeutic response (Bhardwaj et al. 2010). The side effects in long-term use include osteoporosis, high blood sugar concentrations, stomach and intestinal bleeding (Urbańska et al. 2014), and complication of the disease states (Bhardwaj et al. 2010).

DEX is widely investigated and well known drug molecule. It has been used systemically and orally to prevent fibrosis (Rawat & Burgess 2010) and immune response to implantable devices. However, due to serious systemic side effects local administration has shown the most promising results in both acute and chronic inflammation. Therefore, DEX has been incorporated into many different drug delivery applications using many different polymers. For example, PLA, PLGA, poly-ε-caprolactone, PVA, poly (ethylene glycol), HA, chitosan, different copolymers and blends have been used (Urbańska et al. 2014). DDDs including DEX encompass almost

all possible forms of delivery devices, such as microparticles (Hickey et al. 2002), hydrogels (Ito et al. 2007) and hydrogel-microparticle composite materials and scaffolds. Their purpose is to prevent inflammation as such or as sensor coatings (Kim & Martin 2006), or to induce cells (Na et al. 2007). For these reasons it has been chosen to act as model drug in this study.

2.3.4 Muraglitazar

Muraglitazar (Figure 5), is dual peroxisome proliferator-activated reseptor (PPAR)-agonist. PPARs are nuclear reseptors that regulates energy, including lipids and glucose, homeostasis in cells. Three isoforms of PPARs exist: PPARα, PPARγ and PPARβ/γ. PPARγ-agonists are insulin-sensitizing agents and are used in treatment of diabetes. PPARα-agonists are included in lipid metabolism by lowering triglyseride levels in blood and increasing high-density lipoprotein cholesterol. So, they have been used in treatment of dyslipidemia, dysfunction in fat metabolism. (Fernandez et al. 2011; Paukkeri et al. 2013)

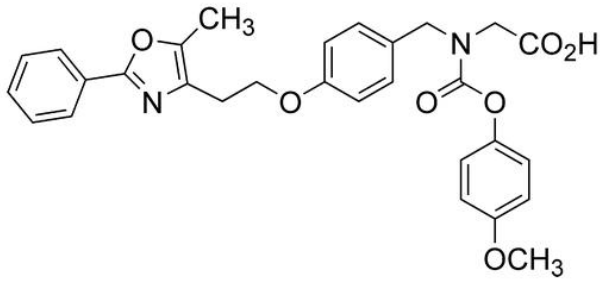

Figure 5. *Chemical structure of the muraglitazar. (modified from (Devasthale et al. 2005)*

Muraglitazar is the first dual PPARγ/α-agonist approved by FDA. It possesses effect to both reseptors, strong effect to PPARγ and moderate effects to PPARα. It was developed for the treatment of type 2 diabetes in order to achieve combined insulin sensitizing and blood lipid concentration lowering effects. (Rubin et al. 2009) In addition, it has shown to have multiple anti-inflammatory properties *in vitro* and *in vivo* models. Anti-inflammatory effects falls upon both reseptors PPARγ and PPARα (Paukkeri et al. 2013). Also other PPARγ and PPARα-agonists have reported to have influence to the regulation of the inflammatory responses in diseases connected to obesity. Furthermore, PPARγ-agonists have ability to reduce inflammatory responses in *in vivo* models in rheumatoid arthritis. (Giaginis et al. 2009)

Safety of the PPAR-agonists has been under concern, as it has been reported to cause the detrimental side effects. Nissen et al. (2005) reported higher risk of harmful cardiovascular events in patients with type 2 diabetes when treated with muraglitazar only relatively short period of time compared with controls. These cardiovascular events included myocardial infraction, stroke, transient ischemic attack and congestive heart failure. In addition, muraglitazar seems to increase risk of bladder cancer in animals

(Tseng & Tseng 2012). Because of these harmful effects, developer of the muraglitazar has decided to stop further development of the drug. (Paukkeri et al. 2013) However, these kinds of cardiovascular complications are common among type 2 diabetes patiences.

Despite these adverse effects, muraglitazar may have a future as treatment of other diseases, as it has shown to have also potential of acting as anti-inflammatory drug in rheumatoid arthritis (Paukkeri et al. 2013) and reducing steatosis in nonalcoholic fatty liver disease (Rogue et al. 2014). At this moment, there is only a little knowledge of treating non-diabetic patients with muraglitazar. Further safety investigations are needed for using muraglitazar in other conditions than diabetes. Due to this potential as local inflammatory suppressor, muraglitazar has been chosen to this study.

2.3.5 Gamma irradiation as sterilization method for biopolymers

All implantable devices, which are introduced into the body, have to be sterile. As a definition, sterility means absence of all living organisms, including bacteria, yeast, molds and viruses. Even one living organism makes the whole product unsterile and may cause serious infection. (Ratner et al. 2004, pp. 754)

In case of chemically labile polymer device, the most preferred way to end up sterile product would be aseptic preparation in a clean room under good manufacturing practice. This way the unchanged properties of the polymer matrix and drug could be guaranteed, but it has naturally higher risk of microbiological contamination. (Martínez-Sancho et al. 2004) The most efficient method to sterilize biomedical devices is steam sterilization. However, this process cannot be used for biodegradable DDDs, because heating and penetrating steam causes deformation of the matrix, and hydrolysis of the matrix polymer. PLGA materials are also very sensitive to dry heat (Carrascosa et al. 2003). Gases, for example ethylene oxide, may leave mutagenic residuals to the device and that is why they are not recommendable to used to sterilize implantable devices (Rothen-Weinhold et al. 1997). At least efficient and long-term ventilation has to be taken care of. Nanoparticles and really small microparticles are successfully sterilized by sterile filtering with 0.22 µm filter, but this method cannot be used to microparticles with size larger or near the pore size of the filter. (Vetten et al. 2014) Hence, gamma irradiation is considered to be suitable sterilizing method for polymeric drug delivery matrices. There are also new, potential methods for sterilize microparticles, such as low-temperature plasma sterilization, but they have to be investigated further (Mohr et al. 1999; Shintani et al. 2010)

Irradiation sterilization comprises gamma irradiation, electron beam, x-rays and UV-light. Electron beam is used to sterilize the particles, but gamma irradiation is more common and they both affect the particles in the same way. UV-light is mainly suitable to sterilize surfaces. (Vetten et al. 2014) Gamma irradiation is the most common of the irradiation sterilization methods and it has many advantages. They include high penetrating power, low chemical reactivity, low residues, small temperature rise and fewer variables to control. (Martínez-Sancho et al. 2004) Possible disadvantages of

gamma irradiation, as ionizing radiation, are harmful effects on the PLGA microparticles. These include radiolytic degradation of matrix polymer and/or incorporated drug dose dependently crosslinking and scission of the polymer chain. (Nuutinen et al. 2002) Chain scission leads to loss of molecular weight, and so on accelerates degradation rate of the PLGA. Acidic degradation products of the PLGA, in turn, change the microenvironment inside the device and catalyses degradation of the polymer matrix even further. Therefore, it may also affect the drug release profiles. Degradation products may crystallize inside the devise consequently either diffusing out or altering the porosity by blocking the pores of the device. Also the stability of the drug may be affected, especially in case of thermal sensitive drugs like peptides. (Rothen-Weinhold et al. 1997; Carrascosa et al. 2003; Martínez-Sancho et al. 2004) Of course, the final effect of the radiation is dependent of the size and the porosity of the device (Martínez-Sancho et al. 2004) as well as nature of the matrix polymer and its composition (Montanari et al. 1998). The minimum adequate dose for sterilizing pharmaceutical product is regarded to be 25 kGy (European Guideline 1992).

Effects of gamma irradiation as sterilization method of PLGA microparticles have been studied and results differ from each other depending on the incorporated drug, the size of the microparticles and irradiation conditions (Montanari et al. 1998; Çalış et al. 2002; Martínez-Sancho et al. 2004). Boix et al. (2003) report improved results of irradiation when sterilization is carried out in decreased temperature. On the other hand irradiation is said to cause crosslinking in absence of oxygen and lowered temperatures (Ražem & Katušin-Ražem 2008). Lactide-glycolide ratio of PLGA has been shown to affect also the stability during gamma irradiation. The higher the lactide portion, the more stable are microparticles (Williams et al. 2006).

3 HYDROGELS IN DRUG DELIVERY

3.1 General about hydrogels

Hydrogels are three-dimensional, hydrophilic and crosslinked polymer networks which are able to absorb large amounts of water or body fluids up to 90 % of their dry weight. They are soft and elastic, and are usually used above their glass transition temperature. They can be prepared theoretically from any hydrophilic polymer that comprises wide range of bulk polymers and also chemically modified polymer derivatives (Hoare & Kohane 2008). Capability to absorb water arises from hydrophilic side groups (hydroxyl, carboxyl, amide and sulfonic groups) attached to the polymer backbone or hydrophilic domains in backbone. Crosslinks between polymer chains make them insoluble in water. In fact, crosslinks have to be present in the hydrogel structure so that hydrophilic polymer chains do not dissolve to the aqueous environment. Water can be removed from the gel without disturbing the gel structure, with freeze dryer or organic solvent extraction. Then, hydrogel becomes highly porous, and is referred as aerogel (Künzler 2002). They have gained interest from researchers in the field of tissue engineering and drug delivery because of their natural, structural similarity with living tissue (compositionally and mechanically), especially extracellular matrix, and ability to promote cell survival and proliferation. (Peppas et al. 2000b; Künzler 2002; Borzacchiello & Ambrosio 2009; Slaughter et al. 2009; Okay 2010)

3.2 Classification and structure of hydrogels

Hydrogels can be classified in several categories depending on the source of material, preparation method, ionic charge, physical structure features, crosslinking method and function. In the simplest way, hydrogels can be classified according to the origin of the polymers: natural or synthetic. (Kishida & Ikada 2001) Hydrogels may be composed also of both natural and synthetic monomers (Hoffman 2002). This kind of hydrogel is called hybrid hydrogel. Synthetic materials for hydrogels include for example from poly (ethylene glycol), poly (ethylene oxide), polyvinyl alcohol, polyacrylates and polypeptides, whereas materials from natural sources include agarose, alginate, chitosan, hyaluronic acid, fibrin and collagen. (Borzacchiello & Ambrosio 2009; Slaughter et al. 2009) Hydrogels prepared from natural polymers possess natural biocompatibility, biodegradability and bioactive site in their structure, which are not easy to mimic in laboratory. In addition, natural polymers are available from reusable sources such as plant, animals, humans, or are synthetized. (Coviello et al. 2007) However, they are not mechanically as strong as hydrogels made from synthetic

polymers, may contain pathogens and can induce immune response. Advantage of synthetic hydrogels is their well-known structures that enable modification in order to obtain tailored properties. (Lin & Metters 2006) Based on preparation method, they may be homopolymer hydrogels when composed of one type of monomer, copolymer hydrogels when composed of two types of monomers, multipolymer hydrogels when composed of more than three types of monomers or interpenetrating polymer networks when one type of polymer network is synthetized or crosslinked in the immediate presence of another swollen polymer network (Sperling 2004; Borzacchiello & Ambrosio 2009). In each case, at least one of the starting monomer types must be hydrophilic. Based on ionic charge of the side groups, hydrogels may be classified as neutral, cationic, anionic or ampholytic hydrogels. On the basis of physical structural features, hydrogels are amorphous, semicrystalline, hydrogen bonded hydrogels, supermolecular structures or hydrocolloidal aggregates. (Peppas et al. 2000b; Kishida & Ikada 2001; Ratner et al. 2004, pp. 100-101)

In addition they can be classified based on the nature of the crosslinks in the network structure, either chemical or physical. However, hydrogel network can also include both chemical and physical crosslinks (Coviello et al. 2007). Crosslinks are defined as connection points of more than one polymer chain. Points may be carbon atoms or small chemical bridges whose molecular weights are much smaller than polymer chains to be crosslinked (Ratner et al. 2004, pp. 101). Chemical crosslinks are covalent bonds and they are permanent, whereas physical crosslinks are reversible including secondary weak bonds like Van der Waals interactions, electrostatic interactions, hydrogen bonds or molecular entanglements. (Hoffman 2002; Borzacchiello & Ambrosio 2009)

Finally, one way to classify hydrogels is divide them into conventional hydrogels and stimuli responsive or in other words smart hydrogels. Conventional hydrogels are usually uncharged, hydrophilic and crosslinked networks that notably swell in water without dissolving but does not change swelling behaviour with response to changed temperature, pH, electric charge, light or other stimulus. Stimuli responsive hydrogels are synthetized in a way that they are able to sense and response to changes to external, usually environmental stimulus by changing the degree of swelling (Lin & Metters 2006). Ability of sensing stimuli arises from tailored chemical structure of the polymer network, for example functionality of the side chain groups, branches or crosslinks. These hydrogels may be charged or uncharged and exhibit significant change in swelling, and so on volume in response to change in temperature, pH, electric charge, magnetic field, light, ultrasound, chemical stimuli or biological compounds. (Peppas et al. 2006) This responsiveness is useful property in drug delivery applications, because drug delivery can be triggered by some external stimulus. Therefore, it converts drug administration from passive to active delivery. (Lin & Metters 2006)

The most used stimuli are pH and temperature. In brief, anionic hydrogels deprotonate and swell more when pH is higher than pK_a of the ionizable groups of the polymer, contrary to the cationic hydrogels which protonate and swell more when pH is

lower than pK_b of the ionizable groups. Swelling and de-swelling of the ionic hydrogels are also depending on the ion movement of the external solution. (Lin & Metters 2006) In case of temperature responsive hydrogels de-swelling of the hydrogel occurs when bulk temperature is higher than lower critical solution temperature. Then polymer chains lose the bound-water, which leads to the hydrophobic interactions between polymer chains and collapse of the gel structure. (Schild 1992) Recently, attempts to design dual-sensitive hydrogels have been made and results are promising. This kind of pH and temperature responsive gel has been done for example by copolymerizing temperature-sensitive monomer (*N*-isoproplylacrylamide) and pH-sensitive monomer (acrylic acid). (Hoffman et al. 2000)

Structure of hydrogels depends on many things, like starting monomers and macromers, synthesis and fabrication method, solvent conditions, degradation, and mechanical loading history. (Slaughter et al. 2009) Hydrogels, neither chemical nor physical, are homogeneous. In chemically crosslinked hydrogels there are always low water swelling and high crosslink density regions as dispersed clusters which within the high swelling and low crosslink density regions. Whereas in the physically crosslinked hydrogels molecular entanglement may form clusters, or domains formed by ionic or hydrophobic association may cause inhomogeneities. Basically, inhomogeneity of the hydrogel increases when crosslinking density increases (Okay 2010). Both, chemical and physical hydrogels have also other defects like free chain end and chain loops, which are introduced below. Nevertheless, the defect does not contribute to the elastic or mechanical properties of the hydrogel. (Hoffman 2002)

The ideal structure of hydrogels comprises of tetra-functional crosslinks formed by covalent bonds (Figure 6A). Structural analysis has shown that this ideal structure is achieved only rarely. In reality, polymer networks may comprise also multifunctional cross-links (Figure 6B), permanent of semi-permanent physical entanglements (Figure 6C). Different crosslinks may also be present in the same gel structure. Also real molecular flaws like unreacted crosslinks (free chain ends) (Figure 6D) or chain loops (Figure 6E) may occur, but they do not affect to the properties of mechanical strength of the hydrogel. (Ratner et al. 2004, pp. 101; Borzacchiello & Ambrosio 2009)

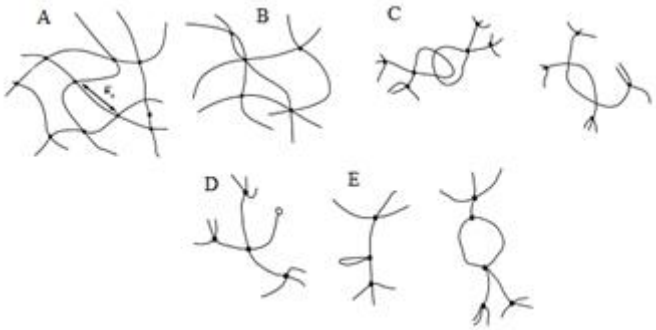

Figure 6: *Hydrogel networks. (A) Ideal hydrogel network. (B) Network including multifunctional cross-links. (C) Physical entanglements. (D) Unreacted polymer chain. (E) Chain loops.(Modified from the source Ratner et al. 2004, pp.101)*

There are several parameters which can be used to depict the structure of hydrogels, but the most important ones are the polymer volume fraction in the swollen state ($\upsilon_{2,s}$), the average molecular weight between crosslinks (M_c), and the correlation length or mesh size (ξ). (Borzacchiello & Ambrosio 2009) The polymer volume fraction in the swollen state, $\upsilon_{2,s}$, indicates the amount of liquid that can be absorbed in hydrogels. It is defined as the ratio of the polymer volume to the volume of the swollen gel as described in equation (1):

$$\upsilon_{2,s} = \frac{V_p}{V_{gel}} = Q^{-1}\,, \tag{1}$$

where V_p is the volume of the polymer, and V_{gel} is the total volume of the hydrogel. It is also the reciprocal of the volumetric swollen ratio, Q, which can be determined by equilibrium swelling experiments. (Borzacchiello & Ambrosio 2009)

The equilibrium swelling level in aqueous solutions of the crosslinked hydrogels may be described as a crosslinking density (Hoffman 2002). The parameter for that is the average molecular weight between crosslinks, M_c. It is a measure of the degree of crosslinking of the polymer and is defined with equation (2):

$$M_c = \frac{M_0}{2X}\,, \tag{2}$$

where M_0 is molecular weight of repeating polymer units, and X is the degree of crosslinking. It may be either chemical or physical in nature. In addition, due to random nature of polymerization process, only average values can be obtained. (Borzacchiello & Ambrosio 2009)

The correlation length, or mesh size, ξ, indicates distance between successive crosslinks, junctions or tie points. It can be defined with equation (3):

$$\xi = a(r_0^2)^{1/2}\,, \tag{3}$$

where a is elongation ratio of the polymer chains, and $(r_0^2)^{1/2}$ is the root-mean-square, unperturbed, end-to-end distance of the polymer chains between two crosslinks. It is the measure for the pore size, in other words, it determines the free space between the polymer chains. (Borzacchiello & Ambrosio 2009)

Depending on the size of the pores, hydrogels can be classified as macroporous, microporous or nonporous hydrogels. The mesh size is important parameter because it determines many physical properties of the hydrogels, such as mechanical strength, degradability and diffusivity of the incorporated molecules (Amsden 1998; Mason et al. 2001). These parameters are derived from two theories that are used to describe the structure of the hydrogels: equilibrium swelling theory and rubber elasticity theory. It must be noted that in biomedical hydrogel applications the experimental measurements

have to be performed in realistic condition, such as phosphate saline solution or culture medium including salts, ions, and nutrients in order to get reliable results (Lin & Metters 2006; Peppas et al. 2006; Borzacchiello & Ambrosio 2009; Slaughter et al. 2009)

3.3 Hydrogel formation

There are many methods for preparing hydrogels. All of them are based on the crosslinking, either chemically or physically. In chemically crosslinked hydrogel, covalent bonds are present between polymer chains. In physically crosslinked hydrogels, crosslinking is obtained through physical interactions. As mentioned above, crosslinks are obligatory part of the hydrogel structure in order to prevent the dissolving of the hydrophilic polymer chains. Initial crosslinked structures of the hydrogel can be prepared in presence of water or in biological fluids. (Peppas et al. 2000a; Hennink & van Nostrum 2012)

3.3.1 Chemical crosslinking

Chemical crosslinking is performed by different methods, including free radical polymerization, chemical reaction of complementary groups, high-energy irradiation and usage of enzymes. Chemical crosslinking means formation of covalent bonds between functional groups of polymer chains. (Hennink & van Nostrum 2012) The reactive groups may be added to pre-polymers as small molecules or by direct conjugation (Hoare & Kohane 2008).

Free radical polymerization crosslinking is obtained using low molecular weight monomers in the presence of bifunctional crosslinking agent. It is commonly used to prepare synthetic hydrogels from the class of vinyl-monomers, acrylates and amides. It can also be used to water-soluble, naturally occurring polymers as long as their backbones or chain ends have been derivatized with radical polymerizable groups. Kim et al. (1999), for example, prepared polymerizable dextran by reaction with maleic anhydride and changed this dextran derivative into a hydrogel by polymerizing formed vinyl-groups. In addition, interpenetrating network hydrogel is preferably prepared using free radical polymerization. With this technique many different water-soluble polymer, synthetic, semi-synthetic and natural, have been used to preparation of the hydrogels. (Künzler 2002; Hennink & van Nostrum 2012) These polymers include for example dextran, albumin, (hydroxyethyl) starch, poly-aspartamide, polyvinyl alcohol and hyaluronic acid (Hennink & van Nostrum 2012). Typical free radical polymerization has four steps: initiation, propagation, chain transfer and termination. These steps lead to the formation of crosslinked hydrogel network. Polymerization may be carried out in solution, neat (bulk polymerization), emulsion or suspension. (Künzler 2002)

Crosslinking via chemical reaction can be performed in case of hydrophilic polymers. Hydrophilic polymers have functional groups (for example hydroxyl,

carboxyl and amine groups) which can be used to create covalent bonds between polymer chains. Two methods are possible, either by the reaction of functional groups with complementary reactivity (polymer-polymer crosslinking), or by using bi – or higher functional crosslinking agent (small molecule crosslinking) in the dilute solution of the polymer. In case of polymer-polymer crosslinking polymers are pre-functionalized with reactive groups and use of toxic crosslinking agents is avoided, but these pre-gel polymers may be toxic themselves. Polymer-polymer crosslinking reactions include formation of an asymmetric Schiff base, hydrazine and Michael reaction to form secondary amine or a sulphide. (Hoare & Kohane 2008) In the latter case, possible chemical reactions include addition reactions, condensation reactions and crosslinking with aldehydes. Reactions are usually performed in a solution. (Hennink & van Nostrum 2012) Sometimes incorporating drug may act as the crosslinker (Nishi & Jayakrishnan 2007). Crosslinking via chemical reactions is suitable method for both, synthetic and natural polymers. In fact, most of the natural polymers can be crosslinked in this way. (Künzler 2002)

Another crosslinking method is using high-energy irradiation such as electron beam or gamma radiation. They are both ionizing radiation, which means that they have enough energy to ionize molecules. Especially, in aqueous polymer solutions, ionizing radiation causes the formation of radicals by C-H scissions to the polymer chains. In addition it causes formation of hydroxyl radicals from water molecules, which may attack the polymer chains leading also to the formation of the radicals. Combining reactive site of the macroradicals leads to the formation of covalent bond and therefore crosslinked structure. Irradiation crosslinking is used to unsaturated, hydrophilic polymers from both, synthetic and natural origin. Irradiation may be performed for pure polymers, monomers or solution of polymers in bulk, solution or emulsion (Rosiak & Yoshii 1999). However, it has to be noted that all the polymers cannot stand ionizing radiation without degrading. In polymer solutions, simultaneous polymer crosslinking and degradation may occur. That is why optimum irradiation conditions for each polymer solution have to be determined. As an advantage, irradiation crosslinking can be performed in mild conditions (room temperature and physiological pH) and absent of toxic crosslinking agents. Possible additional bioactive molecules and cells may be added after irradiation, since final construction of the hydrogel structure occurs without radiation. (Künzler 2002; Hennink & van Nostrum 2012)

Photocrosslinking method to form hydrogels belongs to the same category with high-energy irradiation. In photocrosslinking, covalent bonds between polymer chains are achieved through brief exposure to the ultraviolet or visible light. Photoinitiators create free radicals, which starts polymerization, which leads to the formation of the hydrogel structure. Advantage of photocrosslinking is that it can be performed in physiological conditions *in situ*. Choice of proper photoinitiator and short-term exposure to the low intensity light makes photocrosslinking relatively mild crosslinking method when cells or bioactive molecules are involved. (Jeon et al. 2009) In addition, ability to form hydrogel *in situ* makes complete filling of even irregular shape defects

possible. Also mechanical properties are controllable as well as promoting characteristics of matrix accumulation and cell viability. For example, hyaluronic acid and poly (ethylene glycol) are used as materials for photocrosslinked hydrogels. (Nettles et al. 2004)

Hydrogel properties, like swelling behaviour and permeability, can be modified by changing the amount of crosslinker or radiation dose. Chemical crosslinking gives opportunities for other kind of modifications as well. Stimuli responsive hydrogels may be obtained by adding other polymer molecules to the structure. (Hennink & van Nostrum 2012) Temperature-sensitive hydrogels have been obtained by addition of N-isopropylacrylamide (Cicek & Tuncel 1998), and pH-sensitive hydrogels by addition of methacrylic acid (Bettini et al. 1995). Other examples of additional molecules and chemical structures are immobilized enzymes and genetically engineered proteins, functional groups for enhanced cellular adhesion, and degradable linkers or labile bonds to obtain biodegradable hydrogels (Peppas et al. 2006; Park et al. 2011). Usually, crosslinking agents are very toxic and they may also affect to the bioactive agents, which may be added to the hydrogels. So, careful removal of residual crosslinkers has to be performed. (Hennink & van Nostrum 2012)

3.3.2 Physical crosslinking

Physical crosslinking means the formation of crosslinked hydrogel structure through molecular entanglements or secondary forces, such as ionic bonds, hydrogen bonds and hydrophobic interactions. Physical crosslinks are not permanent and crosslinked structure of the physical hydrogels may be disrupted by changing physiological conditions or applying stress. (Rosiak & Yoshii 1999)

Crosslinking based on the ionic interactions is also called polyelectrolyte complexation. The bonds are forming between pairs of oppositely charged sites in the polymer backbones or between polymer chain and small molecule (Hoare & Kohane 2008). Polyelectrolytes are polymers that possess the net positive or negative charge at neutral pH. Polyelectrolyte polymers may be from synthetic or natural origin. For example nucleic acids, hyaluronic acid, chitosan and polyacrylic acid are polyelectrolytes. (Lankalapalli & Kolapalli 2009) The polyelectrolyte complexes are divided into subclasses depending on the electrochemistry of the components: strong acid-strong base, strong acid-weak base, weak acid-strong base and weak acid-weak base. (Künzler 2002) Formation of polyelectolyte complexes occurs between polycations and polyanions or polycation/polyanion and added ions, for example calcium ions (Xing et al. 2003). As an advantage, biodegradability may occur in this kind of hydrogels as a competitive binding of ionic species of extracellular fluid. Crosslinking may be affected also with change in pH which ionize or protonate the ionic functional groups of the polyelectrolytes. (Hoare & Kohane 2008)

Formation of the hydrogel can also be based on hydrogen bonds. Hydrogen bond is formed between hydrogen atom, that is bonded with strongly electronegative atom and another electronegative atom with unshared electron pair. Hydrogen bonded complexes

can be found in many biological systems and in case of polymers result can be crosslinked hydrogel. (Künzler 2002) For example poly(acrylic acid) and poly(ethylene glycol) form hydrogel that is held together by hydrogen bonds (Eagland et al. 1994) Rheological synergism of polymer blend of two or more natural polymers is explained by hydrogen bonds between the polymer chains and their compatible geometries. It means that viscoelastic properties of the blends resemble more gel than constituent polymers individually. (Hoare & Kohane 2008)

Physical hydrogels can be also formed with hydrophobic interactions. Hydrophobic bonds are formed, for example, in aqueous polymer solutions when hydrophobic domains form aggregates to minimize hydrophobic surface area. The aggregates are dispersed between hydrophilic, water absorbing regions. This kind of crosslinking is called reversible thermal gelation or sol-gel chemistry (Hoare & Kohane 2008). Polymers that usually form hydrogel through hydrophobic bonds are multiblock copolymers or graft copolymers. These kinds of hydrogels have typically low mechanical strength. Nevertheless, they have also advantages compared to other hydrogels. For example these systems have only low economical costs and these polymer blends are usually soluble to organic solvent and flow at elevated temperatures which enable for example injection molding as processing method. (Künzler 2002; Hennink & van Nostrum 2012)

Finally, crystallization can be exploited in the preparation of the hydrogels. For example, aqueous solution of polyvinyl alcohol (PVA) form a gel when stored at room temperature. When PVA solution undergoes freeze-thawing process, the gel formation is enhanced and strong and elastic hydrogel is obtained. Gel formation is described with the formation of PVA crystallites. These crystallites function as crosslinking points in the structure. (Hassan & Peppas 2000; Hennink & van Nostrum 2012) Also less used physical crosslinking methods like stereocomplexation and use of supramolecular chemistry exist. (Hoare & Kohane 2008)

Physical hydrogels have gained interest during these days. The reason is avoiding of the crosslinking agent which are usually very toxic. These chemicals may also affect to the bioactive agents and cells physically entrapped in the gel. So, residual agents have to be removed or extracted before use in different applications. (Hennink & van Nostrum 2012) However, physical crosslinking methods are usually difficult to control, which affect to the network structure and properties (Tan & Marra 2010).

3.4 Drug release applications

Hydrogels have been intensively investigated as drug carriers, devices for controlled drug release, mucoadhesive drug releasing devices and targeted drug releasing devices. Hydrogels may be prepared to obtain many different physical forms, for example solid molded forms, pressed powder matrices, microparticles, coatings, membranes or sheets, encapsulated solids or liquids (Hoffman 2002). For drug delivery applications, both synthetic and natural polymers may be used. Precursor polymers may be used as such or

they may be chemically modified to achieve desired properties. For example, mechanical properties of natural polymers may be improved by crosslinking of the polymer, and incorporation of hydrophobic drug into the hydrophilic hydrogel may be improved by adding hydrophobic sites to the polymer or using cyclodextrins. Biodegradability may be inherent property of the polymers, or it may be designed to the hydrogel via hydrolytic, enzymatic or environmental way. (Hoare & Kohane 2008) These include for example copolymerization of hydrolytically (Metters et al. 2000) or enzymatically (Rice et al. 2006) degradable polymers with non-degradable polymers to form block copolymers and so on biodegradable hydrogels.

Biocompatibility of hydrogels is good due to their high water content. Hydrophilic surface has low adherence of proteins and cells because of low interfacial free energy with body fluids. Additionally, their soft and elastic nature causes only minimal irritation to the surrounding tissue. (Künzler 2002; Hennink & van Nostrum 2012)

The drug release kinetics of the hydrogel may be altered by changing concentration of the polymers in hydrogel preparation and crosslinking density. Polymer concentrations affects directly to the drug release and degradation of the hydrogel. Crosslinking density can be modified by changing the length of polymer chains, polymer composition (Peppas et al. 2000b), and amount of small molecule crosslinker or density of the reactive groups in the polymer. The higher crosslinking density, the smaller the mesh size and slower the drug release. (Hoare & Kohane 2008) Stimuli responsive hydrogels have gained interest as drug delivery applications, because with their volume-changing property, swelling and therefore drug release can be triggered with external stimulus. So, well-designed hydrogel offer versatile material for controlled drug release. Important part of the development is designing of the network structure as well as choosing the right monomers or macromers with well-known chemistry. These yield the hydrogel with desired physicochemical properties and reproducible drug-release profile. Mathematical modelling has been used also to help product development by limiting experiments to understand release mechanism in each system. (Lin & Metters 2006)

Regardless of many advantages of hydrogels, they have also disadvantages when drug release applications are concerned. Typically, hydrogels have relatively small tensile strength, which limits their use in load bearing sites in the body. Low load bearing capacity is not usually problem in drug delivery applications, but it may lead to the early dissolution or flow away of the hydrogel from the administration site. Hydrogel may be designed to protect the drug when it has to maintain its structure until drug is completely released. (Peppas et al. 2000b) Furthermore, drug loading into the hydrogel may be limited and distribution of the drug can be heterogeneous, especially in case of hydrophobic drugs. Drug release of the small molecular drugs may be very fast due to high water content and large pore sizes of the most hydrogels. Hydrogels are also prone to suffer initial drug release. (Hoare & Kohane 2008) Burst release is especially problematic in the case of slow *in situ* gelling hydrogels as drug may be lost before complete gelation (Overstreet et al. 2012).

As mentioned earlier, hydrogels can absorb large amounts of water and that makes drug release from hydrogels totally different compared to hydrophobic matrices. The primary mechanism of the drug release from the hydrogel is diffusion-controlled drug release. Other release mechanisms are swelling and chemically controlled drug release mechanisms. (Lin & Metters 2006) Diffusion occurs through pores, which are spaces between polymer chains. The most important diffusion rate determining factors are crosslinking density and water content. The denser crosslinking, the more hydrogel is able to swell, and the larger are pores between polymer chains. Of course, size of the drug molecule also affects to the release through the pores. Drug release rate from the hydrogel can be controlled also with controlling water content by designing hydrogel of specific hydrophilicity or hydrophobicity. When diffusion of the drug is faster than swelling of the hydrogel, release is regarded as swelling controlled release. Chemically-controlled drug release occurs when drug release results from chemical reactions in the hydrogel matrix. These chemical reactions are usually enzymatic or hydrolytic cleavages of polymer chains or reactions between polymer chain and incorporated drug. (Lin & Metters 2006) Chemically controlled release can be divided into two categories: degradation of the pendant groups through which drug is attached to the polymer, or bulk or surface erosion of the polymer backbone. (Peppas et al. 2000b)

DDDs based on hydrogels can be used as hydrated or dry form. In hydrated form water is still present, but it is removed to obtain dry form of the gel. Incorporated drug is possible to add to the gel in polymerization stage, but it is not commonly used in pharmaceutical applications since drug may be released already during the extraction phase where unpolymerized polymer monomers are removed. Another technique to incorporate drug to the hydrogel is using diffusion methods to preformed devices such as films or tablets. This is performed by soaking device in the drug solution until equilibrium drug concentration is reached. (Künzler 2002)

Hydrogels have been used in peroral, rectal, ocular, epidermal and subcutaneous drug releasing applications. Their excellent properties can overcome problems relating to the conventional ways of drug administration such as tablets and injection. (Peppas et al. 2000a) Hydrogels have advantages compared to hydrophobic polymeric devices, for example in delivering biomacromolecules, like proteins and DNA, since hydrophobic interactions may denature their structure. In addition, processing of the hydrogels requires only rarely chemical solvents and harder processing conditions. (Lin & Metters 2006)

Drug delivery through peroral route, including mouth, stomach, small intestine and colon, is the most common method for pharmaceutical applications, also based on hydrogels. Due to controllable swelling and bioadhesive properties hydrogels are suitable devices for also conditions where rich salivary secretion is present. (Peppas et al. 2000b) Stimuli responsive hydrogels are very useful in controlled drug delivery through oral route. Especially pH responsive hydrogels provide protection to the drugs that are sensitive to the acidic environment. (Lin & Metters 2006)

Many drugs can be administrated through rectal route which advantage is drain straight to the systemic circulation. Bioavailability of the drug may be increased using rectal route if the drug usually suffers from heavy first-pass metabolism. Problem with conventional suppositories is that they melt or soften at body temperature and that is why drugs may not be able to be retained sufficient time in certain part of the rectum or they may migrate upwards to the colon. Hydrogels may be designed to exhibit bioadhesive properties after their rectal administration to ensure sufficient immobility in the certain part of the rectum. (Peppas et al. 2000b)

There are many challenges in ocular drug delivery due to its protective mechanisms including effective tear drainage, blinking and low permeability of the cornea. For this reason drug containing eye drops are usually rapidly eliminated from the eye which leads to the frequent dosing in order to achieve sufficient ophthalmic bioavailability of the drug. Suspensions and ointments as solids and semi-solids may cause uncomfortable feeling even though they may be retained in the eye. Hydrogels' may resist the ocular drainage as an elastic material, and they may also give more pleasant feeling in the eye. In situ gelling hydrogels gives an excellent opportunity in ocular drug delivery, as they are easily administered as a liquid but are retained as a gel after dosing. (Peppas et al. 2000b)

Transdermal route of drug delivery is widening its field from treatment of skin diseases to also systemic drug delivery. Advantage of this route is ability to achieve long term drug release at constant rate, and release is easily interrupted or stopped by removing the device if necessary. In addition, drugs can avoid hepatic first-pass metabolism. Hydrogel based devices offer possibly more comfortable feeling to the skin, due to their elasticity and high water content. (Peppas et al. 2000b)

Subcutaneous drug delivery includes implantable devices, which plays the main role in tissue engineering and regenerative medicine in general. Probably the most important property of implantable DDD is biocompatibility, since foreign materials cause detrimental responses (inflammation and immunogenicity) in the body. Hydrogels offer great opportunities also in this field because of their properties. Hydrogels are considered as biocompatible materials due to their high water content. Their soft and elastic nature causes only minimal mechanical tissue irritation, low interfacial tension prevents protein absorption and cell adhesion, good compatibility with different types of drugs and ability to tailor release kinetics of incorporated drugs. (Peppas et al. 2000b)

In situ gelling hydrogels based on polymers are very attractive systems in sustained and local drug delivery since they can be administrated by injection as solutions into the body where they form locally stable gel and ensures localized drug delivery for a site-specific action. In addition, injectability of the hydrogel provides faster healing time, reduced scarring, decreased risk of infection and there is no need for surgical operation. (Hou et al. 2004) This way hydrogel conform the injection site taking the local anatomy into account. Phase transition leading to gel formation can be also triggered by stimulation from environment, for example temperature, pH, light, biomolecules,

electric current, pressure or ionic strength. (Wu et al. 2006; Kim et al. 2003; Qiu & Park 2001)

Designing of the composite hydrogel delivery system has been under investigation in order to obtain co-delivery system for two or more different drugs (Lin & Metters 2006), to obtain even more sustained delivery system with additional diffusion barrier, and to moderate or even eliminate the burst release (Hoare & Kohane 2008). Two types of composite hydrogel system have been developed, multi-layer and multi-phase systems. For example, during angiogenesis and bone remodelling, simultaneous release of several proteins is advantageous as well as sustained release of anti-inflammatory drug from the hydrogel coated, implanted sensor. (Lin & Metters 2006)

Multi-layer systems are formed from multiple layers. Different drug can be incorporated in each layer or, incorporate same drug into hydrogel layer with different drug release properties (crosslinking densities). (Lin & Metters 2006)

Multi-phase systems consist of two or more different polymer phases. For example preformed or micro/nanoparticle embedded into the hydrogel as in this study. Structure of this kind of composite hydrogel is illustrated in Figure 7. System may be multi-drug system with different drug in each phase or the system may contain only one drug which release is delayed with two diffusional layers, polymer matrix of the microparticles and surrounding hydrogel. Composite hydrogel systems have also been used to overcome pharmacological limitations of the hydrogels, increase biocompatibility of the microparticles (Hoare & Kohane 2008), and immobilize the microparticles in a targeted place therefore avoiding their distribution (Lampe et al. 2011). It may be also solution for delivering hydrophobic drug that is practically insoluble in hydrophilic hydrogel phase as well as aqueous environment outside the hydrogel (Hoare & Kohane 2008).

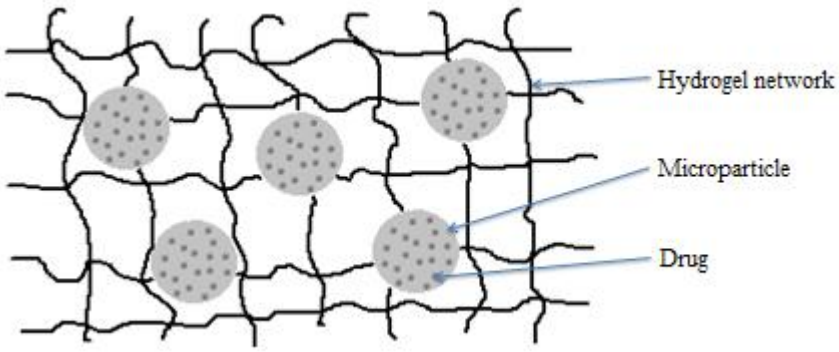

Figure 7: *Structure of the composite hydrogel containing drug releasing microparticles embedded into the hydrogel.*

Multi-phase systems combining micro/nanoparticles and hydrogels have been successfully done using conventional drugs, protein-based drugs and growth factors, and different polymers (Kim & Martin 2006; Hou et al. 2008; Lampe et al. 2011; Gao et al. 2012; Shin et al. 2013).

3.5 Hydrogels based on hyaluronic acid and polyvinyl alcohol

Hyaluronic acid (HA) is the simplest, high molecular weight glycosaminoglycan and it is found in nature in almost every mammalian tissue and fluid as a salt form hyaluronate. It is mainly found in connective tissues and vitreous as lubricant. It has especially role in wound healing and in synovial joints, but plays also role as signalling molecule in cell motility, cell differentiation and cancer metastasis (Prestwich et al. 1998). In nature, molecular weight of HA varies from 1000 Da to 8 MDa (Laurent et al. 1996; Burdick & Prestwich 2011). In industry, it is produced from *streptococci* through fermentation. The chemical structure of hyaluronic acid is shown in Figure 8. It is linear polysaccharide composed of repeating β-1,4-linked D-glucuronic acid and β-1,3-linked N-acetyl-D-glucosamine disaccharide units. (Coviello et al. 2007; Borzacchiello & Ambrosio 2009; Slaughter et al. 2009; Dicker et al. 2014)

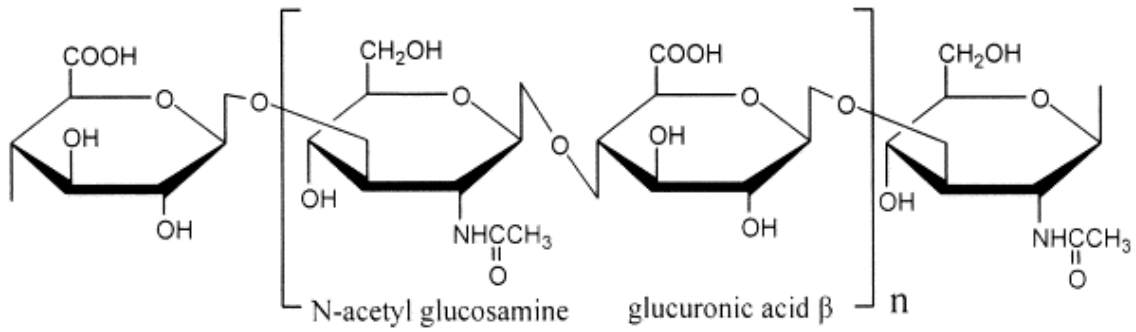

Figure 8: *Chemical structure of hyaluronic acid (Kim et al. 2003).*

In nature, HA is enzymatically degraded by hyaluronidase into smaller oligosaccharides which are further degraded by D-glucuronidase and N-acetyl-hexosaminidase. HA can also degrade by reactive oxygen intermediates. Unmodified HA undergoes fast degradation which leads to its fast removal from the site of administration. In addition, unmodified HA has poor mechanical properties (Segura et al. 2005) and it is incapable to form hydrogels. Therefore, chemical modifications are necessary for both, elongate residence time in the body and enable the formation of the hydrogels. These include chemical attachment of functional groups and crosslinking of HA. HA hydrogels are formed by chemical crosslinking by means of multiple chemical modifications. These modifications involve the carboxylic acid groups, primary and secondary alcohol groups and N-acetyl group of its backbone. (Segura et al. 2005; Prestwich & Kuo 2008) The most commonly carboxylates are modified by carbodiimide-mediated reactions, esterification and amidation: hydroxyls groups by etherification, divinylsulfone crosslinking, esterification and bis-epoxide crosslinking (Prestwich & Kuo 2008). The crosslinking changes the solute transport characteristics and therefore slows the drug release from the hydrogel. In general, preparation of HA

hydrogels can be divided into three categories: direct crosslinking of HA, crosslinking of HA derivatives and crosslinking between two different kinds of HA derivatives (Oh et al. 2010). In case of *in situ* gelling hydrogels, chemical crosslinking of HA has to be performed first and gelling occurs when polymer solutions are mixed. (Peppas et al. 2006; Coviello et al. 2007; Borzacchiello & Ambrosio 2009; Slaughter et al. 2009)

HA as well as other polysaccharides have typically several functional groups or other reaction sites. They can be used to chemical modification of the molecule to give material new biological and physico-chemical properties, but still remaining its natural biocompatibility, biodegradability and lack of immunogenicity. Polysaccharide based hydrogels have also advantage of ability to respond to the changes in surrounding environment (temperature, pH and ionic strength) by modifying the swollen state, and so on the amount of water inside the gel and amount of dissolved and released drug. (Borzacchiello & Ambrosio 2009)

HA is a good material for biomedical applications due to its high viscoelasticity, space filling properties and ability to form hydrated, expanded matrices. In addition it lacks immunogenicity completely, inhibits platelet adhesion and aggregation and stimulates angiogenesis. It has been successfully used in ophthalmic surgery, in the treatment of osteoarthritis, augmentation of soft tissue, applications for wound healing and encapsulation of cells and DDD. (Slaughter et al. 2009) It has been investigated as targeted drug delivery material due to its ability to bind cell surface receptors (Brown 2008 The development of hyaluronan as a drug transporter).

Polyvinyl alcohol (PVA) is synthetic, hydrophilic polymer that is very promising material for biomedical applications due to its nontoxicity, hydrophilicity and good mucoadhesive properties. (Ratner et al. 2004, pp. 105) Hydrophilicity and therefore water-solubility arises from its hydroxyl side group. PVA is easily modified via this hydroxyl group into multifunctional macromer. (Drury & Mooney 2003) The most common modifications are esterification and etherification. (Ossipov & Hilborn 2006) PVA hydrogel can be prepared by physical crosslinking via freeze-thawning method or chemical crosslinking. (Drury & Mooney 2003)

In this thesis, HA based hydrazine crosslinked hydrogels are prepared from aldehyde-modified HA (Ossipov et al. 2008) and hydrazide-modified PVA (Ossipov et al. 2007). Crosslinking method is covalent crosslinking, using pseudo click chemistry, in which small units are joined together in fast chemical reaction (Ossipov & Hilborn 2006). Chemical modification of the HA and PVA polymers have to be performed in order to achieve crosslinking between polymer chains. Multifunctional crosslinker used with HA based hydrogels enable lower degree of functionalization and faster gelation time than for example poly (ethylene glycol). (Ossipov et al. 2008)

EXPERIMENTAL PART

4 MATERIALS AND METHODS

4.1 Materials

Microparticles were prepared using biodegradable poly (lactide-*co*-glycolide) (PLGA) granules (Purasorb PDLG 7507) with lactide-glycolide ratio of 75/25. They were donated by Purac Biomaterials (The Netherlands). They have inherent viscosity of 0.7 dl/g, maximum residual solvent of 0.2 %, residual monomer of 2 % and water content of 0.5 %. Emulsifying agent PVA with molecular weight of 31-50 kDa, 98-99% hydrolysed and sucrose was purchased from Sigma-Adrich (USA). Dexamethasone (DEX) is purchased from Orion (Finland). Muraglitazar is from Immunopharmacy Research Group, University of Tampere School of Medicine. Originally it is synthetized in the laboratory of Dr. Paula H. Aulaskari (University of Eastern Finland, Joensuu, Finland).

Both polymers for hydrogels, hyaluronic acid sodium salt from streptococcus equi (molecular weight 1.5- 1.8 MDa) and poly(vinyl alcohol) (PVA) Moviol® (molecular weight 27 kDa, 98.0-98.8 % hydrolysed) was purchased from Sigma-Aldrich (USA). Hyaluronic acid (HA) was aldehyde-modified (20 mg/mL) after method of Ossipov et al. (2008). PVA was hydrazide-modified (10 mg/mL) after method of Ossipov et al. (2007).

4.2 Dexamethasone loaded poly(lactic-co-glycolic) acid – polyvinyl alcohol microparticles

4.2.1 Preparation of dexamethasone loaded microparticles

Biodegradable, drug loaded PLGA microparticles were prepared with two-step method, which is combination of oil-in-water (o/w) solvent evaporation method and spray drying. Spray drying method was chosen because it is simple and relatively fast method to produce stabilized and dry microparticles. In addition, it can be easily converted into production of larger batches of microparticles. All different kinds of microparticle samples including and excluding dexamethasone prepared in this study are listed in Table 3. In addition, microparticles including muraglitazar were prepared.

Table 3: *Dexamethasone loaded microparticle samples.*

Samples including DEX	***Blank samples***
PLGA-PVA_DEX	PLGA-PVA_BLANK
PLGA-PVA_DEX_sucr0.25%	PLGA-PVA_BLANK_sucr0.5%
PLGA-PVA_DEX_sucr0.5%	
PLGA-PVA_DEX_sucr1%	

First, an aqueous solution including stabilizing emulsifier PVA was prepared. In this study, we used two separate PVA solutions with percentages of 1 % and 0.1 %. PVA polymer was dissolved into distilled water at 70°C with continuous stirring with magnetic stirrer until polymer was fully dissolved and then cooled down to room temperature (RT). Solutions were filtered with 4-12 µm Macherey-Nagel MN 615 filter papers (Germany) to eliminate impurities. Amounts of PVA and volumes of solutions depend on the size of the batch prepared. Ratio of 1% and 0.1 % PVA solutions was 1:5. For example 1000 mg of PVA was dissolved into 100 mL of de-ionized water to prepare 1 % PVA solution, and 500 mg of PVA into 500 mL of de-ionized water to prepare 0.1 % PVA solution. Then, organic solvent solution (oil phase), including polymer PLGA and DEX was prepared. Mixture of 9:1 dichloromethane (DCM) and ethanol (EtOH) was used as a solvent. Ratio of oil phase and aqueous phase (in total) was 1:24, and ratio of the drug and the polymer PLGA was 1:10 every time. According to the previous example, 250 mg of PLGA and 25 mg of the drug was dissolved in 25 mL DCM:EtOH (9:1).

During the emulsification, 1 % PVA solution was continuously stirred with magnetic stirrer 500-700 revolutions per minute (rpm). Simultaneously, oil phase was added drop by drop into 1 % PVA solution with small glass pipet to obtain as small drops as possible (Figure 9A). Then, solution was homogenized with 10 000 rpm for three minutes with IKA Ultra-Turrax T 25 Digital homogenator (Germany) (Figure 9B). Then the resultant first emulsion was added drop by drop into 0.1 % PVA solution, which was again stirred simultaneously (Figure 9C). Emulsification step was done as fast as possible to avoid premature evaporation of the solvent (Figure 9D).

In order to decrease attachment of the microparticles to each other and so on improve formation of homogeneous suspension when re-suspended into distilled water, sugar coating of the microparticles was tested. Ability to re-suspend is important when introducing microparticles into the hydrogel. Sucrose was chosen as coating material as it is used also in hydrogel preparation. Sucrose is added as 20 % solution. The final concentration of sugar is reported as concentration in the emulsion before spray drying. Three different concentrations were tested: 0.25 %, 0.5 % and 1 % (v/v). Sucrose solution was added to the emulsion immediately after emulsification. Emulsion including sucrose was stirred with magnetic stirrer for 10 minutes before drying process.

Difference between previous and new preparation method is solvent evaporation phase. Previously solvent was let to evaporate from the emulsion in room temperature continuously stirring. In the new method, emulsion is spray dried (Figure 9E) immediately after emulsification with Büchi Mini Spray Dryer B-290 (Switzerland) using 0.7 mm two-fluid nozzle and 1.5 mm nozzle cap. Emulsion was continuously strirred during the process. Air flow of the drying chamber is co-current type. The emulsion was fed into the spray dryer via peristaltic pump with flow rate of 2 mL/min. Air flow rate was set to 414 L/h and aspirator rate to maximum 100 % which refers to

gas flow of 40 m^3/h. Inlet temperature was set to the range of 65-75 °C in order to maintain constant outlet temperature of 38 °C during the drying process. Because passing the solvent evaporation completely, new preparation method is called combination of emulsion method and spray drying.

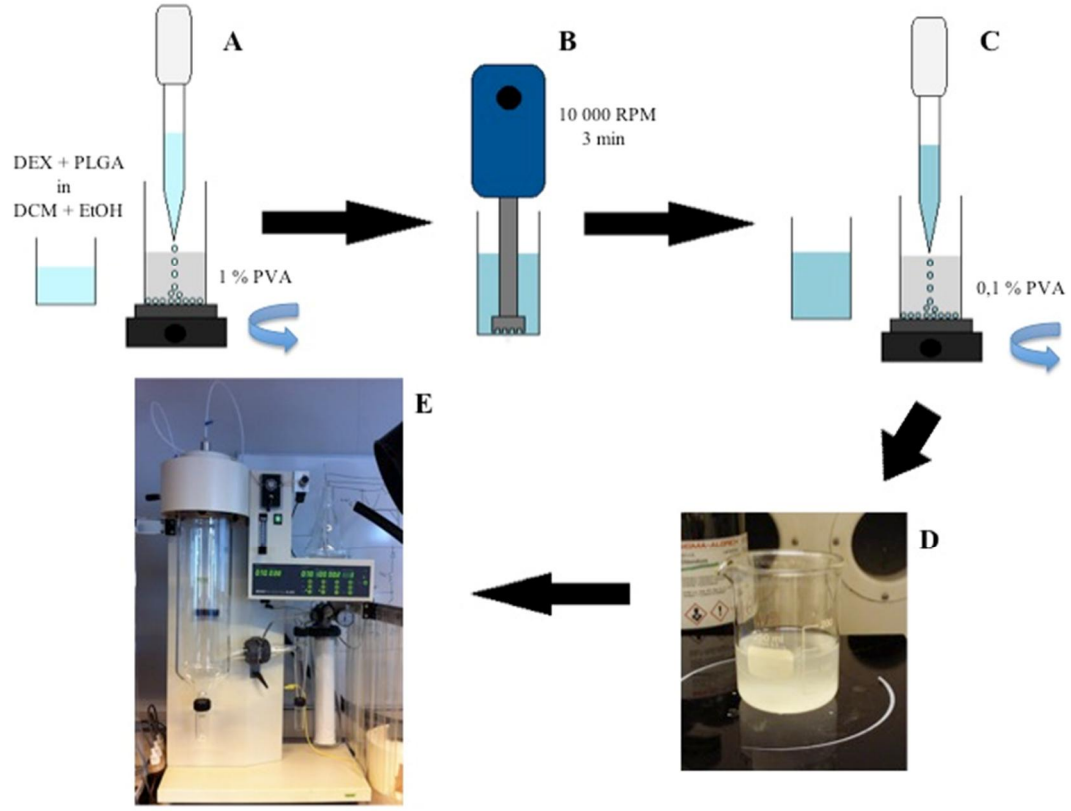

Figure 9: *Preparation of the microparticles. Phases: A) emulsification, B) homogenization, C) stabilization of the emulsion, D) final emulsion/adding of sucrose, and E) spray drying.*

Produced microparticles were collected into the sealed glass containers, wrapped with aluminium foil and stored in desiccator in room temperature until further use.

4.2.2 Yield of the prepared microparticles

Effectiveness of the spray drying process was evaluated by calculation of the yield. It is calculated using equation (4):

$$Yield = \frac{w(mp)}{w(total)} \times 100\% , \quad (4)$$

where w(mp) is the weight of the produced microparticles and w(total) is the total weight of the polymers and the drug used to prepare microparticles.

4.2.3 Characterization of the dexamethasone loaded microparticles by microscope before and after gamma irradiation

The morphology, size and size distribution of the microparticles including DEX was investigated by using scanning electron microscope (SEM), Zeiss ULTRAplus Scanning Electron Microscope (Germany). Samples were prepared by attaching microparticles to the two-sided carbon tape on top of the sample holder and then coated with carbon using sputter coater for SEM. During the imaging, 2 kV acceleration voltage of electrons was used.

Size distribution was achieved by measuring diameters of microparticle population from SEM image with ImageJ software. Based on this measuring data, histogram representing the size distribution was compiled with Microsoft Excel.

4.2.4 Gamma sterilization of the microparticles

Four different batches of microparticles were gamma irradiated for sterilizing the microparticles: DEX loaded microparticles, DEX loaded microparticles covered with sucrose, blank microparticles and blank microparticles covered with sucrose. Microparticles were sent in the sealed glass jars, which were put separately to the sealed plastic bags. Gamma sterilization was performed by commercial supplier. The exact dose that was given to the microparticles was 30.5 kGy. Radiation time was approximately five hours and therefore total radiation dose was given at the rate of 6.1 kGy/h.

4.2.5 *In vitro* drug release study of dexamethasone loaded microparticles

In vitro drug release study was performed at 37 °C in a shaking incubator, 100 rpm in phosphate buffer saline (PBS) solution for cell culturing with 0.05 % (w/v) sodium azide to avoid bacterial growth. Also *in vitro* drug release test were done before and after gamma sterilization. Samples of 40 mg were added to 5 ml of PBS in sealed 15 mL Falcon tubes. Samples were taken at set time points. Tubes were gently centrifuged using 3000 rpm for 10 minutes at 20 °C. Then, 1 mL sample was taken from the surface with pipet. Each time point had an own tube. Three parallel samples were used. All the collected delivery samples were stored at -20 °C before analysis by high performance liquid chromatography (HPLC). Three parallel samples were used. Mean values with standard deviation were reported.

Samples were pre-treated with absolute ethanol in order to precipitate possible impurities from the sample. 100 µL of sample and 200 µL of absolute EtOH was measured to the eppendorf tube, mixed well with vortex and kept in -20 °C overnight. Before analysis, precipitated samples were centrifuged using 10000 rpm for 10 minutes at 4 °C. Then, 80 µL were taken from the surface of the tube with pipet and transferred to the inserts.

Samples were analysed using Agilent-Packard 1100 Series High Performance Liquid Chromatography Value system (Germany) with UV detector. Used column was a Merck Purospher STAR RP-18 encapped (5 μm) column (USA).

HPLC gives chromatograms in which every peak indicates different component. With known retention time, desired compound can be identified. Peak of the certain compound, in this case DEX, is integrated and area of the peak is converted to concentration with calibration curve. Calibration curve is formed when standards with known concentrations are run with HPLC and then curve with concentration and corresponding peak areas. It is important to use same method in HPLC to calibration samples and real samples. In this case there were eight samples from concentration 0.78 μg/mL to 100 μg/mL.

The results from the preliminary drug release test with DEX loaded microparticles indicate degradation of the DEX in the PBS. This problem led to the very serious problems in analysing samples from the prolonged drug release test, as degradation product formed peak in the HPLC chromatogram next to the active DEX peak partly overlapping each other, so on, forming together a double peak. Separation of the peaks formed by different molecules is very important step in order to get reliable drug concentrations from the release samples. That is why several different methods for HPLC was developed and tested. These conditions are listed in Appendix I.

Analyses of the samples including DEX were carried out using two methods. In the first method (Appendix I, Method 1), following conditions were kept constant in every run: injection flow 1 mL/min, injection volume 20 μL and UV wavelength 238 nm. Maximum pressure of the system was set to 250 bar in order to protect the column. Analysis was performed using the gradient elution mode. Two different mobile phases were used: eluent A 20:80 acetonitrile to water and eluent B 80:20 acetonitrile to water. Analysis time was 15 minutes. Gradient elution program, in other words how two different mobile phases A and B are distributed percentually, during this time is listed in Table 4. Data was analysed with ChemStation for liquid chromatography software.

Table 4: *Gradient elution program.*

TIME (min)	***ELUENT A (%)***	***ELUENT B (%)***
0	90	10
1	90	10
10	10	90
10,50	90	10
15	90	10

Mobile phase A: 20:80 acetonitrile:water. Mobile phase B: 80:20 acetonitrile:water.

In the second method (Appendix I, Method 23), following conditions were kept constant: injection flow 60 mL/min, injection volume 20 μL, UV wavelength 238 nm and limit for maximum pressure 250 bar. This method did not use gradient elution

method. Mobile phase composed of 30:70:0.5 acetonitrile to water to phosphoric acid. Analysis time was 30 minutes.

In the results, released drug concentration is expressed as percentage of theoretical maximum amount of the drug. It is calculated using equation (5):

$$\%\, Released = \frac{c(t)}{c(\max)} \times 100\% \quad (5)$$

where c(t) is drug concentration in that time point and c(max) theoretical maximum concentration of the drug.

Drug content in the microparticles was tried to determine before and after drug release. To determine how much drug is still entrapped in the microparticles after the drug release (four weeks), the microparticles were freeze dried. Initial drug content was determined with original microparticles. Then, 25 mg microparticles were dissolved in the 5 mL of 9:1 DCM:EtOH by sonication for two hours. The solvent was chosen according to solubility of the PLGA and DEX. After sonication, solution was centrifuged using 10000 rpm for 15 minutes, 2 mL supernatant was taken from the surface of the tube, and then solvent was evaporated in room temperature. Dry content was re-dissolved in 2 mL of HPLC mobile phase, in case of DEX 30:70:0.5 acetonirtile:water:phosphoric acid solution. Samples were pre-treated with absolute ethanol and analysed with HPLC as described above in this chapter.

4.2.6 Dexamethasone degradation study

The results from the *in vitro* drug release study suggest that DEX degrade in the PBS in a period of time. That is why the incubation of DEX was studied in PBS. DEX solution of 55.6 μg/mL was prepared and 2 mL of the solution was added to the each sealed 15 mL Falcon tube. Initial concentration was determined with HPLC. Conditions were the same as in the *in vitro* drug release test. At each time point one tube was taken out of the incubator and 1 mL sample was taken with the pipet. Samples were stored in -20 °C until analysis by HPLC. Again, samples were pre-treated with absolute ethanol before HPLC analysis.

4.3 Composite of hyaluronic acid - polyvinyl alcohol hydrogel and dexamethasone loaded microparticles

DEX loaded PLGA-PVA microparticles were introduced into the HA-PVA hydrogel for examination of the properties of the composite. Earlier these same microparticles were introduce into the gellan gum hydrogel, but previous studies show the capacity of HA-PVA hydrogel to introduce bigger amount of microparticles than gellan gum hydrogels and that is the reason why HA-PVA hydrogels is subject of experiments in this thesis.

4.3.1 Re-suspension test of spray dried microparticles

When introducing microparticles into the hydrogel, microparticles must be homogeneously distributed in a suspension. For this reason, three different concentrations of sucrose in the microparticle emulsion, 0.25 %, 0.5 % and 1 %, was tested to see sufficient amount of sucrose to keep microparticles separate in the suspension.

Sufficient sucrose concentration was tested with dry powder. 50 mg of each microparticle powder was added to the 500 µL of distilled water, mixed with vortex and left stirring overnight. Formed suspensions were viewed by eye. The lowest sucrose concentration in the emulsion, which was still enough to form good microparticle suspension without aggregates, was chosen.

4.3.2 Preparation of dexamethasone loaded hydrogel-microparticle composite

Hydrogels were prepared to the cut syringes, which worked as a mold. First, all solutions including polymer solutions, aldehyde-modified HA (m-HA) 20 mg/mL, hydrazide-modified PVA (m-PVA) 10 mg/mL, and DEX loaded microparticle suspension were prepared separately. Microparticle suspension was prepared from the dry microparticle powder including 0.5 % sucrose in the wet form. 100 mg of this powder was resuspended into the 1 mL of distilled water. This way, microparticle concentration in the final suspension is 36 mg/mL Then more viscose polymer solution, m-HA, was measured to the syringe with pipet. Microparticle suspension was added to the m-HA solution and mixed carefully. Then, crosslinker m-PVA was added with pipet to the syringe and mixed immediately few seconds with the tip of the pipet and left to the room temperature to gelate (Figure 10).

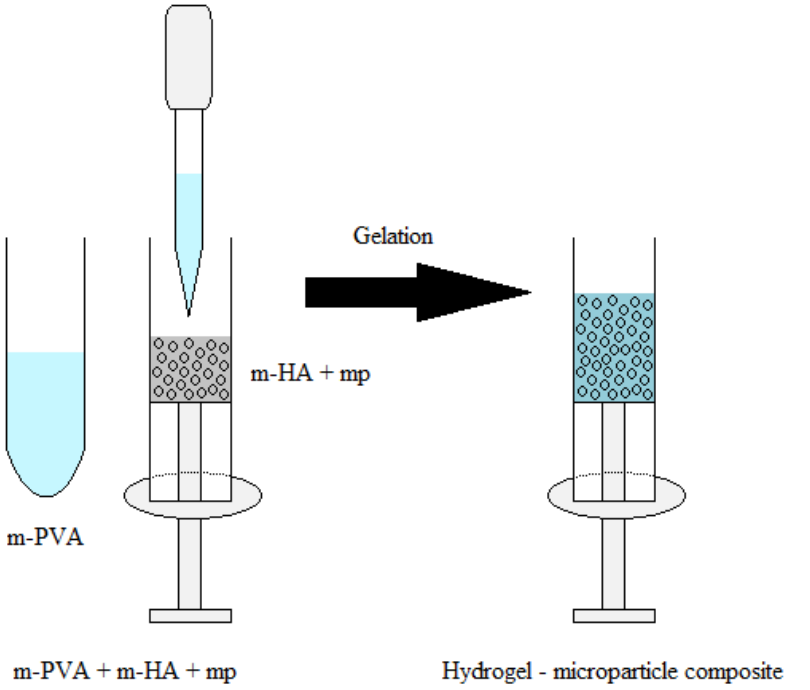

Figure 10: *Preparation of the hydrogel-microparticle composite.*

Gelation time in these hydrogels was fast, only few seconds. Syringes were covered with the parafilm to prevent moisture evaporation and left in room temperature for complete gelation. Total gelation time depends on the volume of the hydrogel. For small gels, one hour was sufficient, but larger gels demanded longer time and they were left to gelate usually overnight.

Blank hydrogel means the hydrogel with same formulation than composite gel but without microparticles. Blank hydrogel were prepared in the same way as hydrogel-microparticle composite, but without microparticles. First, more viscous m-HA solution was measured to the syringe. Then, crosslinker m-PVA was measured and added to the syringe with pipet simultaneously mixing with the pipet tip. Syringes were covered with the parafilm and left to gelate in room temperature.

4.3.3 Designing the hydrogel-microparticle composite

In experimental design, there were two main aspects to taken into account: 1) Best formulation (m-HA and m-PVA concentrations), and 2) Maximum amount of microparticles introduced into the hydrogels. Three different gel formulas and four different percentage microparticle values in the gels were tested. These are listed in Table 5 and Table 6:

Table 5: *Designing the hydrogel-microparticle composite. Tested hydrogel formulations.*

FORMULATION	***[HA] (mg/mL)***	***[PVA] (mg/mL)***	***V(HA) µL***	***V(PVA) µL***	***[Mp]* (mg/mL)***	***V(Mp) mL***
1	5	2,5	50	50	-	-
2	5	2,5	50	50	36	60
3	5	2,5	40	40	36	100
4	10	5	50	50	-	-
5	10	5	50	50	36	60
6	10	5	40	40	36	100
7	20	10	50	50	-	-
8	20	10	50	50	36	60
9	20	10	40	40	36	100

Table 6: *Determination of the maximum amount of microparticles that is possible to introduce into the hydrogel.*

FORMULATION	***[HA] (mg/mL)***	***[PVA] (mg/mL)***	***V(HA) µL***	***V(PVA) µL***	***[Mp]* (mg/mL)***	***V(Mp) µL***	***% of Mps***
1	20	10	50	50	36	60	59
2	20	10	50	50	36	80	66
3	20	10	50	50	36	90	68
4	20	10	50	50	36	110	73

Amount of microparticles in the hydrogel is presented as percentage of the microparticles from the total mass of the hydrogel components. It is calculated using equation (6):

$$\%\ of\ mp = \frac{V(Mp)*[Mp]}{V(HA)*[HA]+V(PVA)*[PVA]+V(Mp)*[Mp]} \times 100\% \quad (6)$$

where units of the volumes and concentrations are the same than in Table 6 above.

First, the best hydrogel formulation was chosen. Then, maximum amount of microparticles in the gel without affecting the structure was determined. Hydrogel-microparticle composites were always compared with the blank hydrogels in order to see difference in the gel structure with and without microparticles.

4.3.4 Characterization of the composite by microscope

Morphology, surface topography and microparticle distribution of the hydrogel-microparticle composite was investigated with SEM as mentioned in Chapter 4.2.3. For SEM analysis, samples were frozen in – 20 °C for one day and freeze dried using Heto Drywinner (Denmark) for two days. Two different samples were analysed with SEM:

newly prepared hydrogel-microparticle composite and hydrogel-microparticle composite after six weeks incubating in shaker plate. SEM images of the hydrogel-microparticle composites were compared to SEM image of the blank hydrogel.

4.3.5 *In vitro* drug release study of hydrogel-microparticle composite

In vitro drug release test of the composite were performed in the same condition as mentioned in Chapter 4.2.5. Three parallel hydrogel-microparticle composites of 2360 µL were prepared. Volume of the composite was chosen so that total microparticle content in the hydrogel was 40 mg, as it was in the *in vitro* drug release test of DEX loaded microparticle as such. Composite hydrogel was placed in the 50 mL Falcon tubes with 5 mL of PBS. At certain time points, 300 µL samples were taken from the tubes and removed volume was replaced with fresh PBS. Composite with blank microparticles was used as a control. Three parallel samples were used. Mean values with standard deviation were reported. Samples were pre-treated with absolute ethanol and analysed with HPLC using second method as mentioned in Chapter 4.2.5.

4.3.6 Mechanical testing of the composite

For testing mechanical properties of the composite, 870 µL composite gels were prepared. Fresh, wet gels were tested with BOSE ElectroForce Biodynamic 5100 test instrument (USA) with WinTest 4.1 software. Gels were compressed in air at room temperature. Displacement of the compression was 65 % of the height of the sample. Heights of the samples were between 6.5-7.2 mm and diameters between 11.0-12.3 mm. Used compression speed was 10 mm/min. Data was collected four times per second. Compressive stress σ is calculated with equation (7):

$$\sigma = \frac{F}{A} \tag{7}$$

where F is applied load which act perpendicular to the sample cross-section and A is initial cross-sectional area of the sample. Strain ε is then calculated using equation (8):

$$\varepsilon = \frac{l - l_0}{l_0} = \frac{\Delta l}{l_0} \tag{8}$$

where l is length, or as in this case height of the sample under certain load, l_0 is initial sample length and Δl deformation elongation of the sample. According to measuring data, stress-strain curve was drawn. Young's modulus which is also called elastic modulus was calculated using trend line drawn to linear viscoelastic region of the curve. Young's modulus was calculated using equation (9):

$$Young's\ modulus = \frac{\Delta stress}{\Delta strain} \tag{9}$$

Then Young's moduli of the composite hydrogels were compared with the Young's moduli of the blank hydrogels. Volumes of the blank hydrogels were exactly the same as composite in order to able to compare the results.

4.4 Muraglitazar loaded poly(lactic-co-glycolic) acid - polyvinyl alcohol microparticles

4.4.1 Solubility of the muraglitazar

In order to determine solubility of muraglitazar to organic solvents, 5 mg of drug powder were added to the 1 mL of four different organic solvents: ethanol, dichloromethane, 9:1 dichloromethane to ethanol mixture and acetone. Solution was mixed with vortex and volume of the solvent was added 1 mL at the time until the same ratio of drug and solvent as in microparticle preparation oil phase was reached. Finally, solution was left stirring with magnetic stirrer overnight. Solvent in which muraglitazar seemed to dissolve best was chosen.

4.4.2 Preparation of microparticles

Muraglitazar loaded microparticles were prepared with the same method as described in Chapter 5.9.1. Only exception is the drug: DEX is replaced with muraglitazar. Shortly, 250 mg of PLGA and 25 mg of muraglitazar was dissolved in 25 mL of DCM:EtOH (9:1). This oil phase was first added drop by drop into the 100 mL of 1 % PVA solution simultaneously stirring. Then, first emulsion is homogenized with homogenator. Finally, first emulsion is added drop by drop into the 500 mL of 0.1 % PVA solution and spray dried.

4.4.3 Characterization of the muraglitazar loaded microparticles by microscope

The morphology, size and size distribution of the microparticles including muraglitazar was investigated with SEM as mentioned in Chapter 4.2.3.

4.4.4 *In vitro* drug release study of muraglitazar loaded microparticles

In vitro drug release was performed in the same conditions as mentioned in Chapter 4.2.5. Microparticle samples of 10 mg were added to 2 mL of PBS in sealed 15 mL Falcon tubes. Microparticles were centrifuged same way as mentioned above and 1 mL samples were taken with pipet at certain time points. Three parallel samples were used. Mean values with standard deviation were reported.

Samples were pre-treated with absolute ethanol as described in Chapter 4.2.5. Samples were analysed with HPLC with mobile phase of 60:40 acetonitrile to water including 0.06 % of trifluoroacetic acid (TFA). Following conditions were kept constant

in every run: injection flow 1 mL/min, injection volume 10 μL and UV wavelength 280 nm.

Initial and residual drug contents in the microparticles were tried to determined also with muraglitazar loaded microparticles. Preparation of the samples was performed in the same way as with DEX loaded microparticles (Chapter 4.2.5.) with few improvements. In brief, 25 mg original microparticles or freeze dried microparticles from the drug release tests were dissolved into the 10 mL dichloromethane and stirred over night with magnetic stirrer. Then, samples were sonicated for three hours and centrifuged using 9000 rpm for 15 minutes. 2 mL of the supernatant was taken from the surface of the tube and solvent was evaporated in room temperature. Dry content was re-dissolved in 1 mL of HPLC mobile phase. Here, sample was concentrated in order to achieve equal drug concentration in the sample as with DEX samples. Finally, samples were pre-treated and analysed with HPLC as described above in this chapter.

5 RESULTS

5.1 Dexamethasone loaded microparticles

Drug loaded microparticles were successfully prepared using combination of two methods, emulsion method and spray drying with Büchi Mini Spray Dryer B-290 using selected parameters in the spray drying process adopted from previous study (Ugur 2014).

5.1.1 Effectiveness of the preparation method

Effectiveness of preparation method was evaluated based on yield of the microparticles. In the previous studies performed with the same method and same equipment yield of the microparticles remained very low, only 48.6 % of the initial solid content. Differences in the spray dryer between the current results compared to previous results are new, high performance cyclone for Büchi Mini Spray dryer B-290 (Switzerland), thorough service and cleaning to the spray dryer machine. Yield of each produced batch of the microparticles was calculated using equation (4) presented in Chapter 4.2.2. Results and standard deviations are reported in Table 7.

Table 7: *Yield results for drug loaded microparticles with and without added sucrose.*

Composition	***Yield (%) ± s.d.***
PLGA-PVA microparticles	77.1 ± 1.3
PLGA-PVA microparticles + sucrose	78.5 ± 3.3

Here, yield of microparticles alone and microparticles including sucrose are presented separately. This is due to high difference in solid contents in the emulsion.

5.1.2 Size and morphology of dexamethasone loaded microparticles

Visually observed, produced microparticles seem white powder. More precise observation was performed with scanning electron microscope (SEM). Microparticles form large clusters and are tightly adhered to each other as shown in Figure 11A and 11B. Microparticles have spherical appearance and smooth surface topography without visible pores or cracks, which can be seen with higher magnifications (Figure 11C and 11D). Few irregularities can be seen on the surface of the largest microparticles.

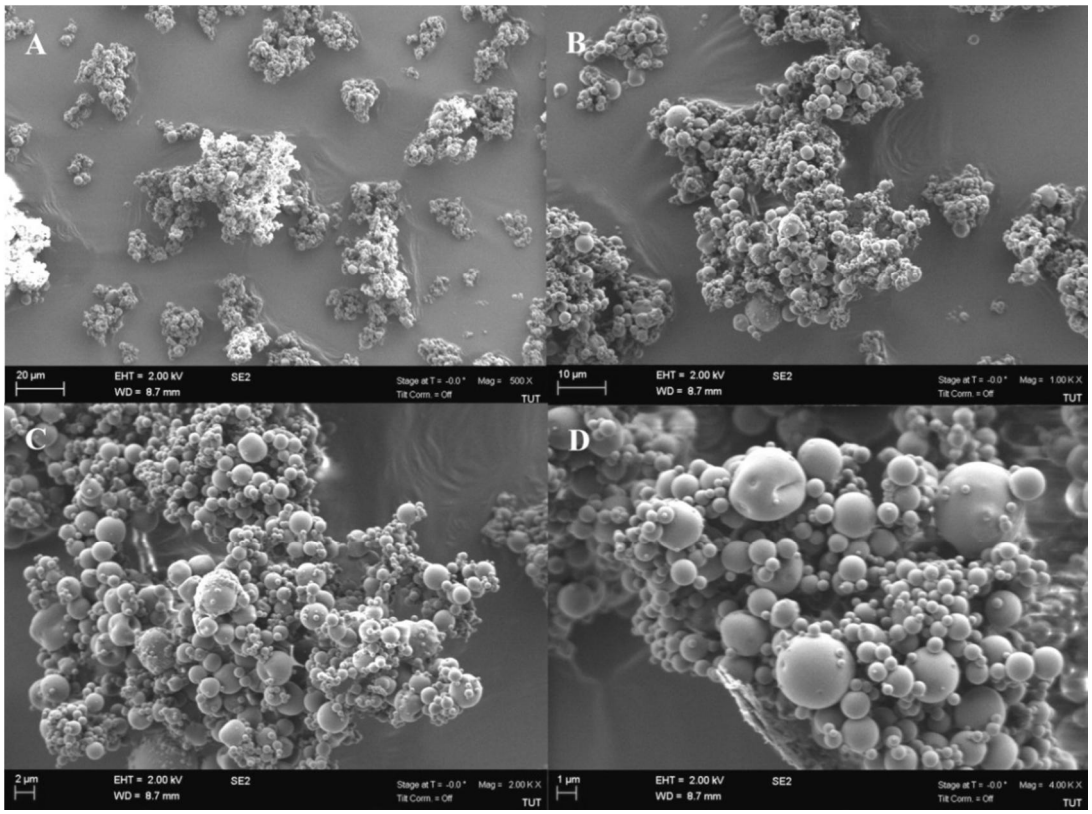

Figure 11: *SEM images of dexamethasone loaded microparticles. A)-D) Microparticles imaged with different magnifications. Scale bar is A) 20 µm, B) 10 µm, C) 2 µm and D) 1 µm.*

Size distribution of the DEX loaded PLGA-PVA microparticles is shown in Figure 12. The frequency is presented as percentage of measured population. In this case, population of 209 microparticles was measured from the SEM image using ImageJ software. Diameters of the microparticles in this sample varied from 145 nm to 4.7 µm. The most frequent size range of the microparticles was 501-1000 nm.

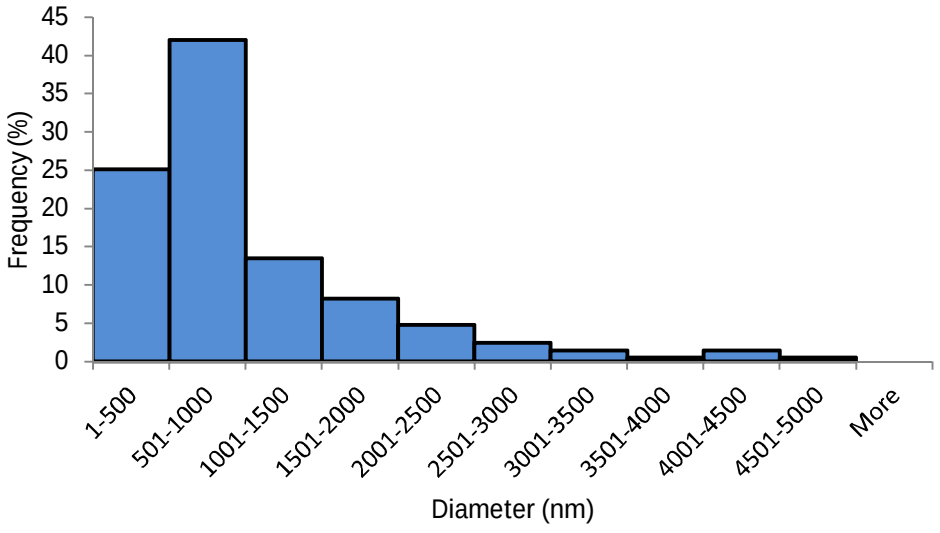

Figure 12: *Size distribution of the dexamethasone loaded microparticles.*

It has to be highlighted that measuring method used here is not accurate. For accurate diameter measurements from SEM images, sample has to be prepared in the way that there is monolayer of separate microparticles. Then it is possible to use tools that automatically measure and form size distribution of the sample. Here, microparticles form clusters, which are three-dimensional structures. That is why microparticles are in the different focus planes. This affects to the measured diameters, as some microparticles are near microscope and the others are farther behind. In addition, every single microparticle is measured separately by hand using the straight tool of the ImageJ software. Scale is set to match the scale bar of each SEM image. Still, picture quality together with method for measuring diameters causes significant inaccuracy to the results. This is why microparticle size distribution has to consider approximate.

Sample of DEX loaded microparticles coated with sucrose has same kind of appearance than microparticles without sucrose. Also these microparticles form aggregates (Figure 13A), and are smooth and spherical (Figure 13B and Figure 13C). Only visual difference between microparticles with and without sucrose is amount of small microparticles (Figure 13 D). When compared Figures 12 and 13 it can be seen that there are more nanoscale particles.

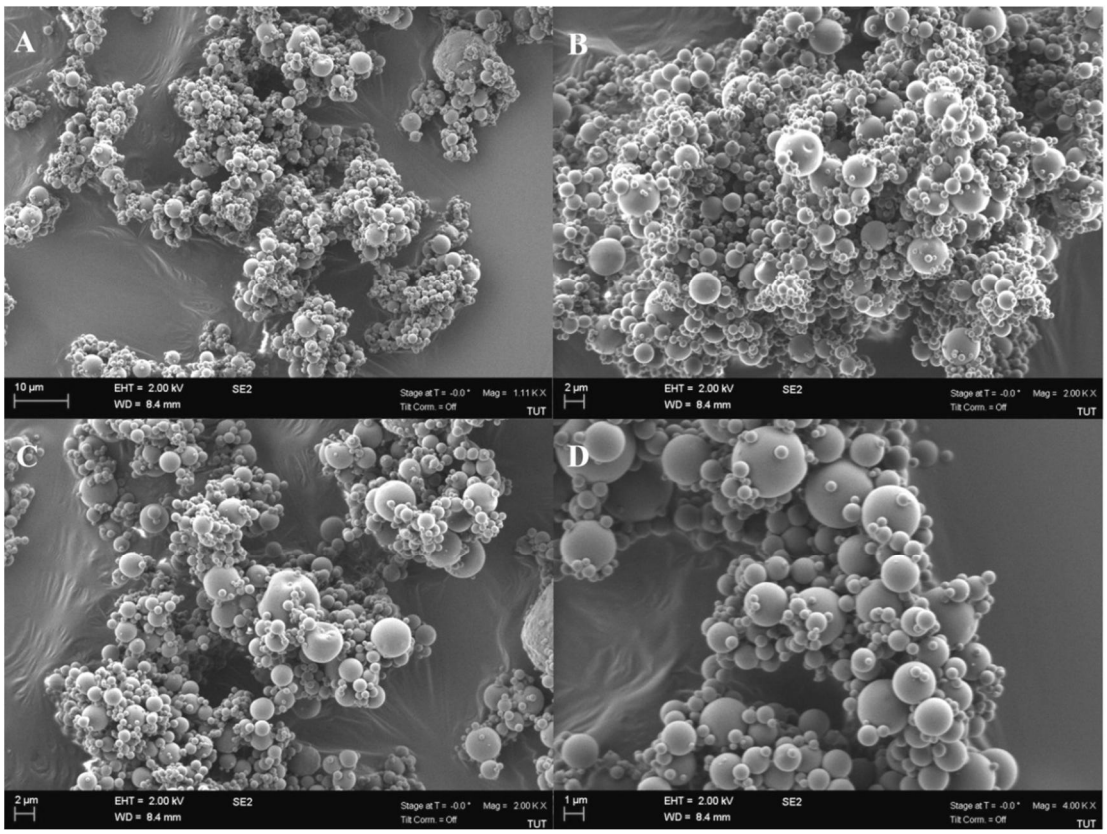

Figure 13: *SEM images of dexamethasone loaded microparticles coated with sucrose. A)-D) Microparticles imaged with different magnifications. Scale bar is A) 10 µm, B) 2 µm, C) 2 µm and D) 1 µm.*

Size distribution of sucrose coated microparticles is shown in Figure 14. Here, population of 203 microparticles was measured. Diameters of the microparticles in this sample varied from 181 nm to 4.9 μm. The most frequent size range of the sucrose coated microparticles was 1-500 nm

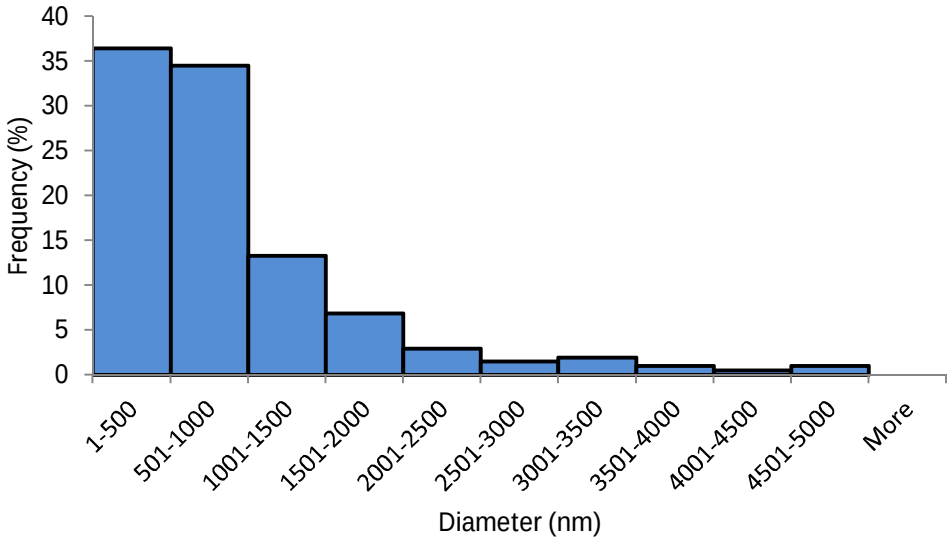

Figure 14: *Size distribution of the dexamethasone loaded microparticles coated with sucrose.*

DEX loaded microparticles were analysed before and after exposure to the gamma irradiation as sterilization method. Compared batches of the microparticles are otherwise identical. They have been prepared in the same way, and sucrose was not added to these batches. Visual look of microparticle powder did not change after gamma irradiation, but more detailed observation with SEM revealed serious changes in morphology of the microparticles (Figure 15). Figures 15A and 15B shows microparticles with lower magnifications, and Figures 15C and 15D different kinds of changes in the morphology with higher magnification.

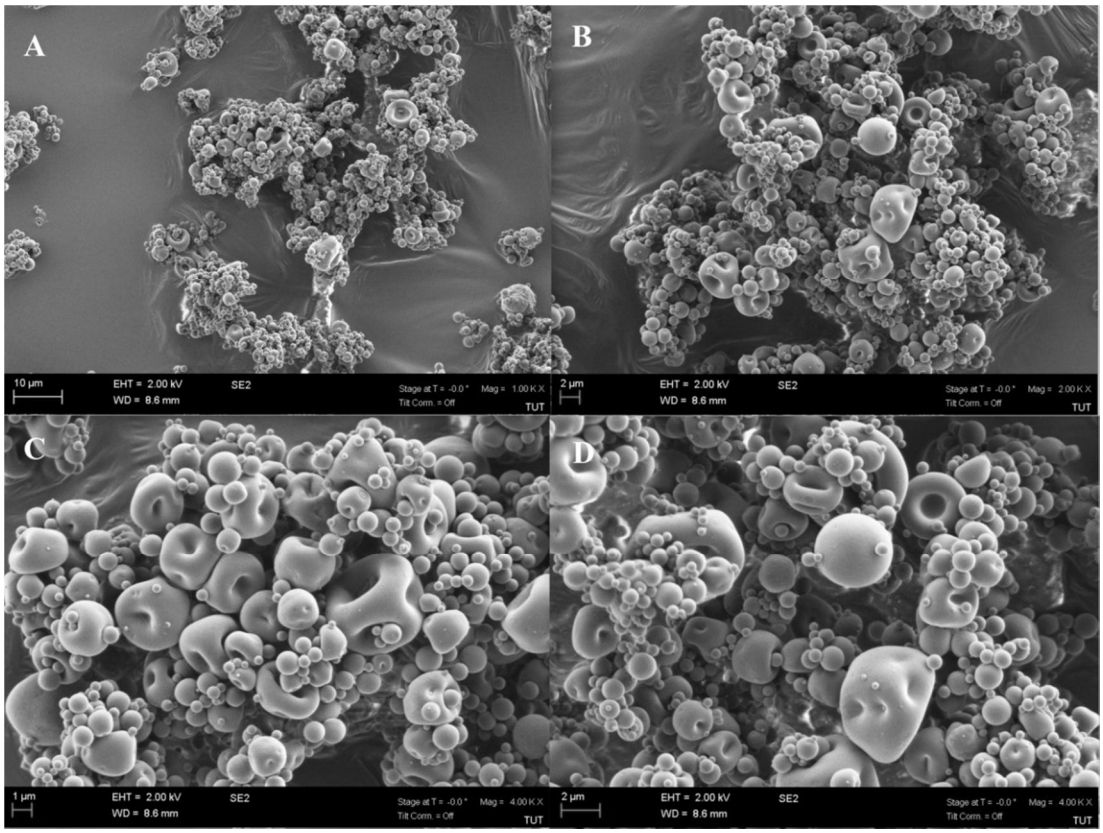

Figure 15: *SEM images of gamma sterilized dexamethasone loaded microparticles. Scale bar is A) 10 µm, B) 2 µm, C) 1 µm and D) 2 µm.*

All the microparticles with diameter larger than one micrometre show irregular shape with either fully collapsed structure or smaller pits on the surface of the microparticles. Instead, smaller microparticles with diameter less than one micrometer have still spherical shape without irregularities.

5.1.3 Developing high performance liquid chromatography method for microparticles loaded with dexamethasone

Problems of analysing DEX drug release samples with HPLC caused by degradation of the DEX lead to the developing new method. All different methods that were tested are listed in Appendix I starting with the original method, used in previous studies and ending with the best, chosen method for the DEX analysis.

Chromatograms of the starting situation are presented in Figure 16. In Figure 16A and 16B, there are chromatograms of drug release sample at time points two hours (A) and four days (B). Samples are from preliminary drug release test. Used method is the original one, where samples are only centrifuged to eliminate possible microparticles and then directly analysed. The small secondary peak can be seen already beside active DEX peak at the time point of four days. In Figure 16C and 16D method is slightly improved with ethanol precipitation, which eliminates impurities from the sample and therefore disruptive peaks from the chromatogram. In sample at the time point two weeks (Figure 16D) secondary peak is already so obvious that it is impossible to

integrate the peaks separately. In other words, concentration of the DEX in the sample cannot be determined using this method for HPLC.

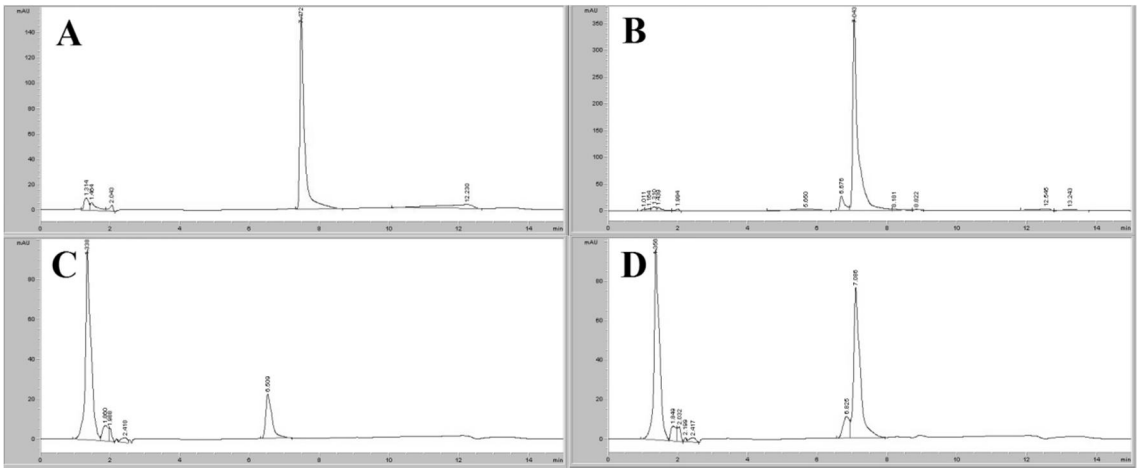

Figure 16: *Chromatograms of dexamethasone drug release samples from the starting situation. A) Sample as such at time point two hours, B) Sample as such at time point four days (first drug release test lasted only four days), C) Sample pre-treated with ethanol at time point two hours, and D) Sample pre-treated with ethanol at time point two weeks.*

In Figure 17, there are chromatograms of chosen steps of HPLC method development. Developing was done in order to separate peak of DEX degradation product from the peak of active DEX.

First, the simplest possible changes in measuring conditions were tested. These include changing the ratio of water and acetonitrile in eluents, prolonging the gradient elution method for HPLC and decreasing eluent flow rate.

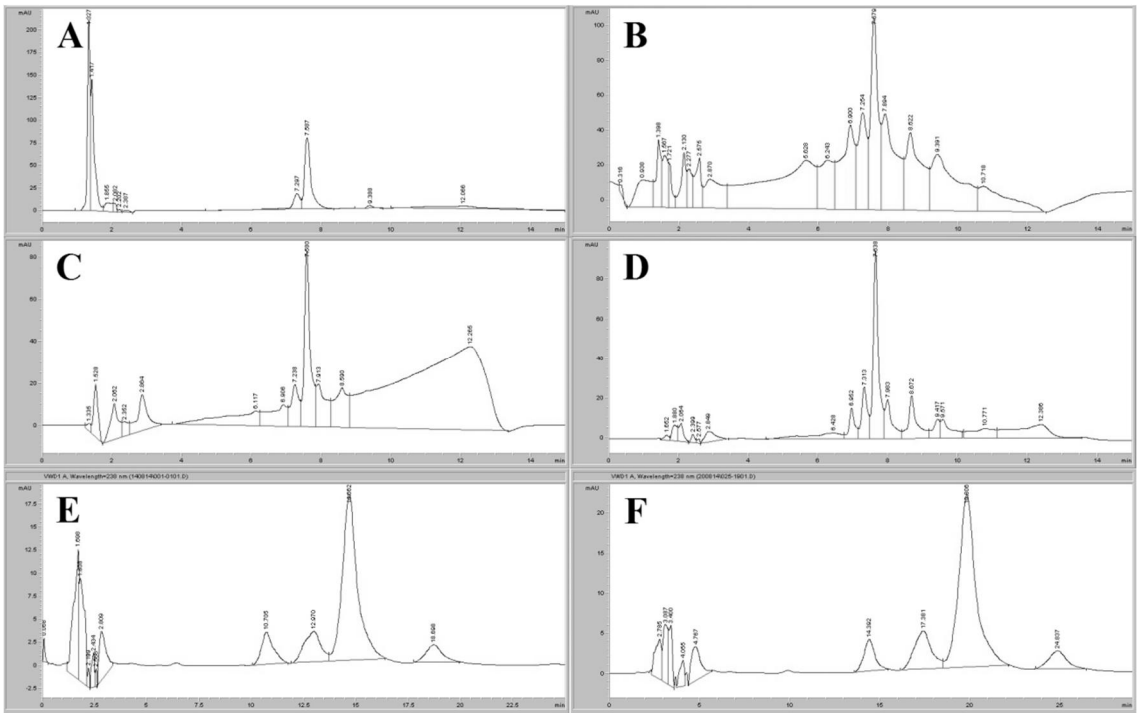

Figure 17: *Chromatograms of dexamethasone drug release samples using different methods. All samples are first pre-treated with ethanol. A) Sample is evaporated and then re-dissolved into eluent A, B) Same sample than previous but 0.1 % TFA is added to the eluents, C) Sample is filtered with Amicon 0.3 kD centrifugal filter and 0.1 % TFA is added to the eluents, D) 0.5 % H_3PO_4 is added to the eluents, E) Only one 30 % ACN + 0.5 % H_3PO_4 eluent, F) Same sample than previous but flow rate of the eluent decreased to 0.6 mL/min.*

Next step was comparing the two solvents for precipitation and evaporating the solvent from analysed sample (Figure 17A). Also adding acid to the eluents were tested, trifluoroacetic acid (TFA) as first acid (Figure 17B). These were followed by avoiding the precipitation by using Amicon 0.3 kD centrifugal filters to eliminate degraded polymer chains and other impurities. Filtered samples were analysed without and with TFA (Figure 17C) in eluents. Then, second acid, phosphoric acid (H_3PO_4) was added to the eluents but still using gradient method (Figure 17D). It can be seen that shape of chromatogram improved a lot. Finally, completely different method with only one eluent was tested (Figure 17E) and fine-tuning by decreasing eluent flow rate was done (Figure 17E) to achieve separated peaks.

5.1.4 *In vitro* drug release from dexamethasone loaded microparticles

Cumulative drug release profile of DEX from microparticles is shown in Figure 18 (blue diamonds). Profile follows bi-phasic drug release mechanism, which is typical for PLGA drug delivery devices (DDDs). Also, typical for PLGA devices is burst release that is quick release of the drug in the beginning of the drug release period. In this study, drug release profile reveals initial burst release followed by slower release period.

During the initial burst, which occurs over the first four hours, approximately 21.5 % of the theoretical drug loading is released. After burst release, drug is released more slowly and 53.0 % of theoretical drug loading is released within 28 days.

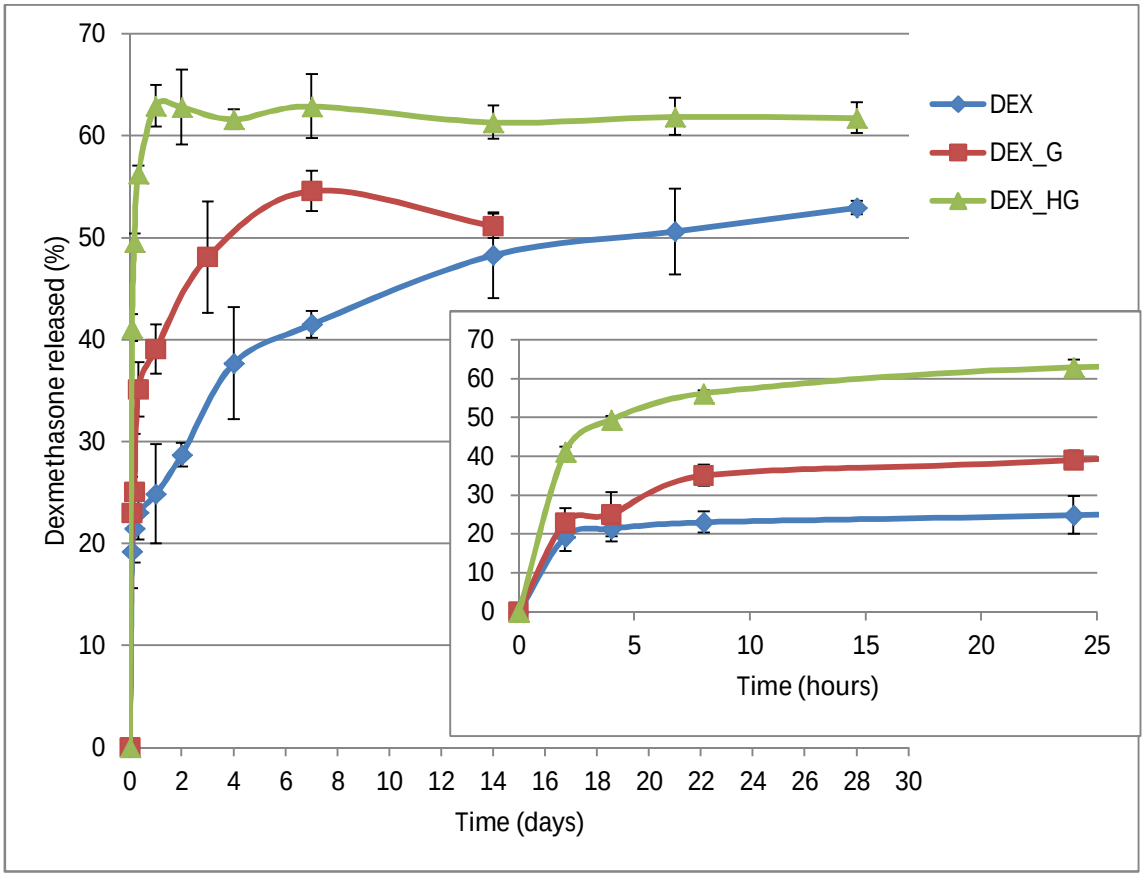

Figure 18: *Cumulative release of dexamethasone at 37 °C in PBS buffer, pH 7.4. Reported as percentual release with standard deviations, n = 3. Blue diamond present drug release of dexamethasone from microparticles (DEX), red square from gamma sterilized microparticles (DEX_G) and green triangle from hydrogel-microparticle composite (DEX_HG). Time interval 0-1 days, 0-24 hours is expanded to separate chart.*

There were attempt to determine drug content in the microparticles before and after drug release test. Samples were prepared as described in Chapter 4.2.5. Destroying the microparticles turned out to be more complicated than literature implies. Despite preliminary tests with different solvents and methods, determination of the drug content failed. Obviously, method used in this test was not strong enough to break microparticles down. Solvent was chosen according to solubility of PLGA and DEX, which are main components in the microparticles. But, PVA is insoluble in organic solvents and it must affect to the results. After sonication, solution had still a milky appearance, which proves incomplete dissolution of the microparticles. According to this test microparticles would contain only 21 % ± 0.8 %. This result cannot be true, as drug release tests indicate that drug content in the microparticles is higher.

Residual drug content test failed as well due to same reason. There was strong suspicion that after four-week drug release test, there would still be some drug left in the microparticles. Still, there was not peak at all in the HPLC chromatogram. With this method possible small drug amount could not be released to the solution but it is still left entrapped into the PLGA-PVA structure.

5.1.5 *In vitro* drug release from gamma sterilized dexamethasone loaded microparticles

Cumulative DEX release from gamma sterilized DEX loaded microparticles is shown in Figure 18, Chapter 5.1.4 (red squares). For the purpose of comparison, drug release profiles from all different drug release tests concerning DEX is shown in the same figure. Duration of this release test was 14 days due to size of the batch that was sent to gamma sterilization. Also this drug release profile follows bi-phasic profile starting with burst release and followed with slower release phase. However, compared to drug release profile of non-sterilized microparticles, drug release is much faster from the gamma sterilized microparticles. In this case, burst release during first four hours is 25.1 % of theoretical drug loading. Release seems to be completed in seven days when maximum DEX concentration of 54.6 % in the sample is reached. Slight descent in the trend line after these seven days can be explained by degradation of the free DEX in the PBS.

5.1.6 Degradation of the dexamethasone

As mentioned earlier, results of initial *in vitro* drug release tests from DEX loaded microparticles suggest that DEX degrade at 37 °C in PBS. Secondary peak in the HPLC chromatogram started to appear after around one week of hydrolysis and it increased in size with increase of elapsed time. The secondary peak was situated beside the active DEX peak partly overlapping each other (see Figure 16, Chapter 5.1.3), which made determination of DEX concentrations unreliable and finally impossible. In order to confirm the degradation of the DEX and eliminate possible interactions with polymers and decrease in pH because of the polymers, the incubation test of DEX in PBS was done as described in Chapter 5.1.3. Samples were taken once a week. The results are shown in Figure 19 and they show clearly reduction of DEX concentration in the sample. In this experiment there were not parallel samples. Initial DEX concentration was 55.6 μg/mL and it decreases linearly as indicated by R^2-value (≈ 1). After four weeks, concentration was only 23.4 μg/mL, which is 42.1 % of the original concentration.

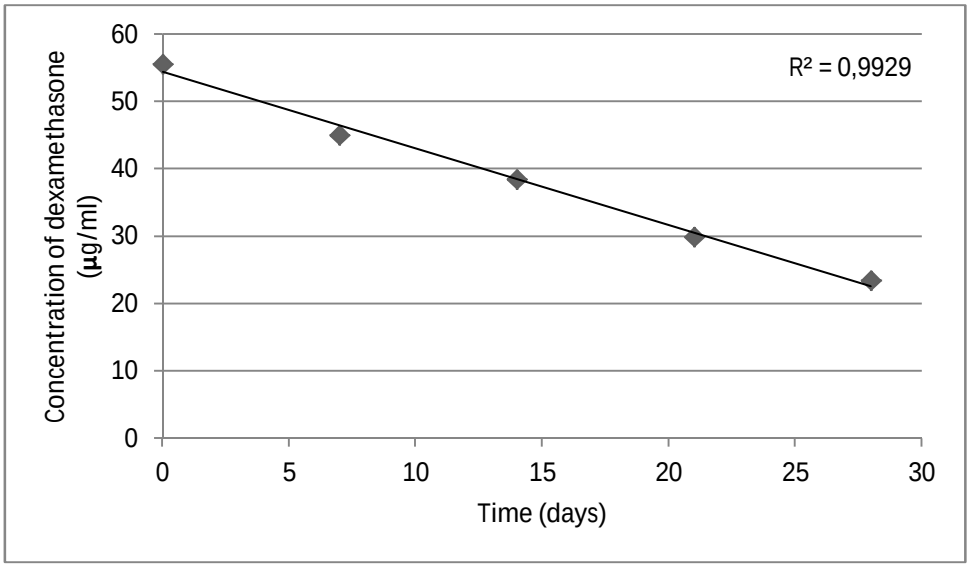

Figure 19: *Degradation of dexamethasone with time at 37 °C in PBS buffer, pH 7.4.*

Degradation may happen due to reduce in pH, exposure of light in the incubator or drug-polymer interaction. In the drug release samples, there are two different polymers, PLGA and PVA, and at least degradation products of the PLGA are acidic. The pH of the PBS in the drug release tests was measured after 28 days of hydrolysis and it was still 7.17, in the range where buffer solution should maintain its buffering capacity. This decrease of pH in PBS does not take local acidic environment inside the microparticles, and its affect to the degradation of the DEX into account.

5.2 Hydrogel-microparticle composite

This hydrogel-microparticle composite combining DEX loaded PLGA-PVA microparticles incorporated in hyaluronic acid (HA) – polyvinyl alcohol hydrogel (PVA) was designed with the aim to prolonged drug releasing period. Hydrogel-microparticle composites were prepared with the method described in Chapter 4.3.2.

Microparticles are added to the hydrogel as a certain volume of the microparticle suspension during the preparation of the hydrogel. In order to get homogeneously distributed microparticles in the hydrogel, also microparticle suspension has to be homogeneous without large microparticle aggregates. Spray dried, DEX loaded PLGA-PVA microparticles as such do not form homogeneous suspension with water easily. In the previous studies, microparticles were suspended in 10 % sucrose solution, but with this method, concentration of the microparticles in the suspension remained quite low level. In this study, new method, where microparticles are spray dried with the sucrose as described in Chapter 4.2.1 was investigated. Three different concentrations of sucrose in the emulsion were tested: 0.25 %, 0.5 % and 1 % as mentioned in Table 3, Chapter 4.2.1. Dry powders ability to re-suspend into the water was tested as described in Chapter 4.3.1. Sucrose concentration of 0.5 % was chosen, as being the lowest

concentration that was still enough to form visibly homogeneous microparticle suspension.

First step in designing the hydrogel-microparticle composite was choosing the best gel formulation, in other words m-HA and m-PVA concentrations, for the composite. All tested formulations are listed in Table 5, Chapter 4.3.3. In this case the best formulation is tough, elastic hydrogel, which maintain its structure after removing it from the mould. Hydrogel formulation with polymer concentrations of 20 µg/mL of aldehyde-modified HA (m-HA) and 10 µg/mL of hydrazide-modified PVA (m-PVA) was chosen. This hydrogel formulation showed the toughest structure and the most importantly structure was the least affected by added volume of microparticle suspension.

Second step in designing the composite was determining the maximum amount of microparticles that is possible to introduce into the hydrogel. In practice, this means maximum volume of microparticle suspension that can be added into the hydrogel in preparation method without affecting the gels toughness. Four different percentages of the microparticles were tested and they are listed in Table 6, Chapter 4.3.3. Chosen percentage of microparticles in the hydrogel was 68 %. Percentage is calculated using equation (3). This relates to 47 % volume percentage of microparticle suspension in the total volume of the hydrogel. This was the maximum amount of microparticles in the hydrogel with maintained toughness and elastic structure.

5.2.1 Morphology of hydrogel-microparticle composite

Visual appearance of the hydrogel-microparticle composite and control hydrogel without microparticles is illustrated in Figure 20. Composite gel has regular cylindrical shape due to adopted shape of the mold, into which it is prepared. Colour of the gel is white and translucent. In comparison, shape of the control hydrogel composing only of m-HA and m-PVA is more irregular and surface is grainy even though it has been prepared into the same kind of mold as composite hydrogel. Control hydrogel is throughout transparent due to absence of the microparticles.

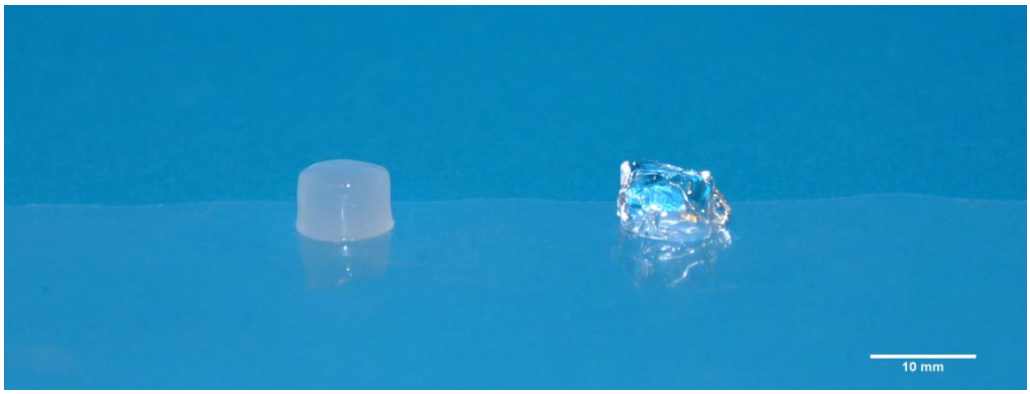

Figure 20: *Photograph of the composite and control gel. Scale bar is 10 mm.*

More detailed observation of the composite hydrogel was done with SEM (Figure 21). Hydrogels were freeze dried for two days before SEM imaging. Figure 21A illustrates well the smooth structure of the control HA-PVA hydrogel without microparticles. Rest of the images in the Figure 21 demonstrate freshly prepared composite hydrogel. General, porous structure of the hydrogel can be seen in Figure 21B and more detailed images with higher magnifications in Figures 21C-21E. Microparticles can be seen as small bulges in otherwise smooth walls of the gel. Higher amount of visible microparticles was expected, but the problem is small size of the microparticles. As majority of the microparticles have diameter of less than one micrometre, they are easily embedded into the thicker walls of the gel. In Figure 21F, there is cross-section of the hydrogel wall and small bulges inside the wall, only largest microparticles are visible through the walls.

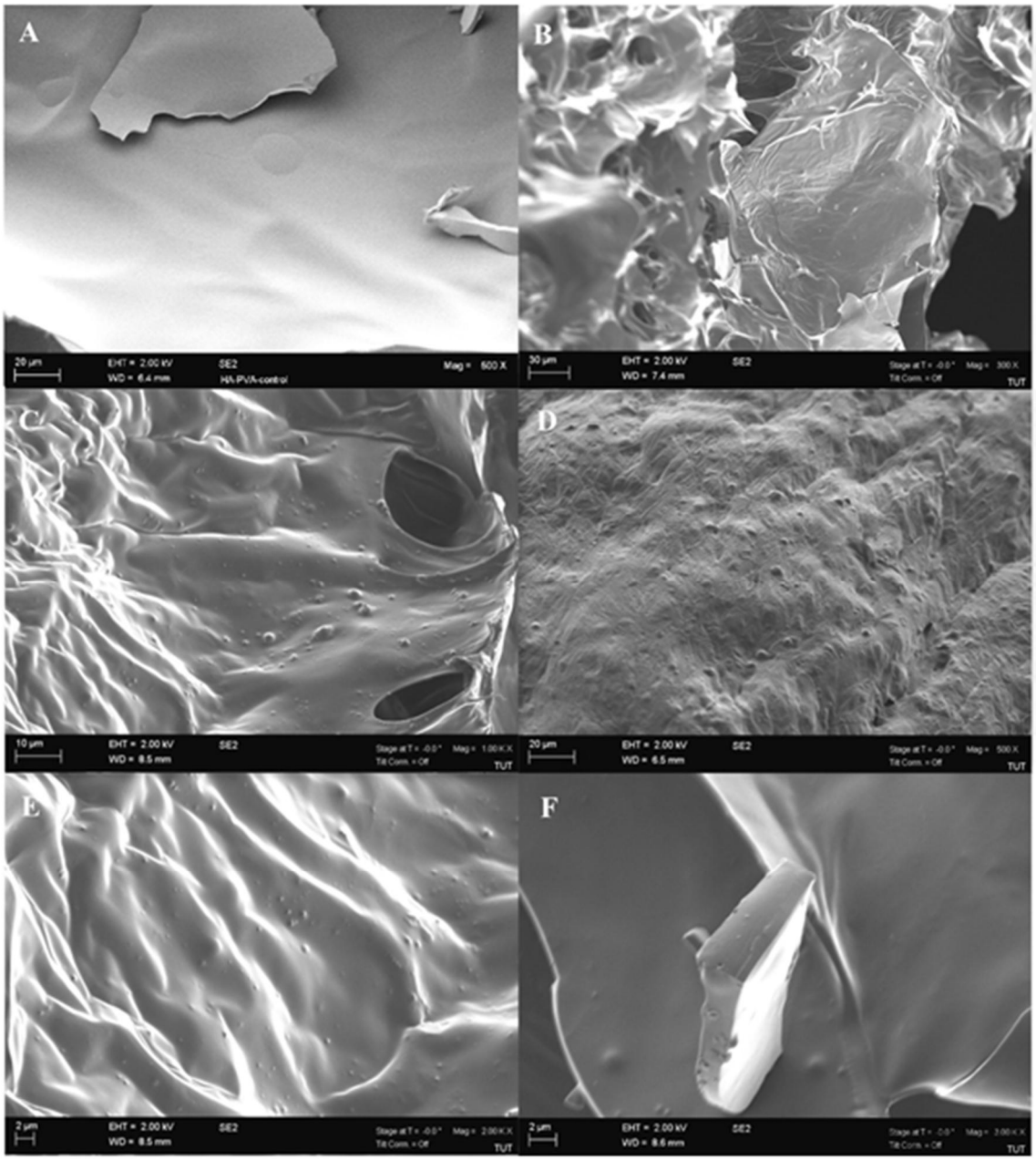

Figure 21: *SEM images of hydrogel-microparticle composite. A) Control gel, B) Porous structure of the freeze dried hydrogel, C)-E) Embedded microparticles in the hydrogel imaged with different magnifications, and F) Wall thickness of the dried hydrogel. Scale bar is A) 20 μm, B) 30 μm, C) 10 μm, D) 20 μm, E) 2 μm and F) 2 μm.*

Microparticles-hydrogel composite was observed with SEM before and after drug release test. Difference can be seen clearly when comparing Figures 21 and 22. Microparticles are much more visible after five weeks of hydrolysis, because hydrogel around the microparticles has started to degrade, which result in thinner walls of the pores than in fresh hydrogel (Figure 21). This proves that amount of microparticles in the hydrogel is higher than could be expected according to the Figure 21. Higher amounts of microparticles in the hydrogel with different magnifications are shown in Figures 22A-22D.

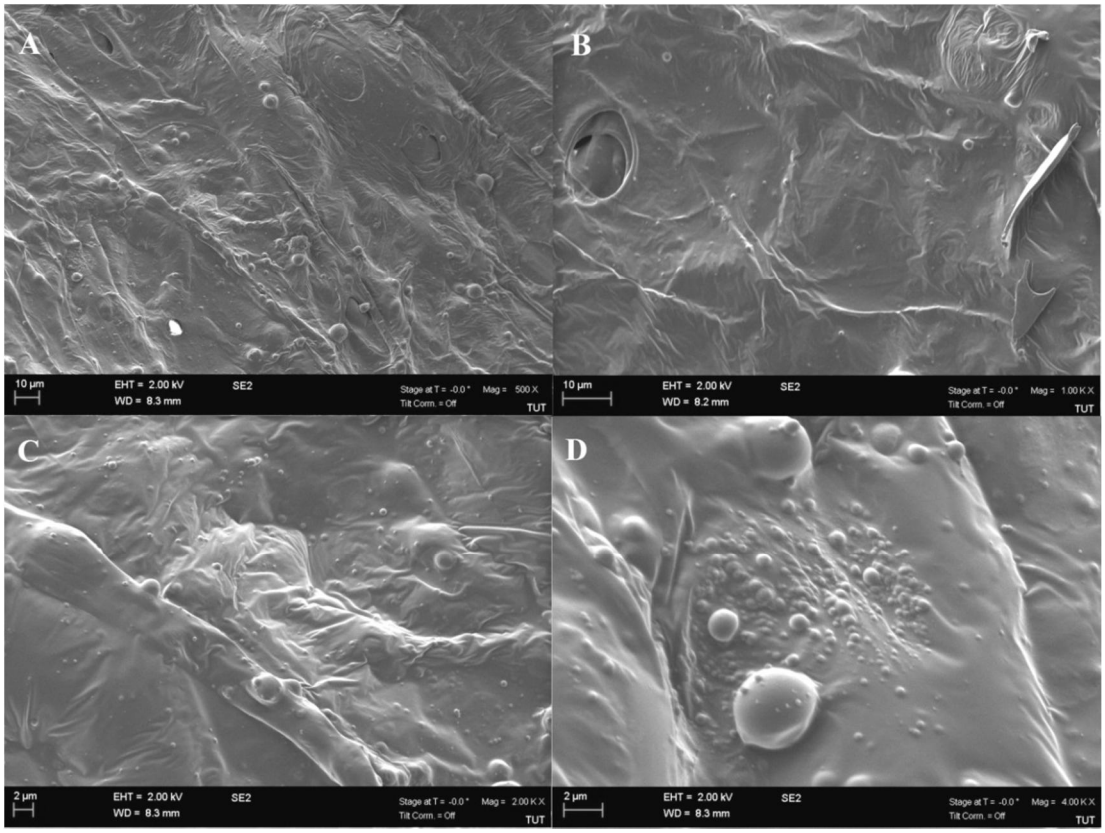

Figure 22: *SEM images of hydrogel-microparticle composite after five weeks of hydrolysis. A)-D) Microparticles in the degrading hydrogel with different magnifications. Scale bar is A) 10 μm, B) 10 μm, C) 2 μm, and D) 2 μm.*

5.2.2 *In vitro* drug release from hydrogel-microparticle composite

Cumulative release of DEX from the hydrogel-microparticle composite is shown in Figure 18, Chapter 5.1.4 (green triangles). Drug release profile follows the same bi-phasic profile as drug release profiles of the microparticles and gamma sterilized microparticles. But, release is faster than in either microparticle drug release

experiments. These results are confusing, as even more sustained drug release was expected. During the burst release, first four hours, 49.6 % of the theoretical drug loading is released. The maximum DEX concentration, 62.0 % of theoretical drug loading, is achieved already in 24 hours. Therefore this is also the time during which drug release is completed.

Chromatogram of four week drug release sample is shown in Figure 23. Depart from chromatograms of microparticle drug release samples seen in Figure 16 and Figure 17, Chapter 5.1.3, there is no peak for degradation product of the DEX.

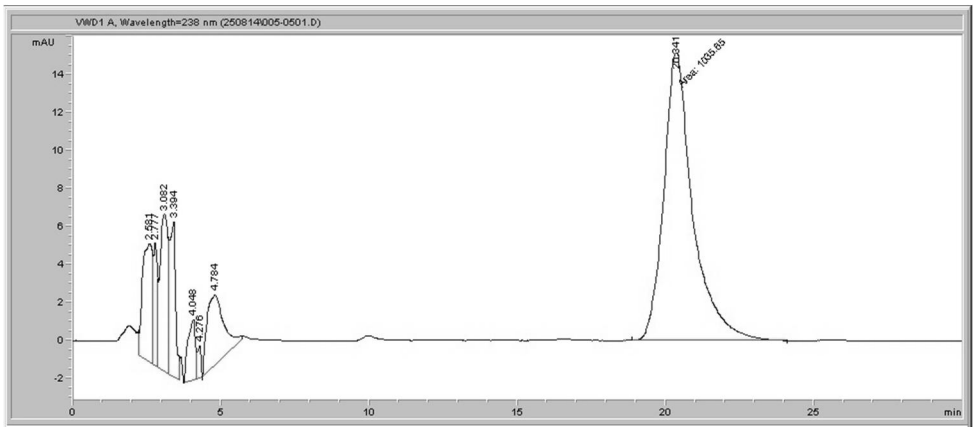

Figure 23: *HPLC chromatogram of hydrogel-microparticle composite drug release sample at time point 28 days.*

It may be supposed that DEX does not degrade the same way in the presence of the hydrogel. In the absence of hydrogel, active DEX starts to degrade in the PBS at 37 °C.

5.2.3 Mechanical analysis of the hydrogel-microparticle composite

Mechanical properties of the hydrogel-microparticle composite were evaluated by compression test as described in Chapter 4.3.6. Results of the mechanical tests were compared with the results of the control hydrogel without microparticles. Stress-strain curves of the most representative samples of the hydrogel-microparticle composite and control hydrogel populations are shown in Figure 24.

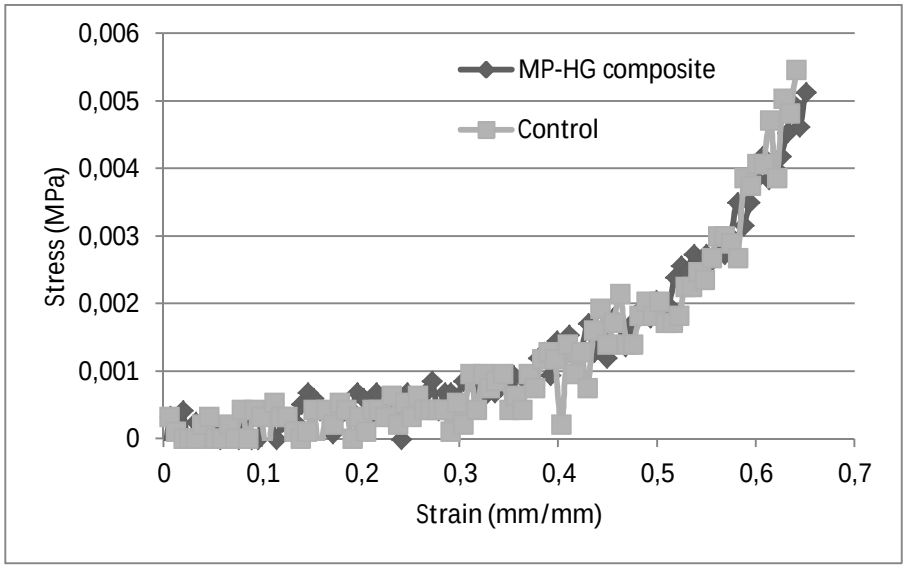

Figure 24: *Stress-strain behaviour of the hydrogel-microparticle composite and control hydrogel. Curves represent measurement of the most representative samples of four parallel samples in both cases.*

In both cases, there were five parallel samples of which one was excluded because of strange behaviour of the sample. As seen from the Figure 24, curves follow very similar pattern, which is typical to the HA-PVA hydrogels. Either of these two hydrogels did not fracture.

In the beginning of the curve there is area, where sample do not yet resist the increasing stress. First, sample is settled equally between the measuring probes and then polymer chains orient themselves. Both hydrogels started to resist force in the same strain value of 0.25 mm/mm. Slope rises linearly from this strain value to the approximately stress value of 1.5 kPa. It is the region into where trend line was drawn and according to this trend line Young's modulus was calculated. This linear region is called linear viscoelastic region of the stress-strain curve and thereafter slope starts to raise steeper. Average Young's moduli with standard deviations of the composite and control gel are presented in Figure 25.

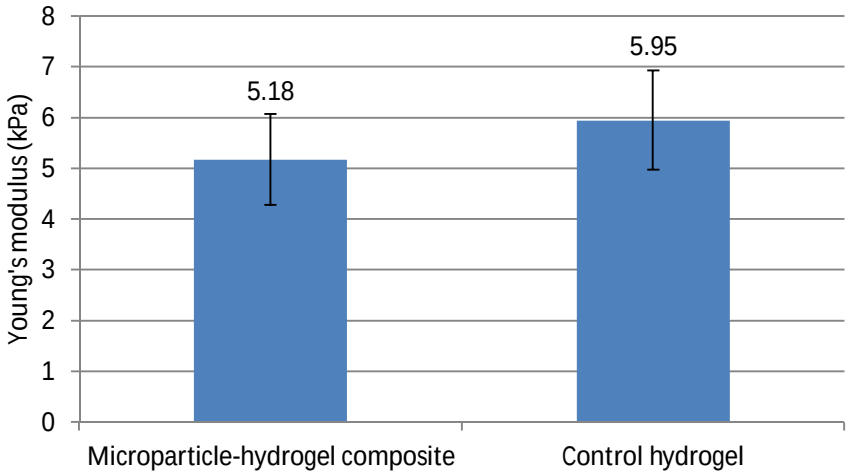

Figure 25: *Compression test results of hydrogel-microparticle composite and control hydrogel. Young's moduli and standard deviations are reported. In both cases n = 4.*

Values of the Young's modulus are very similar, 5.18 kPa for the hydrogel-microparticle composite and 5.95 kPa for the control hydrogel. So, similarity in mechanical properties, which were seen in stress-strain curves, is obvious also when values of Young's modulus are compared.

5.3 Muraglitazar loaded microparticles

In this study, peroxisome proliferator-activated receptor (PPAR)-agonist drug muraglitazar was encapsulated into the PLGA-PVA microparticles. This study is unique, because it is not published in the literature before. The microparticles were prepared exactly the same way as DEX loaded microparticles using combination method of emulsion method and spray drying.

The preceding study before microparticle preparation was test of muraglitazar's solubility in organic solvents. Solvents into this test were chosen according to the preparation method and they are listed in Chapter 4.4.1. In this study, 9:1 dichloromethane to ethanol mixture was used to prepare DEX microparticles as DEX dissolves to ethanol. That is the reason why the mixture as well as pure ethanol is chosen to solubility study. Protocol of the test is described in Chapter 4.4.1 and solutions were viewed by eye. Muraglitazar was dissolved into the ethanol fastest, but it cannot be used solely to prepare microparticles. Between rest of the solvents, no significant difference was noticed. Mixture of dichloromethane and ethanol was chosen because ethanol improves solubility of the drug and dichloromethane is trustworthy solvent in preparation of the microparticles. As a great advantage, no changes in preparation method are needed.

5.3.1 Size and morphology of muraglitazar loaded microparticles

Visual appearance of muraglitazar loaded microparticles did not differ from other microparticles prepared in this study. They seemed white powder as expected. Changes was not expected either in more detailed imaging with SEM (Figure 26). Also here, microparticles form aggregates (Figure 26A). Mostly, microparticles are smooth and spherical (Figure 26B and 26C), but some irregularities can be seen in shape (Figure 26D). As mentioned before, effects of the electron beam of the microscope were noticed which may cause some error in shape of the microparticles. Also processing temperature during the spray drying varies from batch to batch, and higher temporary outlet temperature in the process may affect to the microparticles.

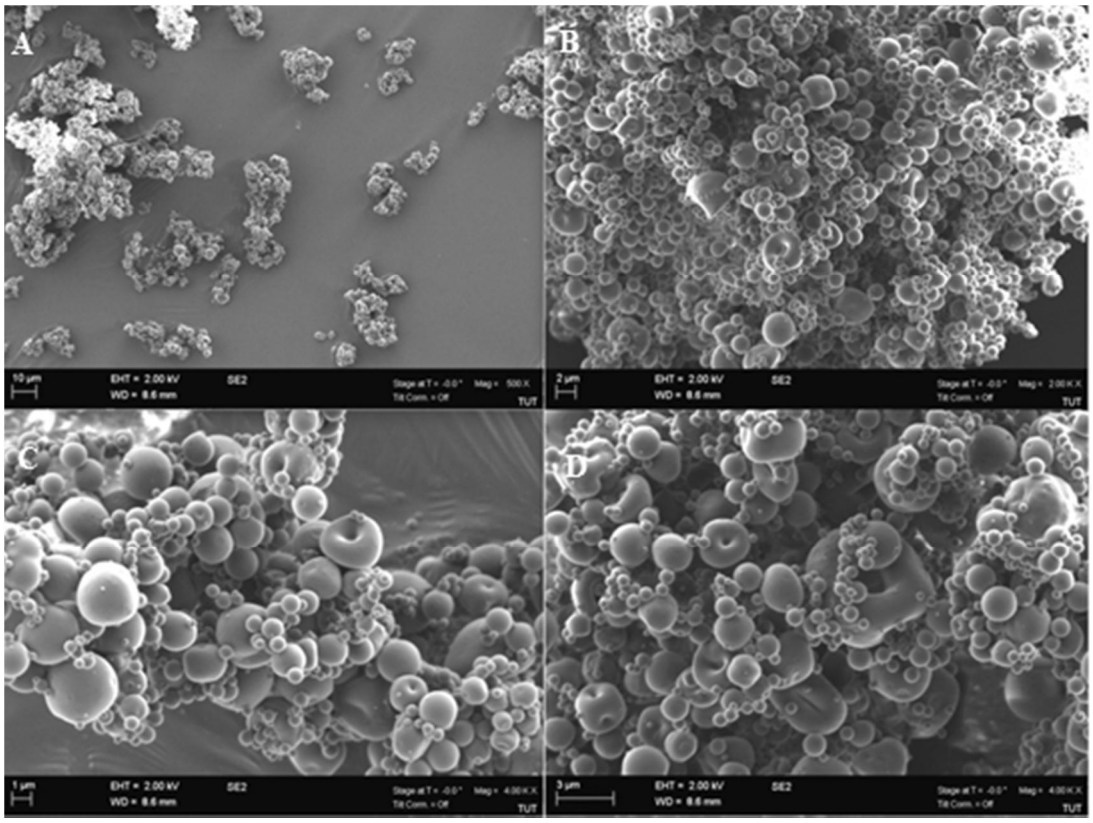

Figure 26: *SEM images of muraglitazar loaded microparticles. A)-C) Microparticles imaged with different magnifications, and D) Irregularities in the surface of the microparticles. Scale bar is A) 10 µm, B) 2 µm, C) 1 µm and D) 3 µm.*

Size distribution of the muraglitazar loaded microparticles is presented in Figure 27. Population of 209 microparticles was measured from SEM images. Diameters of the muraglitazar loaded microparticles varied from 172 nm to 4.3 µm and the most frequent size range was 501-1000 nm.

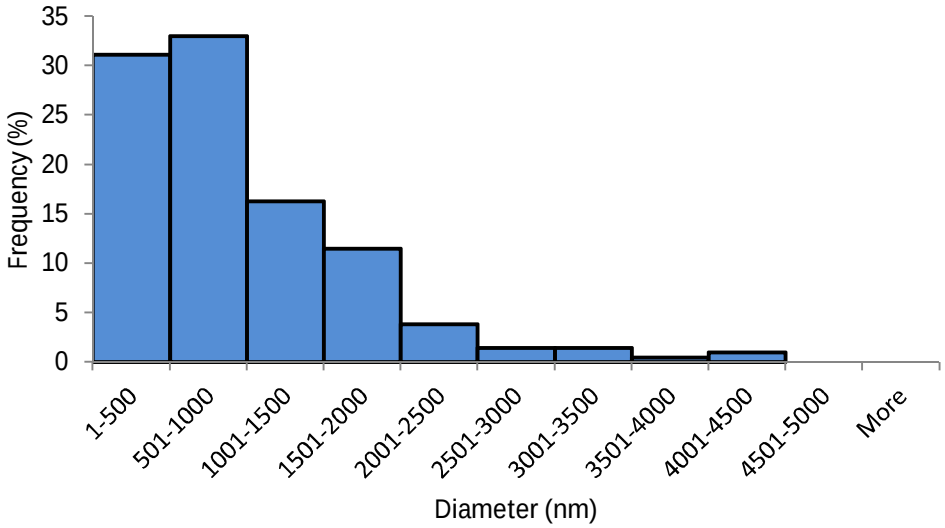

Figure 27: *Size distribution of muraglitazar loaded microparticles.*

Size distribution of the muraglitazar loaded microparticles follows same trend as DEX loaded microparticles. This is desirable result, as only change in preparation method was the drug.

5.3.2 *In vitro* drug release from muraglitazar loaded microparticles

In vitro drug release test was performed as described in Chapter 4.4.4. Cumulative drug release profile of muraglitazar from the microparticles is shown in Figure 28. Release profile follows again bi-phasic release. During burst release, first four hours, 38.8 % of the theoretical drug loading is released to the surrounding PBS. Again, amount of drug released during first few hours includes free, non-encapsulated drug. Burst release is followed by slower release period. Drug release test period in this study was 28 days, but release seems to be completed after seven days after which drug concentration remains at the same level, at approximately 74.0 % of theoretical drug loading.

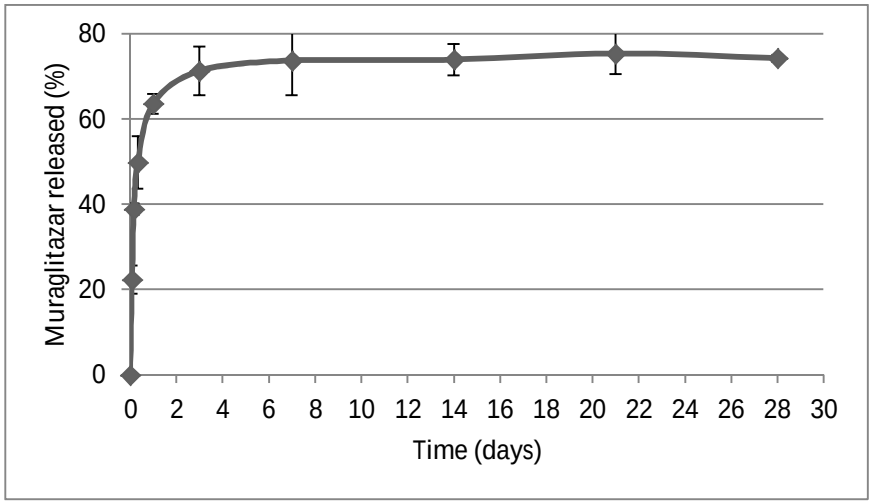

Figure 28: *Cumulative release of muraglitazar at 37 °C in PBS buffer, pH 7.4. Reported as percentual release with standard deviations. N = 3.*

The chromatogram of muraglitazar drug release sample is presented in Figure 29. Chromatogram shows clear peak, which was verified as muraglitazar peak with standard sample. Additional peaks indicating degradation products of impurities are not detected.

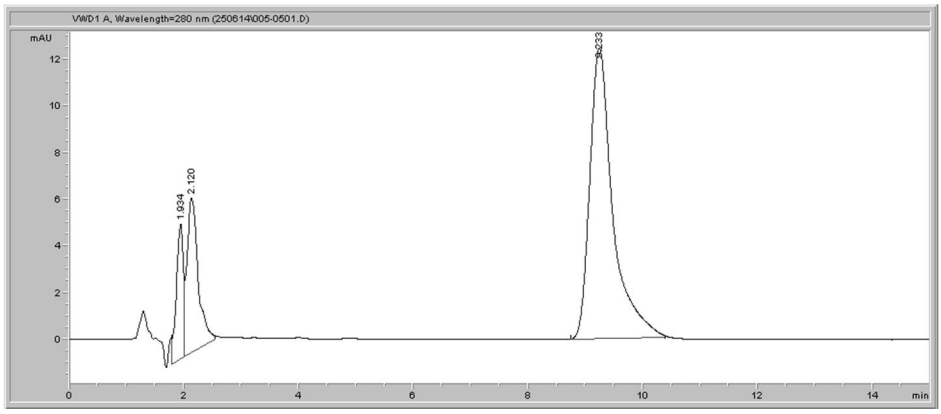

Figure 29: *HPLC chromatogram of muraglitazar drug release sample at time point 28 days.*

Muraglitazar content in the microparticles before and after drug release was attempted to determine. Because the corresponding test with DEX loaded microparticles failed, improvement to the method for sample preparation was made. Despite longer exposure to the solvent, stirring overnight and sonication, microparticles did not disintegrate completely. Improved destroying method resulted in more homogeneous solution, but still appearance was milky instead of clear. According to HPLC analysis, initially the microparticles would contain 68.2 % ± 2.8 % of theoretical muraglitazar loading. However, it is again lower value than drug release test indicates. Concentration near 100 % was expected, as also non-encapsulated drug is included in the powder. Residual drug content test did not show any peak in the HPLC chromatogram. So, residual drug content could not be determined.

6 DISCUSSION

6.1 Dexamethasone loaded microparticles

In this thesis, dexamethasone (DEX) loaded PLGA-PVA microparticles were prepared using combination of emulsion method and spray drying according to the protocol of Ugur (2014). This microparticle system (including both drugs, DEX and muraglitazar) were developed and investigated as possible inflammation relieving system for articular applications. Slight modifications were done to the preparation process after previous study, because during solvent evaporation step, crystals started to appear on the surface of the emulsion. Likely, these crystals were PLGA, which would lead to the loss of the matrix polymer during the process. In order to avoid forming crystals, solvent evaporation step was passed completely and emulsion was spray dried immediately after emulsification.

The efficiency of the preparation method was evaluated by means of yield of the process. In previous studies (Ugur 2014) yield remained really low when using the standard cyclone in the spray dryer. Yield of the produced microparticle batches in this study, increased substantially, approximately 60 % in both cases, microparticles solely and with sucrose. The new, high performance cyclone played an important role in this improvement of the yields, as it is possible to separate smaller particles and particles on the whole more effectively from the air flow than with larger standard cyclone. Maury et al. (2005) reported 20-35 % higher yields with high performance cyclone than standard cyclone. However, the spray dryer was not working properly before cleaning the aspirator and changing the filter of the device. These service operations enabled more efficient and unimpeded air flow in the spray dryer, which affected to the efficiency of the whole process. Also time required for spray drying process decreased. Despite the improvement of the process after these service operations, there is concern that equipment is not working perfectly. That is because, processing parameters were hard to keep constant, and especially outlet temperature varied a lot even when all other variables maintained unchanged. In addition, inlet temperature in this study had to be adjusted relatively high (70 – 80 °C) to achieve outlet temperature of 38 °C. Compared to literature (Mu & Feng 2001; da Silva-Junior et al. 2009; Ungaro et al. 2012) higher outlet temperature should have been reached using these parameters. Temperature difference between inlet and outlet temperature affects to the residual moisture content in the microparticles. The higher the difference, the higher is the amount of moisture in the product (Büchi 2002). Especially with long storing times this moisture content may affect to the microparticles, because water plasticizes amorphous polymer such as

PLGA (Bouissou et al. 2006). Of course, outlet temperature is depended also on other factors than inlet temperature. Still, difference in spray dryer behaviour was not noticed during drying microparticles without sucrose compared to microparticles with sucrose even though solid content of the feed solution was higher solid content. It is very important to bear in mind the importance of the regular service of the equipment.

Yield near 100 % would be desirable, but with spray dryer it is impossible in practice as always part of the drying microparticles adhere to the glass walls of the drying chamber. Also some microparticles can be seen in glass parts leading from drying chamber to the cyclone and also in the walls of the cyclone. These adhered microparticles are impossible to collect from the walls. It is also impossible to collect all the microparticles from the collection vessel due to antistatic charges even if collection was done near ionizer antistatic system (Mettler-Toledo, Switzerland). There are also different kinds of antistatic mats on the market and they may be useful aid for this purpose.

SEM images of the DEX loaded microparticles (Chapter 5.1.2) revealed smooth and spherical surface. Some irregularities were seen on the surface of the larger microparticles. They may have been formed during the spray drying process, but also changes in the samples were noticed when exposed to the electron beam during the imaging (Loo et al. 2005). With the help of these pictures, any conclusions of the inner structure of the microparticle cannot be drawn even though outer surface is smooth and dense. Still, underneath the surface, structure may be dense, porous or even hollow. It would have been interesting to see SEM pictures of these same microparticles after drug release. There are many factors that affect to the structure of the particles, but probably the most important is the preparation method (Yushu & Venkatraman 2006). Here, combination of two methods is used. First, oil-in-water (o/w) emulsion is prepared followed by immediate spray drying. At least in case of o/w solvent evaporation method, solvent evaporation affects the structure of the forming particles significantly. The faster the solvent evaporation rate is, the more porous the structure of the microparticles will be (Freitas et al. 2005; Yushu & Venkatraman 2006). In previous studies (Ugur 2014) solvent was let to evaporate and microparticles solidify by stirring it before spray drying. In this study, method was changed a bit due to forming crystals during solvent evaporation and fear of losing the matrix polymer. Solvent evaporation phase was passed completely. So, forming microparticles were still oil droplets among aqueous phase during spray drying. Solvent evaporation rate during spray drying is much faster due to heat. Therefore, this change in the method may have influence on the inner structure of the microparticles.

DEX loaded, sucrose coated microparticles showed similar morphology than microparticles without sucrose. Morphology studies about spray dried sucrose were hard to find, but results about spray dried mannitol are promising. Outlet temperature during spray drying and droplet size affects to the surface topography of the particles. Lower outlet temperature (60 °C) results smooth surface whereas, higher temperatures caused rough surface due to formation of the larger mannitol crystals (Maas et al. 2011).

In addition to effect of the outlet temperature, Littringer et al. (2013) reported also the smooth topography when diameter of the microparticle was 10 µm compared to 80 µm. These results may explain unchanged, smooth surface topography of the microparticles in this study. However, sucrose and mannitol are not directly comparable, since other is disaccharide and other is monosaccharide with higher T_g. Instead, gamma sterilized microparticles showed very different morphology compared to unsterilized microparticles. It is well known that gamma irradiation has harmful effects to the polymers, especially when size of the device is small. It also explains structural changes of the microparticles. Gamma irradiation causes scission of the polymer chains, which decreases the molecular weight of the polymer. This, together with increasing temperature caused by irradiation leads to faster degradation of the polymer. (Martínez-Sancho et al. 2004) Especially larger microparticles with diameter more than one micrometer had high degree of irregularities, smaller tips on the surface or collapsed structure. Thus, polymer matrix inside the microparticles has either melted or in case of porous inner structure, walls between pores have collapsed. There is no doubt; gamma irradiation has adverse effects on the microparticles. Interestingly, microparticles with diameter less than 1 µm had no visible marks of degradation or melting, and seemed to maintain unharmed. This could refer to denser inner structure in small microparticles than in larger ones. Porous structure is more vulnerable to collapse than dense structure when exposed to conditions that accelerate degradation of the matrix (Martínez-Sancho et al. 2004). Assumption, that smaller microparticles have denser structure than larger microparticles can be made.

The change in preparation method of the microparticles have also affected to the size distribution of the microparticles. Microparticles obtained by the old method with solvent evaporation, had diameters between one and five micrometers. In this study, diameters varied from approximately 200 nm to 5 µm. Most frequent size range being 501-1000 nm. The widening of the size distribution can be explained with the absence of the solvent evaporation phase and so on microparticle-hardening phase. Previously, feed solution for the spray dryer contained already hardened microparticles in PVA solution. In this study, feed solution contains oil droplets in the aqueous phase, as mentioned before. When this kind of emulsion is fed through the spray dryer, the pneumatic nozzle disperses feed solution, not only aqueous phase but also oil droplets containing polymer and the matrix. Therefore, desired microparticle size achieved by homogenator is lost, and new, smaller droplet size of the oil droplets determines the size of the microparticles. There are still larger microparticles (diameter near five micrometers) in the product, because spray drying process lasts few hours and solvent from the feed solution can evaporate during that time. The emulsion was continuously stirred, and even though container was covered with parafilm, dichoromethane is able to evaporate. So, at the end of the process some of the microparticle may be hardened and cannot be dispersed further.

Sucrose covered microparticles showed interesting size distribution. In this sample, solid content in the emulsion preceding the spray drying is higher because of the

sucrose. Higher concentration of the feed solution should result in larger size of the microparticles (Büchi 2002). Some of the dissolved sucrose is probably covering the PLGA-PVA microparticles some of it may have formed separate sucrose particles. Larger sizes of the microparticles were expected, because of this extra layer around the microparticles. Instead, the diameter of most of the microparticles is between 181-500 nm. Results are somewhat surprising because amount of large microparticles (> 2 μm) in this sample is in the same range than in sample without sucrose. It means that adding sucrose does not result in microparticle loaded sucrose particles when solution is spray dried. Another important observation is that sucrose particles have also very smooth surface without any marks of sugar crystals. Sucrose particles cannot be distinguished from any other microparticles prepared in this study. Nevertheless, sucrose coated microparticles differ from microparticles without sucrose in amount of particles near nanoscale. There seems to be slightly more microparticles with diameter less than 1 μm than in other samples. The most probable explanation is water-soluble sucrose that is fully dissolved into the continuous, watery phase, which surrounds oily droplets including solvent, polymer and drug. When this emulsion is sprayed through atomizing device of the spray dryer, watery solution may be spread to smaller droplets than more viscous oil droplets due to frictional forces. Unfortunately, this kind of study could not be found from the literature. Instead, similar studies but using mannitol exists. For example, Takashima et al. (2007) reported suppressed aggregation of PLGA nanoparticles when 1.79 % mannitol was added to the nanoparticle emulsion before spray drying. Size of the nanoparticles decreased with increasing mannitol concentration, which means that with higher concentrations (more than 1.79 % w/v) fewer aggregate were formed. In addition, uniform sugar layer around the nanoparticles did not affect to the size of the microparticles. It was approximately the same before spray drying and after spray drying with mannitol. Also Adi et al. (2010) reported unchanged size of the drug particles with and without mannitol. If this is true with sucrose, it would explain why microparticle size did not increase after adding sucrose to the spray dried emulsion.

According to the literature, sucrose is not the best excipient for preventing aggregation of the microparticles during spray drying. Due to its low T_g (62 °C), the final product is relatively sticky powder, compared to sugars with higher T_g (Chaubal & Popescu 2008). Sucrose was chosen into this study, because it is also used in preparation of hydrogel, and microparticles including sucrose were prepared exclusively to facilitate preparation of hydrogel-microparticle composite. Since the composite gel included already many different components, choosing the sucrose did not increase the amount of components.

In vitro drug release profile from the DEX loaded microparticles followed bi-phasic drug release profile. Including burst release within first few hours, followed by slower release phase (Figure 18, Chapter 5.1.4). This is typical for PLGA microparticles, especially with small diameter (Berkland et al. 2003; Berchane et al. 2007; Fredenberg et al. 2011). Compared to the release profile of previous study (Ugur 2014), prolonged

drug release profile is achieved (53 hours against 28 days) with lower burst release (21.5 % against 57% within first four hours). The *in vitro* results from all DEX related drug release tests are collected to the Table 8. Burst release is fast release phase in the beginning of the release period. The amount of drug released during burst release in this case includes drug located near the surface, but also non-encapsulated drug, and drug that is adhered to the surface of the microparticles. When using this preparation method for microparticles, in which the emulsion without solvent evaporation is spray dried, isolation of the microparticles from the surrounding PVA solution and free drug cannot be done. Therefore, final product includes also free, non-encapsulated drug and PVA.

Table 8. *Collection of dexamethasone related drug release results.*

Drug release from	**Initial burst (4h)**	**$[DEX]_{max}$**	**$T_{[DEX],max}$**
MP_DEX	21.5 %	53.0 %	28 days
MP_DEX (gamma)	25.1 %	54.6 %	7 days
HG_DEX	49.6 %	62.0 %	1 day

MP indicates microparticle,
$[DEX]_{max}$ maximum DEX concentration reached and
$T_{[DEX],max}$ time required to achieve the maximum concentration.

In case of drug delivery device, it is important to remember that PLGA with different molecular weights, lactide-glycolide ratios and end-group capping behave very differently. In addition, properties of the PLGA change during the degradation. (Fredenberg et al. 2011) Time required until onset of the degradation ranges from weeks to months. For of PLGA with lactide-glycolide ratio of 75:25, Resomer® gives 270 days during which weight of the microparticles has decreased 50 %, and Medisorb® 4-5 months. (Yeo & Park 2004) In drug loaded microparticles, the most used lactide-glycolide ratio of PLGA is 50:50 and drug release period can vary from days to months (Rawat & Burgess 2010). Typically, DEX has been released from PLGA microparticles during 20-40 days, with higher lactide amount even 80 days. Diameters of the microparticles ranging from few hundred nanometer to few dozen micrometers. (Galeska et al. 2005; Kim & Martin 2006; Zolnik & Burgess 2008; Bhardwaj & Burgess 2010; Rawat & Burgess 2010; Shen & Burgess 2012) As concluded in the literature, DEX release from the nanoparticles is faster than from microparticles (Song et al. 1997; Gómez-Gaete et al. 2007). In this study, release period was prolonged from few days to four weeks by making changes to preparation method, but still drug release rate is relatively fast considering the copolymer composition. There are several reasons for fast drug release from the microparticles.

First is preparation method. The most important factor influencing the properties of the DDD is the preparation method. (Yushu & Venkatraman 2006) Microparticles prepared in this study cannot be directly compared with either microparticles prepared by o/w solvent evaporation method nor spray drying method because both preparation

methods affect to the microparticle properties in their own way. In the literature there are no studies where hydrophobic drug would have been encapsulated into the microparticles by combination of these two methods as in this study. Spray drying process expose the feed emulsion and forming microparticles into the stress conditions, such as shear stress in the nozzle and thermal stress in the drying chamber, which may affect to the polymer-polymer interactions during and after preparation. (da Silva-Junior et al. 2009) On the other hand, also emulsion preparation phase create own challenges to the microparticle preparation, since parameters such as polymer concentration, ratio of dispersed phase and continuous phase influences to the properties of the microparticles. Change in preparation method in this study leads to faster solvent evaporation rate. This change has been noticed to affect to the other properties listed here below.

The second reason is small size of the microparticles. Diffusion plays important role in drug release from PLGA microparticles. When device dimensions increase, in other words, size of the device increases, diffusion pathways become longer and drug release has reported to be slower. (Siepmann & Göpferich 2001) So, drug release becomes faster with decreasing diameter of the microparticles. Microparticles in this study have smaller diameters and wider size distribution than in previous study. Interestingly, drug release period was longer even though diameter of the microparticles decreased. Change to the preparation method was done because of the crystals during the solvent evaporation. Therefore, matrix polymer was not lost which may be the reason for improved drug release profile.

Third reason is possible inner porosity. Again, preparation method affects to the structure of the microparticles as mentioned in this chapter earlier. In general, faster solvent evaporation rate results in more porous structure of the microparticles. Still, there are conflicting results. For example faster solvent evaporation caused by increased temperatures leads to the dense surface due to fast solidifying of the surface, but porous or hollow core. (Freiberg & Zhu 2004) This may be the case when oil droplets are hardened during spray drying process. On the other hand, in solvent evaporation method, low solvent evaporation rate leads to the diffusion of aqueous phase to the dispersed phase causing more porous structure (Yeo & Park 2004). Drug release rate is faster through porous matrix compared to dense matrix.

Fourth reason is heterogeneous drug distribution. Location of the drug in the device, may affect to the release profile. Surface associated drug is the main reason for the burst release (Yeo & Park 2004). In case of microparticles, their size may affect to the drug distribution within the matrix. (Berkland et al. 2003) Location on the other hand, may be affected by physico-chemical properties of the drug (Sandor et al. 2001) In addition, the use of method in which both, drug and polymer is completely dissolved into the solvent enables homogeneous distribution of the drug and the polymer in the solution. Therefore drug is homogeneously distributed in the droplets in the solvent evaporation step of the spray drying process, which may lead also to the homogeneous distribution of the drug in a polymer matrix in the microparticles. After all, drug distribution

depends on the rate of the solvent evaporation and hydrophobicity/hydrophilicity of the drug and the polymer. (da Silva-Junior et al. 2009)

Fifth reason is polymer-polymer interactions. In this study final product includes also emulsifier polymer PVA. Amount of PVA in the powder is high and it may have effect on the drug release properties of the microparticles. PVA interferes with the dichloromethane in the solvent evaporation phase and forms a layer around the forming microparticles. In addition, microparticle powder probably includes PVA microparticles. Presence of PVA has been reported to affect to the wettability of the microparticles, making the surface more hydrophilic (Lee et al. 1999). In addition, it may lead to slower microparticle degradation and slower drug release. Or contrary, PVA layer may act as barrier that prevents diffusion of acidic degradation products of PLGA out from the microparticle catalysing degradation. (Panyam et al. 2003) In addition, PVA may act as plasticizer and affect to the mobility of the PLGA. Polymer chain mobility, on the other hand affects to the interactions between other polymers, incorporated drugs and any additive.

Sixth reason is small drug molecule. Low molecular weight drugs are more likely to have burst release profiles because of the small size of the drug molecule. (Huang & Brazel 2001) For hydrophobic drugs, the burst effect depends on the affinity of the drug for the solvent–water phase versus the solvent–polymer phase. If the drug has a higher affinity for the solvent–water phase, which initially surrounds the device, then a high burst effect will be observed. (Hatefi & Amsden 2002)

Predicting the drug release is difficult, since there are so many different factors affecting to the drug release from the biodegradable device influencing simultaneously to the degradation of the microparticles and drug release mechanisms as described in Chapter 3.3.2. However, release profile can be modified. Several methods exist. For example drug release from PLGA microparticles has been modified successfully by blending end-capped and uncapped PLGA (Friess & Schlapp 2002). Blending resulted more sustained release than from either polymer alone. One possibility is to change matrix polymer completely, by coating the microparticles with other more slowly degrading polymer (Berkland et al. 2004), or by coating drug loaded microparticles with the same but drug-free polymer (Ahmed et al. 2012). Spray dryer used in this study (Büchi Mini Spray Dryer B-290) enables simultaneous preparation of microparticles and their coating with another solution when using four-fluid nozzle. Crosslinking of the outer surface of the microparticles is also one way to delay the drug release (Thote et al. 2005). Slower drug release is achieved also by changing the solvent during microparticle preparation. Darbandi & Zandkarimi (2012) replaced part of the dichloromethane with ethyl acetate, achieved higher density microparticles with slower drug release rate. Finally, it would be interesting to investigate these microparticles after isolation from the excess of PVA. It is true, that PVA stabilizes the emulsion and the microparticles in the preparation phase, but it also increases the amount of microparticle powder in order to reach certain drug concentration. Unfortunately, the isolation would require further changes in preparation method, because aqueous phase including PVA is

not possible to remove when microparticles are still in the form of soft oil droplets. However, with Inert Loop B-295 equipment, it would be possible to exclude PVA completely or at least decrease the amount of PVA. In this study, Inert Loop was not possible no use because it is not working. In the future, reparation of this accessory is recommended.

Drug release test with DEX loaded, gamma sterilized microparticles shows accelerated drug release profile. Drug released during burst release is only slightly increased (25.1 % against 21.5 %). Instead, maximum DEX concentration is reached already within one week. This kind of affected drug release profile was expected based on the SEM images. So, drug release profile confirms that microparticles are seriously affected due to gamma irradiation and/or temperature rise during the irradiation. Others report accelerated drug release profiles compared to unsterilized microparticles as well (Mohr et al. 1999; Çalış et al. 2002; Carrascosa et al. 2003). They noticed acceleration of drug release rate to be dose dependent. There are also plenty of studies were gamma irradiation has significant effects on neither morphology nor drug release rate of the microparticles (Montanari et al. 2001; Boix et al. 2003; Martínez-Sancho et al. 2004). According to these studies, adverse effects of the gamma irradiation on the PLGA microparticles can be minimized by using decreased temperatures (Montanari et al. 1998; Martínez-Sancho et al. 2004) or by giving the radiation dose slowly (Montanari et al. 2001; Keles et al. 2014). Cooled environment were achieved by using dry ice or liquid nitrogen. Dose rate in this study was approximately 5-9 kGy higher than in slow rate studies. Given dose was approximately the same (30.5 kGy against 25 kGy). Thus, the microparticles in this study were exposed to the tough radiation conditions. Milder conditions may not have resulted as severe effects.

Drug release tests with sterilized and unsterilized microparticles show that only 50 % of theoretical drug loading is released from the microparticles. Probably in published studies released amounts of drug are compared to real drug loading of the microparticles and real drug loading is only rarely near theoretical drug loading. In this study, theoretical drug loading is used because initial drug loading test failed as described in Chapter 5.1.4. The drug release from hydrogel-microparticle composite shows, that higher drug concentration can be achieved.

The most likely reason for such low maximum drug concentration in the sample is degradation of the DEX. First prolonged drug release tests with DEX loaded microparticles revealed the time dependently growing secondary peak next to peak of active DEX (Figure 16, Chapter 5.1.3). The assumption of degrading DEX was made, and it was confirmed with degradation test of DEX. Figure 19 (Chapter 5.1.6) shows linearly decreasing concentration of active DEX in the PBS. The reason for degradation is unclear. The chemical stability of DEX is said to be relatively high (Chen et al. 2008). There are huge amount of drug release studies concerning DEX, but only Hickey et al. (2002) and Zolnik & Burgess (2008) mention degradation of the DEX. Hickey et al. (2002) reported degradation of the DEX during drug release test and secondary peak in HPLC chromatogram. They showed similar descending slope as in this study to confirm

the degradation. They also tested the effect of sodium azide (NaN_3) and it was not affecting the results. Their drug release results resemble ours in every way. They achieved also maximum drug release level of 50 % of the drug loading. Their microparticles have diameter of 11 µm, which explains slightly longer release period. Unfortunately, they did not determine the chemical structure of the degradation product. Zolnik & Burgess only mention that drug release product is included to the cumulative drug release. Nevertheless, many possible degradation products as well as impurities have been reported. According to Spangler & Mularz (2001) the main degradation product of the DEX would be 17-ketone of DEX. In contrast, in human body DEX is metabolized in liver by cytochrome P450 3A4 enzyme. Hence, *in vivo* the main metabolite would be 6beta-hydroxydexamethasone. (Gentile et al. 1996) Possible reason for only one report about degradation of the DEX during drug release may be different methods for drug release tests. Most of the researchers have replace most of the PBS at certain time points and are probably avoiding the degradation. In this study, separate tube in each time point is used. Therefore, drug is very long time in the same tube and in the same PBS solution. That is why, for example, oxidation of the DEX cannot be excluded. According to chromatograms from this study, the only idea about the degradation product is that it is more hydrophilic than active DEX, because elution time for more hydrophilic products is shorter. Degradation means, that drug concentration cannot be accurately determined after one-week time point. Without degradation, drug concentrations would be higher, and so on, the slope of the curve would be steeper at the end of the drug release period.

There are also other possible reasons for low maximum drug concentration in drug release samples. One possible reason is secondary crystallization of PLGA degradation products during incubation (Yoshioka et al. 2010; Fredenberg et al. 2011). These crystals may entrap the drug inside. Shorter oligomers from PLGA may also react with PVA and prevent rest of the drug coming out from the system. This may also explain failing of the residual drug content test. Microparticles were even more difficult to destroy by dissolving after incubation than before incubation. Especially in case of hydrophobic drugs, affinity of the drug to the test tubes may cause error (Wischke & Schwendeman 2008)

The degradation of the DEX lead to serious problems with HPLC, since concentration of the active DEX could not be determine when partly overlapping secondary peak started to appear. Original method for HPLC was adopted from previous study. That included only 53-hour drug release test and that is why this problem did not emerged then. The huge amount of effort and time was demanded to resolve the problem and finally it resulted the development of totally different method. All different changes to the method are listed in Appendix 1. In this study also revealed that despite filtering the drug release sample, they precipitated with eluent. Precipitate may block the column and destroy it. First change to analyzing method was pretreatment of the sample with absolute ethanol in order to precipitate possible impurities from the sample. This change was done before first longer drug release test

that revealed the degradation. First the simplest ways to separate the peaks from each was tried. These included changing the water-acetonitrile ratios in eluents, prolonging gradient elution method and changing the flow rate of the eluent. Next step was verifying that ethanol is not affecting to the samples. Acetone as precipitant was tested, as well as evaporation of precipitant and filtration with 3 kD Amicon filters. Then, polarity of the eluent was increased by adding acid (Spangler & Mularz 2001; Arthur et al. 2004; Lu et al. 2010). At that time, trifluoroacetic acid (TFA) was available. After TFA, phosphoric acid (H_3PO_4) was tested. Finally, gradient elution program was completely abandoned, and widely used program using only one eluent including water, acetonitrile and phosphoric acid was tested (Acarregui et al. 2014; Bhardwaj & Burgess 2010; Shen & Burgess 2012). Finally, slight modification was done by reducing eluent flow rate. With this method separation of active DEX from degradation product was finally achieved.

6.2 Hydrogel-microparticle composite

In this study, a DEX hydrogel-microparticle composite was designed in order to modify drug release profile of the microparticles. Used hydrogel was prepared from aldehyde-modified hyaluronic acid (m-HA) and crosslinked with hydrazide-modified PVA (m-PVA). Microparticles were introduced in a form of microparticle suspension during preparation step of the hydrogel. Preparation of the hydrogel-microparticle composite is presented in Figure 10, Chapter 4.3.2. In previous study, preliminary tests with HA-PVA hydrogel with microparticles were done. Only microparticles including sucrose were used to prepare the composite gel. Analysis of these microparticles without hydrogel is handled in Chapter 6.1 DEX loaded microparticles.

Visual appearance of the composite gel compared to control gel is shown in Figure 20, Chapter 5.2.1. Control gel had typically very irregular shape, whereas composite gel is beautiful, regular cylinder. Difference between the composite and control gel is volume of microparticle suspension, which is practically extra volume of liquid. The total volume of the gels were every time the same. Therefore, polymer concentration is lower in composite gel than in control gel. This may be the reason for different shape of the gels. The same amount of liquid without microparticles cannot be added because it would affect to the gelation too much. In this case liquid would be distilled water. Despite the difference in polymer concentration, control gel and composite gel behaved very similarly in compression test as can be seen from results of mechanical tests (Figure 24 and Figure 25 in Chapter 5.2.3). Stress-strain curves are almost identical and Young's moduli, which is calculated using stress-stain curve with equation (9), does not show significant difference. The result is desired as substantial liquid volume of microparticle suspension is added to the hydrogel and still difference in compression test is not considerable large. Stress-strain curve shows also very elastic nature of these gels, since fraction point was not reached. Samples were compressed until 60 % of the heights of the samples were reached. This value was chosen because all the other

samples are compressed same way by now. May be the fraction point would have been reached with higher degree of compression, for example 80 %.

Distribution of the microparticles within the hydrogel was evaluated with SEM images. The gels were freeze dried before SEM imaging. Figure 21, in Chapter 5.1.1, presents fresh hydrogel-microparticle composite. Figure 21A shows structure of the control gel. Surfaces in control gel are very smooth. Any roughness and spots in composite gel (Figure 21B-21F) is caused by microparticles. Surprisingly, more visual microparticles within the gel were expected. It can be explained with the diameter of the microparticles and thickness of the walls of the dried hydrogel (Figure 21F). Small, microparticles with diameter less than one micrometer are easily embedded into the wall with thickness of two micrometers. Fortunately, all the hydrogel walls are not that thick, and microparticles can be seen in some extent even though most of the microparticles are in size range of 500-1000 nm.

Hydrogel was imaged with SEM also after five weeks degradation test. There, microparticles are much more visible than before hydrolysis. This indicates that walls between the pores of the hydrogel have become thinner due to degradation of the hydrogel structure. That makes also smallest microparticles visible through the walls. This proves that amount of microparticles in the gel is high, but they cannot be seen through the walls when diameter of the microparticles is only a fraction compared to thickness of the wall. In this point, some of the microparticles can also be degraded, so initial amount of microparticles in the hydrogel may be even higher than could be expected based on Figure 22 in Chapter 5.2.1.

Drug release test from the hydrogel-microparticle composite showed very fast drug release rate. It was much faster than with microparticles alone. This result was not expected, since more sustained drug release was expected. Even though drug release is hard to predict due to large amounts of possible interaction between components, hydrogel forms extra barrier between PBS and drug. Therefore, reasonable expectation is slower drug release profile. There is couple of explanations.

First is microparticle suspension. There is high probability that preparation of the homogeneous microparticle suspension required too much force and effort. Microparticle powder was stirred in distilled water with magnetic stirrer overnight to obtain smooth suspension. After getting results from drug release test, assumption, that drug was already released from the microparticles before preparation of the gel, arose. So, it is likely that drug release test does not show the reliable drug release of hydrogel-microparticle composite gel. Unfortunately, further testing with composite gel was not possible due to problem of analysis with SEM. The system may be improved by using higher concentration of sucrose, other sugar or other cryoprotectant in order to facilitate formation of suspension. This drug release test may be compared with drug release from hydrogel alone. Typical mesh sizes of the biomedical hydrogels are from 5 to 100 nm (Cruise et al. 1998; Mason et al. 2001). This is much larger than most of the small-molecule drugs which means that in swollen stage, diffusion of the drug is not significantly delayed. The case would be totally different with macromolecules like

proteins and oligonucleotides. Their diffusion, and so on release can be retained with hydrogel. (Lin & Metters 2006). For example, DEX was completely released from crosslinked, HA-based hydrogel films within approximately 90 minutes (Luo et al. 2000).

Second reason may be polymer-polymer interactions. If it is hard to predict drug release from microparticles, it is definitely more complicated in case of composite gel in which amount of different components is doubled. Composite gel includes four different polymers, PLGA, unmodified PVA, modified PVA and modified HA. In addition, there is sucrose. Any of these components may interact with some other and therefore accelerate the degradation of the matrix and the drug release.

Although, drug release test results are not reliable, other interesting results came up when drug release samples were analyzed. First of all, the maximum DEX concentrations in the hydrogel-microparticle composite release samples are almost ten percentage units higher than in microparticle drug release samples. That may be explained by another interesting result that came up when the composite drug release samples were analysed with HPLC. As shown in Figure 23 (Chapter 5.2.2), peak of the degradation product lacks completely. For the comparison, chromatogram of the microparticle drug release sample at same time point is shown in Figure 17F (Chapter 5.1.3).

There are two possible reasons for this phenomenon. First possibility is that degradation product is entrapped into the hydrogel or reacting further. Degradation product is probably very similar molecule with active DEX, but because of the difference it may be able to react with some component of the hydrogel. If it is the case, it is not visible in the chromatogram. Also here, active DEX is still released to the surrounding PBS, because it can be detected with HPLC. However, this theory does not explain the higher drug concentration reached in the samples if DEX would degrade and form a complex with other component. So, lack of secondary peak in the chromatogram cannot be explained by degradation product that is entrapped into the hydrogel. That is why higher DEX concentration and undegraded DEX, are probably interconnected to each other.

So, second and possibly more likely explanation is that DEX does not degrade in the same way in the presence of hydrogel, in other words, some of its component protects DEX from degrading. Concentrations of the active DEX in the composite release samples can be higher because DEX does not start to degrade when released from the composite. Possible protecting component must be m-HA, m-PVA or sucrose. The evidence that HA or PVA would protect DEX could not be found from the literature. Instead, sucrose is widely used stabilizer in the drug delivery.

Microparticle suspension was prepared using sucrose coated microparticles. In *vitro* drug release tests with microparticles were performed only with microparticles without sucrose. Sucrose including microparticles was used only to prepare hydrogel-microparticle composite, in more detailed to prepare homogeneous microparticle suspension. For protein stabilization during spray drying process, protective excipients

such as polyols, sugars, polymers, surfactants amino acids and proteins are used. Especially disaccharides sucrose and trehalose protects the native structure of the proteins effectively. Among these, sucrose has been reported to protect certain proteins even better than trehalose. (Liao et al. 2002) Sucrose acts as protective component also in nature, for example in seeds (Koster & Leopold 1988). The protective effect of sucrose is based on the hydrogen bonding with the active agent. Thus, it serves as water-substituent. It may also form glassy matrix with the active agent protecting it that way. (Akers 2002)

In DEX molecule, there are also functional groups that enable hydrogen bonding. Usually, these kind of protective excipients are necessary only with unstable molecules, like proteins. However, even though DEX is stable in air, long-term exposure to aqueous solution may affect to the drug. For example, if reason for DEX degradation is oxidation, sucrose molecules that surround the drug molecule immediately after release from the matrix may be very potential protector against free radicals and degradation.

So, more investigations concerning drug release from composite hydrogel is needed in order to find out the real effect of hydrogel on the drug release profile. Still, more sustained release is expected. In the literature, there are several publications about DEX releasing PLGA microparticle embedded into different hydrogels. More sustained drug release profile, compared to drug release from microparticles alone, has been achieved using alginate hydrogel (Kim & Martin 2006), PVA hydrogels (Patil et al. 2007; Bhardwaj et al. 2010) and PVA hydrogels including polyacids (Galeska et al. 2005). Burst release has been able to avoid with the composite hydrogel, even though more sustained drug release is not achieved (Norton et al. 2005). This study well establish difference in drug release from hydrogel (certain pore size) when using small molecular DEX and large molecular growth factor. In addition to DEX, many other drugs have been used in the composite gels with promising results. For example retinoids (Gao et al. 2012). Hydrogel-microparticle composites have also been investigated as co-delivery devices of two different drugs and/or growth factors (Patil et al. 2007; Lampe et al. 2011; Shin et al. 2013). However, hydrogel not necessarily delay the release from the microparticles. In case of poly (lactide) (PLA) microparticles in poly (ethylene glycol) – poly (ε-caprolactone) – poly (ethylene glycol) hydrogel slightly accelerated release (Fan et al. 2013).

According to the literature, hydrogel-microparticle composites based on HA hydrogels have not been designed before. That is the main reason for interest toward the composite gel designed in this study. Nevertheless, the problem with the preparation of the microparticle suspension must be resolved, first. For example, increasing the amount of sucrose in the emulsion, or testing some other compound, for example mannitol may work. Also, further investigation of possible stabilizing effect of sucrose is highly recommended. By performing drug release test with sucrose coated microparticles without presence of hydrogel, would verify, whether DEX maintain active due to sucrose of some component of the hydrogel. Polymers for hydrogel are dissolved into

the 10 % sucrose solution. Thus, degradation/stability test of DEX in presence of hydrogel will not be valuable.

6.3 Muraglitazar loaded microparticles

Completely new approach in use of muraglitazar is encapsulation into the microparticles. It is interesting field of research, as it offers new possibilities to the drug that is withdrawn from the market because of adverse cardiovascular effect in systemic circulation. In this study, muraglitazar was successfully encapsulated into the PLGA-PVA microparticles using exactly the same preparation method than in encapsulation of DEX.

One batch of muraglitazar loaded microparticles was prepared. SEM image (Figure 26, Chapter 5.3.1) as well as size distribution (Figure 27, Chapter 5.3.1) shows very similar shape, surface topography and frequency of diameter of the microparticles in the sample. May be a little more small pits on the surface of the microparticles were observed, but they may be explained by electron-beam in the SEM imaging. Also during preparation of these microparticles, outlet temperature of the spray dryer rose up to 42 °C for a short period of time. As mentioned earlier, the parameters in this spray dryer are not completely stable. The reason would be worth to find out. This short-term temperature raise may have influence to the part of the microparticles. Though, even higher outlet temperatures are used in preparation of PLGA microparticle without problems (da Silva-Junior et al. 2009). Morphology and size of the microparticles are discussed in more detailed in Chapter 6.1. All the aspects are valid also in case of muraglitazar loaded microparticles.

In vitro drug release profile of muraglitazar loaded microparticles follows the same kind of bi-phasic drug release profile than all other drug release profiles in this work. However, with muraglitazar, drug release is faster than with DEX. During burst release (the first four hours) 38.8 % of theoretical drug loading is released. That is almost doubled, compared to DEX. Slower release phase follows the burst release. The maximum drug concentration in the samples is achieved within seven days. Typical drug release profile and factors affecting to the drug release profile are discussed in more detailed in Chapter 6.1. Again these same factors are valid also in case of muraglitazar. There are still few aspects to point out, because muraglitazar has not been encapsulated before.

As mentioned earlier, drug distribution affects to the drug release. Complete dissolution of the drug into the solvent used in microparticle preparation is important in order to achieve homogeneous distribution of the drug. Muraglitazar is withdrawn from the market and therefore even basic chemical information is not available in the literature. Dichloromethane is probably the most popular solvent used in preparation of the PLGA microparticles, but also acetone is used some extent. Solvent used in this study was chosen according to solubility test of muraglitazar (Chapter 4.4.1). Though, solubility of the muraglitazar into the dichloromethane or ethanol cannot be guaranteed.

The drug molecules may be distributed near surface of the microparticles, and are released fast for that reason. In addition, if drug is not molecularly dispersed within the microparticles, but are still in the form of crystals, these crystals dissolve when particles are disposed to the aqueous environment and fluid is uptaken into the microparticles. Then, concentration gradient drives dissolved drug into the surrounding medium. (Klose et al. 2006) Drug type is also important. Muraglitazar molecule is larger than DEX molecule and that may have influenced to the drug release and also drug distribution within the polymer matrix. Possible interactions of the drug and the PLGA have to be taken into account (Wischke & Schwendeman 2008).

The maximum concentration in the muraglitazar samples was 74.0 % of theoretical drug loading. That is almost 20 percentage units higher than in DEX samples. In this case, there were no problems with degrading drug. Clear peak in the chromatogram was achieved (Figure 28, Chapter 5.3.2). Unfortunately, initial drug loading and residual drug content tests failed also with muraglitazar loaded microparticles. So, released drug concentrations are compared to the theoretical drug loading.

As mentioned several times in this thesis, it is extremely hard to predict the drug release profiles of the drug delivery devices. It is especially hard with totally new drug. It has been proved that type of the drug affects to the polymer degradation and drug release rate, but no clear relationship has been found (Siegel et al. 2006). That is why each drug release system has to be investigated separately, and preliminary drug release test has to be performed before starting to modify the release profile.

7 CONCLUSIONS AND FUTURE PROPOSITIONS

Instead of one larger aim, there were four smaller aims in this thesis. They were: 1) improvement of the previously developed PLGA-PVA-DEX microparticle system for drug release, 2) to study possibility to use gamma irradiation as sterilization method for these microparticles and its effects to the drug release, 3) to develop hydrogel-microparticle composite material in order to modify the drug release profile, and 4) encapsulation of hydrophobic drug, muraglitazar into PLGA-PVA microparticles. All of these four goals are aiming to the same, final aim: developing the more efficient drug release system for hydrophobic, anti-inflammatory drugs.

The efficiency of the combination of emulsion method and spray drying was successfully improved. The yield of the microparticles increased significantly, and simultaneously processing time decreased, which refer to the better air flow of the system. Thus, modified preparation method was confirmed to be suitable for microparticle preparation. Also drug release period of dexamethasone (DEX) was successfully prolonged from 53 hour to 28 days, even though the modified microparticle preparation method decreased the size of the microparticles. Depending on the application this drug release rate may be still too fast and burst release too big. Gamma sterilization had adverse effects on both morphology and drug release. Surface topography of the microparticles showed irregularities, even collisions, and drug release rate was significantly accelerated with shorter release period. Thus, gamma irradiation with the standard dose and procedure is not recommended as sterilization method to the microparticles. Also, DEX loaded hydrogel-microparticle composite was developed. The composite gel was stable and showed same kind of behaviour than control gel. The amount of microparticles were managed to increase compared to preliminary tests by adding sucrose to the o/w emulsion before spray drying. Drug release from the composite gel was really fast which is likely due to problems with preparation of the microparticle suspension. Still, analysis of the drug release samples revealed another interesting aspect: possible stabilizing effect of some component of the composite. In addition, analysis of the DEX with HPLC was improved, since unexpected degradation of the DEX appeared. Original gradient elution method was eventually, after several different trials, changed to the single eluent method including phosphoric acid.

Encapsulation of the hydrophobic drug, muraglitazar was succeeded using exactly the same preparation method than with DEX loaded microparticles. Drug release rate of the muraglitazar loaded microparticles was really fast. The release period of seven day may be enough for certain, short-term applications.

In the future, it would be sensible to make some changes to the microparticles in order to sustain drug release even further. Some other, slower degrading polymer may be better for the encapsulation. Alternatively, microparticles could be coated with another polymer or even same, but drug-free polymer. Acquirement of the automated drop formation device would ensure the repeatability of the preparation process. It would be worth to try producing microparticles with lower PVA content. In addition, further testing of hydrogel-microparticle composite is worthwhile. There are many published articles about modified, more sustained drug release profiles about microparticles embedded in the hydrogel. That was also expected result in this thesis because of this great potential. However, concentration of the sucrose has to be increased or changed into totally other cryoprotectant in order to facilitate the preparation of the microparticle suspension. That way it could be ensured that drug is not released from the particle during preparation of the suspension.

In this thesis, only preliminary drug release test was performed with muraglitazar loaded microparticles. In the future, repetition of the drug release test is recommended to ensure the results.

There are further plans for *in vitro* cell tests with DEX and muraglitazar loaded microparticles, and also hydrogel-microparticle composite. In addition stability testing of the composite gel can be performed.

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APPENDIX 1. DEVELOPING HPLC METHOD FOR DEXAMETHASONE FROM PLGA-PVA-DEX DRUG RELEASE SAMPLE.

METHOD	ELUENT				GRADIENT RUN TIME	FLOW RATE	PRECIPITATION	EVAPORATION + DISSOLVING	OTHER
	ACN	H_2O	TFA	H_3PO_4					
1	20 % 80 %	80 % 20 %	-	-	Yes 15 min	1 mL/min	-	-	-
2	20 % 80 %	80 % 20 %	-	-	Yes 15 min	1 mL/min	Ethanol	-	-
3	20 % 80 %	80 % 20 %	-	-	Yes 30 min	1 mL/min	Ethanol	-	-
4	20 % 60 %	80 % 40 %	-	-	Yes 15 min	1 mL/min	Ethanol	-	-
5	40 % 80 %	60 % 20 %	-	-	Yes 15 min	1 mL/min	Ethanol	-	-
6	20 % 80 %	80 % 20 %	0.1 %	-	Yes 15 min	1 mL/min	Ethanol	-	-
7	20 % 80 %	80 % 20 %	-	-	Yes 15 min	1 mL/min	Ethanol	Yes Eluent A	-
8	20 % 80 %	80 % 20 %	0.1 %	-	Yes 15 min	1 mL/min	Ethanol	Yes Eluent A	-
9	20 %	80 %	-	-	Yes 15 min	1 mL/min	Acetone	-	-

APPENDIX 1. DEVELOPING HPLC METHOD FOR DEXAMETHASONE FROM PLGA-PVA-DEX DRUG RELEASE SAMPLE.

10	20 % 80 %	80 % 20 %	0.1 %	-	Yes 15 min	1 mL/min	Acetone	-	-
11	20 % 80 %	80% 20 %	-	-	Yes 15 min	1 mL/min	Acetone	Yes Eluent A	-
12	20% 80%	80 % 20%	0.1 %	-	Yes 15 min	0.8 mL/min	Acetone	Yes Eluent A	-
13	20 % 80 %	80 % 20 %	0.1 %	-	Yes 15 min	0.7 mL/min	Acetone	Yes Eluent A	-
14	20 % 80 %	80 % 20 %	-	-	Yes 15 min	1 mL/min	-	-	Amicon filter 3 kD
15	20 % 80 %	80 % 20 %	0.1 %	-	Yes 15 min	1 mL/min	-	-	Amicon filter 3 kD
16	20 % 80 %	80 % 20 %	-	0.5 %	Yes 15 min	1 mL/min	Ethanol	-	-
17	20 % 80 %	80 % 20 %	-	0.5 %	Yes 15 min	1 mL/min	Ethanol	Yes Eluent A	-
18	20 % 80 %	80 % 20 %	-	0.5 %	Yes 30 min	1 mL/min	Ethanol	-	-
19	20 %	80 %	-	0.5 %	Yes 30 min	0.7 mL/min	Ethanol	-	-

APPENDIX 1. DEVELOPING HPLC METHOD FOR DEXAMETHASONE FROM PLGA-PVA-DEX DRUG RELEASE SAMPLE.

20	30 %	70 %	-	0.5 %	No 15 min	1 mL/min	Ethanol	-	-
21	30 %	70 %	-	0.5 %	No 15 min	1 mL/min	Ethanol	Yes Eluent A	-
22	30 %	70 %	-	0.5 %	No 30 min	0.7 mL/min	Ethanol	-	-
23	30 %	70 %	-	0.5 %	No 30 min	0.6 mL/min	Ethanol	-	-