Biodegradable Non-aqueous in situ Forming Microparticle Drug Delivery Systems

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Disputation am 07. September 2011

Dedicated to my eternal wife Vivian
and my daughters Emma and Anna Maria

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1 Introduction

1.1 Emulsions

1.1.1 General

Emulsions are thermodynamically unstable systems consisting of two immiscible liquids, one of which is dispersed as small spherical droplets (approx. 0.1µm to 100 µm) in the other liquid, stabilized by the presence of at least one emulsifying agent (Sinko 2006).

Emulsions can conveniently be classified according to the spatial organization of their phases into 1) simple emulsions, e.g. *oil-in-water* (O/W) emulsions or *water-in-oil* (W/O) emulsions, 2) multiple emulsions, e.g. *oil-in-water-in-oil* (O/W/O) or *water-in-oil-in-water* (W/O/W) emulsions, and 3) more sophisticated emulsions such as *oil-in-oil* (O/O) emulsions or *water-in-water* (W/W) emulsions (McClements, Decker et al. 2007).

Emulsion systems are widely used in the pharmaceutical field, e.g. for external use as creams or lotions and for internal use as orally, intravenously or parenterally applicable emulsions (Sinko 2006).

1.1.2 Emulsion instability

Emulsions are kinetically unstable due to the increase in the interfacial area following emulsification. They are subjected to changes in their physicochemical properties over time. Given sufficient time, any emulsion will collapse as the two phases attempt to minimize contact area (Rousseau 2000).

Emulsion instability mechanisms (Figure 1) can be classified into creaming, sedimentation, flocculation, coalescence, Ostwald ripening and phase inversion (Rousseau 2000; Sinko 2006). Gravitational separation is the process whereby droplets move upward (‘creaming’) or downward (‘sedimentation’) having a lower or higher density than the

surrounding liquid, respectively. Flocculation means that two or more droplets form a grape-like aggregate in which each of the initial droplets retains its individual integrity. Coalescence, on the other hand, is the process whereby droplets merge together to form a single larger droplet. Partial coalescence is a special case which occurs when partly crystalline droplets merge together to form a single irregularly shaped aggregate due to the penetration of solid crystals from one droplet into a fluid region of another droplet. The aggregate partly retains the shape of the original droplets from which it was formed. Ostwald ripening describes the growth of larger droplets at the expense of smaller droplets due to mass transport of dispersed phase material through the continuous phase. Phase inversion involves the change of the emulsion type, e.g. from o/w to w/o (McClements 2007).

The rate at which an emulsion breaks down is strongly influenced by composition, environmental conditions (e.g. temperature) and processing conditions (e.g. shear rate) (Rousseau 2000).

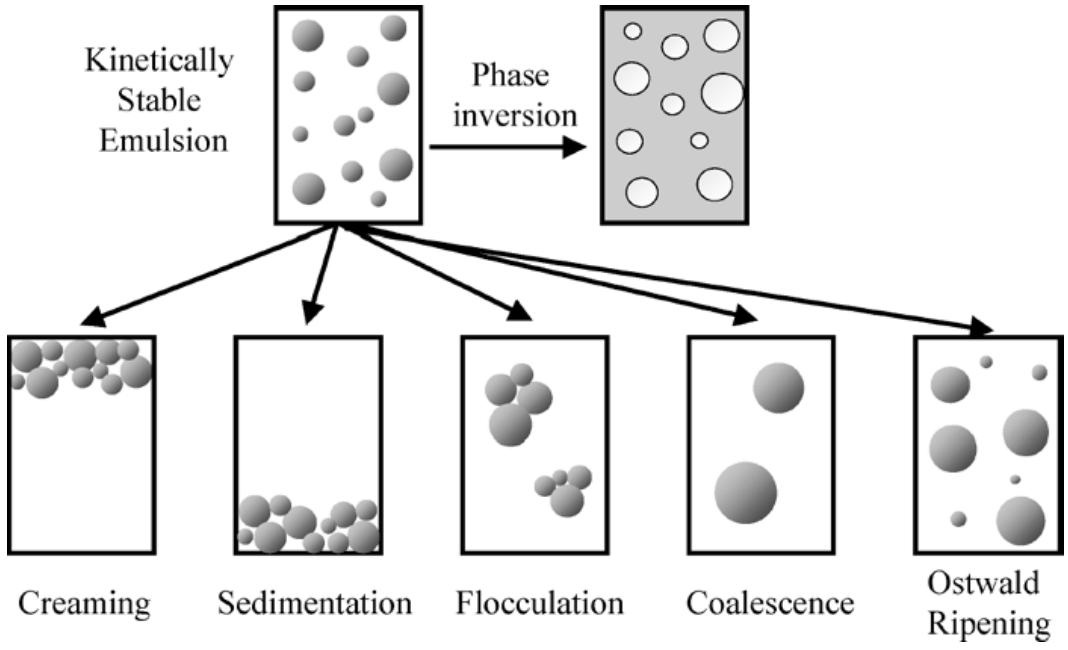

Figure 1 Schematic diagram of common instability mechanisms that occur in emulsions (McClements 2007)

1.1.3 Stabilization approaches

The preparation of kinetically stable emulsions over a practical time period can be achieved by the addition of stabilizing agents. In general, these substances either stabilize the droplet interface or modify the texture of the continuous phase (McClements 2007).

Substances stabilizing the interface form a mechanically cohesive film around the droplets and/or decrease interfacial tension. They can be divided into 1) surfactants which form a flexible monolayer and lower the interfacial tension (e.g. low molecular weight emulsifier such as Tween 80 or Pluronic F 68 and proteins such as sodium caseinate (Wilde 2000)), 2) amphiphilic molecules which form elastic liquid crystalline layers (e.g. high concentrations of low molecular weight emulsifier such as Tween 40 or Span 40 (Pilpel and Rabbani 1987), phospholipids (Rydhag and Wilton 1981) and mono –and diacylglycerols (Macierzanka, Szelag et al. 2009)), 3) hydrocolloids which form an interfacial multilayer (e.g. natural polysaccharides such as carrageenans, cellulose ethers (Sinko 2006)) and 4) solid particles also referred to as 'Pickering agents' which form a solid layer at the interface (e.g. fat crystals (Hodge and Rousseau 2005) or silica (Kargar, Spyropoulos et al. 2011)).

Texture modifiers thicken or gel the continuous phase which improves emulsion stability by retarding or preventing the movement of droplets (McClements, Decker et al. 2007). Examples are polysaccharides (e.g. starches, cellulose ethers, chitin), proteins (e.g. milk or whey proteins), silica, clays or mono –and diacylglycerols (Rousseau 2000; Ojijo, Neeman et al. 2004b; Sinko 2006). Beside an increase of the continuous phase viscosity, also density adaption of the two phases or a decrease in particle size may further minimize gravitational destabilization processes (Yao, Maris et al. 2005). This can be derived from Stoke's equation:

$$v_s = \frac{2(\rho_p - \rho_m)}{9\mu} g R^2$$

which originally described the velocity v_s of a falling hard sphere of radius R in a continuous liquid medium of viscosity μ where g is the gravitational acceleration and $\rho_p - \rho_m$ is the density difference of the sphere material and the liquid medium (Derkach 2009).

However, each emulsion is unique. Thus, the selection of the most appropriate stabilizer(s) is one of the most important factors determining the shelf-life and physicochemical properties of emulsions (McClements, Decker et al. 2007).

1.1.4 Evaluation of emulsion stability

Many techniques have been developed to monitor the physical changes which can result into emulsion instability.

Coalescence can be characterized by determining increases in particle size or particle size distribution over time. This can be done simply via a microscope or more sophisticated via particle size analyzing techniques such as light scattering, electrical pulse-counting ('Coulter Counter'), ultrasonic spectrometry or NMR (McClements 2007). Other methods like the film trapping technique allow the determination of coalescence stability of single, micrometer-sized drops (Tcholakova, Denkov et al. 2002).

Partial coalescence can be monitored with optical/electron microscopy or indirectly by determining changes in the flow behaviour of the emulsion (Hodge and Rousseau 2005).

Ostwald ripening can be distinguished from coalescence by measuring the full particle size distribution over time. Coalescence usually leads to a bimodal distribution (larger droplets coalesce faster than smaller ones), whereas Ostwald ripening leads to a monomodal distribution with the cube of the mean particle diameter increasing linearly with time (Kabalnov 2001).

Gravitational destabilization processes like creaming and flocculation are characterized by the temporal change in droplet concentration or particle size distribution with emulsion height. Visual observation, video imaging, physical sectioning, light scattering, ultrasonic spectrometry, electrical conductivity or magnetic resonance imaging measurements have been used for this purpose (McClements 2007).

Flocculation of emulsion droplets can be determined via microscopy, particle size analysis or via changes in the emulsion flow behaviour. The three-dimensional structure of the aggregates can be investigated with newer microscopy techniques such as confocal laser scanning microscopy (Bushell, Yan et al. 2002). The strength of the attraction between flocculated droplets is characterized by the energy that must be supplied to pull the droplets apart. The flocculation strength can be measured with, for instance, atomic force microscopy or estimated by determining the change in the flow behaviour of an emulsion under increasing shear stress (Quemada and Berli 2002).

Phase inversion can be monitored with similar techniques as being used to determine the emulsion type: dying of one phase, dilution of the emulsion with the liquid of the continuous phase or electrical conductivity measurements (Sinko 2006).

The effectiveness of a substance to stabilize a certain emulsion can be evaluated by determining the emulsifying capacity or the emulsion stability index. The emulsifying capacity of an emulsifier is defined as the maximum amount of internal phase that can be dispersed in the continuous phase containing a specific amount of the emulsifier (Sherman 1995). The emulsion stability index indicates changes in the particle size of an emulsion after storage for a specified length of time or after exposure to environmental stress like heating, freezing or stirring. The smaller the increase in particle size, the better the emulsifier (McClements 2007).

Emulsion instabilities may occur within minutes or after months. Thus, often the evaluation of emulsion stability is performed under accelerated phase separating conditions to identify instabilities in relatively short time periods. Such environmental stress tests may include freezing, thaw-freeze cycles, storage at increased temperatures, shearing and centrifugation.

Each technique mentioned above has its advantages but also its limitations. More detailed information is given in comprehensive reviews from McClements (McClements 2007) and Bushell et al. (Bushell, Yan et al. 2002)

1.1.5 Non-aqueous emulsions

The replacement of the aqueous phase by various other solvents (e.g. dimethyl sufoxide, N-methyl-2-pyrrolidone, formamide or dimethylformamide) offers the great potential of developing emulsion systems exhibiting other physico-chemical properties than conventional water based emulsion systems. However, non-aqueous emulsions have received very little attention in the literature (Suitthimeathegorn, Turton et al. 2007).

The earliest accessible reports about the stabilization of non-aqueous emulsions have been published from three workgroups in the 1960s and 1970s. Petersen et al. evaluated the stabilization of emulsions composed of non-toxic, pharmaceutically relevant substances where olive oil or mineral oil were used as non-polar phase, glycerin as polar phase and conventional emulsifiers as stabilizing agents. After comprehensive screening of existing low molecular emulsifiers, they found that non-aqueous emulsions could be stabilized with amphiphilic surfactants which have attraction for the polar and the non-polar phase.

Increasing the viscosity of the continuous phase improved emulsion stability. McMahon et al. suggested that these emulsions may serve as application bases for dermatologicals (McMahon, Hamill et al. 1963; Petersen, Hamill et al. 1964; Hamill, Olson et al. 1965; Hamill and Petersen 1966a; Hamill and Petersen 1966b; Reichmann and Petersen 1973). Molau stabilized two immiscible polymer solutions with self-synthesized two block polymers consisting of one polymer from each solution (Molau 1965a; Molau 1965b). Periard et al. adopted the idea of Molau and stabilized two immiscible non-aqueous liquids with graft polymers, where one block of the polymeric emulsifier was selectively soluble in one of the liquids (Periard, Banderet et al. 1970).

Based on these findings, some other groups have developed non-aqueous emulsion systems which could be used for non-pharmaceutical applications such as 1) a medium for organic reactions like Diels–Alder (Ray and Moulik 1994), 2) for polymer synthesis (Cameron and Sherrington 1996), 3) in cleaning systems that are sensitive to formation of rust such as engines and other mechanical systems (Imhof and Pine 1997), 4) for the polymerization of latices (Schubert, Lusvardi et al. 1996; Landfester, Willert et al. 2000; Klapper, Nenov et al. 2008), 5) templates for the production of monodisperse macroporous materials (Imhof and Pine 1997b) and 6) the formation of elastomeric films containing droplets of confined disinfecting liquids used as barrier materials in the biomedical area (Riess, Cheymol et al. 2004). The utilization of non-aqueous emulsions in pharmaceutical applications is even less common. Non-aqueous emulsions could be used as a topical drug delivery system (Sakthivel, Jaitely et al. 2001), to prepare nano –and microparticles (Wang, Schmitt et al. 1991; Kobašlija and McQuade 2006), as a parenteral drug delivery system its self (Suitthimeathegorn, Jaitely et al. 2005; Suitthimeathegorn, Turton et al. 2007) or in non-aqueous in situ forming microparticle (ISM) systems (Kranz and Bodmeier 2007).

The low interest in non-aqueous emulsions in the pharmaceutical field could be related to the utilization of organic solvents considering their toxic potential or the use of mostly unapproved and newly synthesized emulsion stabilizers. Paygan et al. gives a comprehensive overview about investigated potential stabilizers (Payghan, Bhat et al. 2008).

The fabrication of stable non-aqueous emulsions is not as advanced as the stabilization of aqueous emulsions. The selection of suitable stabilizers is still challenging due to a lack of general knowledge about the underlying stabilization mechanisms (Cameron and Sherrington 1996; Suitthimeathegorn, Jaitely et al. 2005; Payghan, Bhat et al. 2008). The

hydrophilic-lipophilic balance (HLB) introduced by Griffin for aqueous emulsions in 1949, for instance, does not apply (Petersen, Hamill et al. 1964).

Summarizing the data from all available literature, stabilization has been achieved by either utilization of a suitable oil-immiscible polar liquid that can substantially replace water, e.g. formamide or by designing surfactants having two incompatible blocks, each of which is selectively soluble in one of the immiscible liquids (Imhof and Pine 1997). Theoretically, stabilization mechanisms observed in aqueous emulsion systems should also be considered as potentially valid for non-aqueous emulsion systems, i.e. formation of a viscous network in the continuous phase or interface stabilization via solid particles or liquid crystals.

1.2 Biodegradable parenteral drug delivery systems

1.2.1 General

Over the last two decades successful developments in biotechnology have led to a growing use of proteins and peptides as therapeutic agents and have become the therapeutics of significance in the 21st century. For instance, between 1982 and 1992 14 biopharmaceuticals were approved world-wide, whereas a total of 65 new biopharmaceuticals reached the marked between 1999 and 2006 (Tsuji and Tsutani 2008). In the year 2010, more than 2000 macromolecular candidates have been under investigation in clinical trials (Maeder and Weidenauer 2010). Furthermore it has been estimated that by 2014 half of the top 100 selling pharmaceuticals will be therapeutic proteins (Strohl and Knight 2009). Challenges associated with biopharmaceuticals are often a very low oral bioavailability, short biological half-lives and the high susceptibility to physical and chemical instability reactions (Giteau, Venier-Julienne et al. 2008).

Furthermore a large number of the new low molecular pharmaceutical substances are confronted with a low solubility and/or low gastrointestinal permeability (BCS classes 3 and 4)**,** making an oral administration very challenging (Wischke and Schwendeman 2008; Patel, Solorio et al. 2010). Thus, especially a large proportion of new active compounds has to be administered via an alternative route. Various modes of administration have been investigated, such as the nasal, pulmonary or transdermal route. However, the parenteral administration of active substances still remains the most common alternative (Cázares-Delgadillo, Ganem-Rondero et al. 2011). Beside the intravenous, intramuscular or subcutaneous injection of drug solutions, the sustained or controlled release from parenteral depot systems represents an attractive way in the long-term treatment of many diseases (Brannon-Peppas 1995). Such depot systems contain the active ingredient encapsulated in a polymeric matrix and release the drug over weeks to months in a continuous manner.

Potential advantages of parenteral depot systems can be summarized as: 1) higher bioavailability for BCS class 3 or 4 compounds compared to oral administration, 2) sustained drug release with constant plasma levels in the therapeutic window und thus

reducing side effects, 3) reduced frequency of drug administration in long-term treatments, which may increase patient compliance, 4) dose reduction in comparison to daily administration, 5) if applicable, local delivery at the site of action, e.g. to the brain or into tissue cavities after tumor resection, and 6) possible stabilization of drugs with a short serum half-life within the polymer matrix, e.g. proteins, like human growth hormone (Elstad and Fowers 2009; Wischke, Zhang et al. 2010).

1.2.2 Polymers

Polymers used in drug-eluting depot systems can be grouped into two main categories: non-degradeable and biodegradeable polymers. Non-degradable polymers, such as silicone elastomers, poly(ethylene–co-vinyl acetate) and polyacrylates, have been used in implants (Exner and Saidel 2008). The DUROS® technology, an implantable osmotic pump (ALZA corporation, CA, USA) is an example for such systems (Wright, Johnson et al. 2003).

The primary advantage of depot systems based on biodegradable polymers in comparison to non-degradable polymers is that there is no need of a surgical removal of the device at the end of treatment. The removal of non-degraded devices is often invasive and painful due, in part, to a fibrous encapsulation of the foreign body over time (Exner and Saidel 2008).

Biodegradable materials are capable of being cleaved into biocompatible fractions, then oligomers and finally monomers through chemical or enzyme-catalyzed hydrolysis within the body. Thereby incorporated drug can be released in a sustained manner into the surrounding body fluid mainly controlled by diffusion and erosion processes (Park, Ye et al. 2005).

Erosion of the biodegradable polymer matrix is a consequence of the polymer degradation process. It is characterized by the formation of fragments which are able to dissolve into the surrounding aqueous media leading to a mass loss of the polymer matrix. Degradable polymers can be classified into surface (or heterogeneous) and bulk (or homogeneous) eroding materials (Figure 2). In the case of polymers that degrade in bulk, the rate of water penetration into the matrix is higher than the rate of polymer degradation. This is a homogeneous process in which degradation occurs at a uniform rate throughout the polymer matrix. For surface-eroding polymers, hydrolysis of the polymer is confined to the outer surface while the interior of the matrix remains essentially unchanged. In order to

have purely erosion-controlled systems, polymer erosion has to be faster than water uptake or drug diffusivity (Alexis 2005).

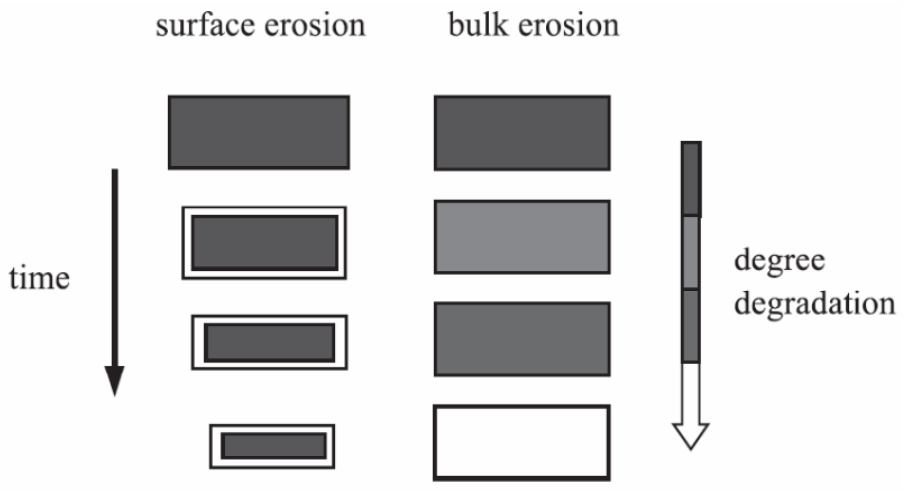

Figure 2 Schematic illustration of surface vs. bulk erosion of a polymer matrix (Burkersroda, Schedl et al. 2002).

In recent years especially tremendous advancements in the synthesis of new polymers have led to the development of a large number of promising delivery systems (Chitkara, Shikanov et al. 2006). They can be classified into solid matrices (e.g. implants, wafers), solid particles (e.g. microparticles, nanoparticles), semi-solids (e.g. hydrogels, liposomes, microemulsions) or injectable in situ forming depot systems (e.g. in situ crosslinked systems, in situ polymer precipitation based systems, thermoplastic pastes, thermally induced gelling systems) (Packhaeuser, Schnieders et al. 2004; Heller 2009).

Biodegradable polymers are either based on natural materials, such as proteins (e.g. albumin, collagen, fibrin, gelatine), polysaccharides (e.g. alginates, cellulose, chitosan, dextran, hyaluronic acid and starch) and lipids or synthetic substances, such as poly(amides), poly(amino acids), poly(anhydrides), poly(cyanoacrylates), poly(dioxanones), polyesters (e.g., poly(ε-caprolactones) (PCL), poly(carbonates), poly(glycolic acids) (PGA), poly(hydroxybutyrates), poly(lactic acids) (Vanapalli, Palanuwech et al.), poly(tartrates)), poly(ortho esters), poly(phosphazenes) and various copolymers (Middleton and Tipton 2000; Park, Ye et al. 2005).

Table 1
Examples of currently marketed biodegradable parenteral drug delivery systems (May 2011).

Dosage form	Product	Drug	Company	Polymer	Drug delivery, month	Indication
Implant	Gliadel®	Carmustin	MGI Pharma	Polifeprosan 20	<1	Malignant glioma
	Leuprone® HEXAL	Leuprolide acetate	Hexal	PLGA or PLA	1 and 3	Prostate cancer
	Ozurdex®	Dexamethasone	Allergan Pharmaceuticals	PLGA	>2	Retinal vein occlusion
	Profact® Depot	Buserelin acetate	Aventis Pharma	PLGA	2 and 3	Prostate cancer, endometriosis
	Zoladex®	Goserelin acetate	AstraZeneca	PLGA	1 and 3	Prostate cancer
In situ implant	Atridox®	Doxycyclin	Atrix/Curasan	PLA (NMP)	4	Periodontal disease
	Eligard®	Leuprolide acetate	Astellas Pharma	PLGA (NMP)	1 and 3	Prostate cancer
Microparticle	Arestin®	Minocycline	OraPharma	PLGA	0.5	Periodontal disease
	Decapeptyl® Depot	Triptorelin acetate	Ferring Pharmaceuticals	PLGA	1	Prostate cancer
	Enantone®	Leuprorelin acetate	Takeda	PLGA	1	Prostate cancer, endometriosis
	Lupron Depot ®	Leuprolide acetate	Abbott	PLGA	3 and 4	Prostate cancer
	Pamorelin® LA	Triptorelin acetate	Ipsen Pharma	PLGA	1 and 3	Prostat cancer
	Risperdal® consta	Risperidone	Janssen Pharmaceuticals	PLGA	0.5	Schizophrenia
	Salvacyl®	Triptorelin embonate	Pfleger	PLGA	3	Men sexually-deviant behaviour
	Sandostatin® LAR	Octreotide acetate	Novartis	PLGA	1	Acromegaly, carcinoid syndrome
	Sixantone®	Leuprorelin acetate	Takeda	PLA	6	Prostate cancer, endometriosis
	Somatuline® LA	Lanreotide	Ipsen Pharma	PLGA	0.5	Acromegaly
	Suprecur® MP	Buserelin acetate	Mochida Pharmaceuticals	PLGA	1	Prostate cancer
	Trelstar® Depot	Triptorelin pamoate	Watson Pharma	PLGA	1, 3 and 6	Prostate cancer
	Trenantone®	Leuprorelin acetate	Takeda	PLA	3	Prostate cancer, endometriosis
	Vivitrol®	Naltrexone	Cephalon	PLGA	1	Alcohol dependence

Although numerous promising biodegradable polymers have been discussed in the literature, only a few have led to marketed products. The majority of all approved formulations are based on the polyesters poly(lactic acid) (Vanapalli, Palanuwech et al.) and the copolymer poly(lactic acid-co-glycolic acid) (PLGA) (Table 1). However, the number of clinical useful systems based on other polymers has been increasing in recent years. Some of the major developments are briefly discussed in the next chapter.

More detailed information about biodegradable polymers and investigated devices are given in excellent reviews by Park et al. (Park, Ye et al. 2005), Shi et al. (Shi and Li 2005), Chitkara et al. (Chitkara, Shikanov et al. 2006), Kim et al.(Kim, Kim et al. 2009), Luten et al. (Luten, van Nostrum et al. 2008), and Van Tomme et al. (Van Tomme, Storm et al. 2008)

1.2.3 Promising non-poly(lactic/glycolic acid) based systems

The polyanhydrid polifeprosan 20 (poly[bis(*p*-carboxyphenoxy) propane-co-sebacic acid] 20:80) is the only polymer which has been marketed in a parenteral product beside PLA and PLGA (Figure 3). Wafers of polifeprosan 20 (Gliadel® from MGI Pharma, Bloomington, USA) loaded with carmustine are placed in the brain cavity created by the surgical removal of a tumor to destroy cancer cells which were left behind after surgery (Heller 2009). More than 70 % of the copolymer degrades by 3 weeks. Approximately 70 % of carmustine was released after 48 h in brains of normal rats (Dang, Daviau et al. 1996). In general, polyanhydrides are hydrophobic polymers with hydrolytically labile anhydride linkages which primarily undergo surface erosion (Park, Ye et al. 2005). This makes them very attractive for protein encapsulation**,** maintaining stability and activity of the macromolecules in solid state in the hydrophobic core of the polymer matrix (Jain, Modi et al. 2005).

The polyester poly(ε-caprolactone) is a semi-crystalline polymer which has shown good biocompatibility**,** resulting in FDA approval. Unfortunately, there is no parenteral product on the market. Clinical trials of Capronor®, a 1 year levonorgestrel releasing implant developed in the 1980s, were cancelled after phase II (Ma, Song et al. 2006). In recent years, however, there has been a growing interest in PCL especially in the field of tissue engineering (Woodruff and Hutmacher 2010). A PCL implant containing naltrexone to treat alcohol or opioid dependence is currently under clinical investigation. The implant

releases the drug over 5 to 9 month and completely degrades after 1 to 2 years (http://www.naltrexoneimplant europe.com/en/naltrexone-implants, April2011).

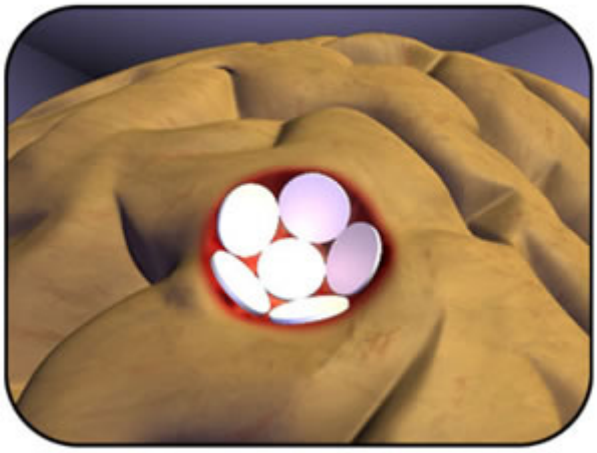

Figure 3 Up to 8 Gliadel® wafers can be implanted in a brain cavity created after a tumor has been surgically removed (from http://www.gliadel.com).

ReGel® is a thermosensitive triblock copolymer comprised of PLGA and polyethylene glycol (PEG) with the basic structure of PLGA–PEG–PLGA. The polymers transform from a free-flowing water soluble low viscosity solution (sol-state) at temperatures between 2 and 15 °C to a viscous, water insoluble biodegradable controlled release gel (gel-state) at body temperature. Preclinical studies showed a biocompatibility similar to biodegradable sutures and microspheres based on PLGA. ReGel® is a non-ionic surfactant that spontaneously forms polymeric micelles containing a hydrophobic core. Thus, it has the ability to increase the solubility of in particular hydrophobic small molecules such as paclitaxel. Paclitaxel loaded ReGel® (Oncogel®) was developed for the local treatment of esophageal cancer. Thereby the anti-cancer drug is continuously released directly to the tumor and surrounding tissue for 6 weeks (Elstad and Fowers 2009). Unfortunately, primary promising clinical trials were discontinued after phase IIb in the beginning of the year 2011. However, ReGel® is still investigated as a possible delivery system for other active substances (http://www.btgplc.com, April 2011).

The SABER® system consists of sucrose acetate isobutryate (SAIB) dissolved in a biocompatible organic solvent. Upon injection of for instance a low viscous ethanol based polymer solution**,** the solvent rapidly diffuses away which results in the formation of a

highly viscous depot at the injection side. This system is best suited for the delivery of small molecules. For instance Posidur® containing bupivacaine has been developed to control post-operative pain providing local analgesia for up to 3 days (Okumu, Dao et al. 2002; Heller 2009). Posidur® is currently in clinical phase III (http://www.durect.com, May 2011). On the other hand, less viscous depots using e.g. benzyl benzoate as organic solvent have shown continuous protein and peptide release up to one month in preclinical studies.

The Medusa® drug delivery platform is based on a polyaminoacid based nanogel depot system which can be used to release drugs (e.g. small molecules as well as peptides and proteins) from once a day up to 2 weeks. The nanogel has been proven to be safe and biocompatible. A drug master file (DMF) was filed with the FDA in 2011 (http://www.flamel.com, May2011). For instance, the Medusa® II system consists of a poly L-glutamate backbone grafted with hydrophobic α-tocopherol molecules, creating a colloidal suspension of nanoparticles (10 – 50 nm) in water. The sustained drug release is based on reversible drug interactions with hydrophobic nanodomains within the nanoparticles. In vivo, it was suggested that the therapeutic protein is displaced by endogenous proteins present in physiological fluids, leading to a slow drug release. The polyaminoacid nanogel degrades into the biocompatible compounds glutamic acid and vitamin E (Chan, Meyrueix et al. 2007). The Medusa® technology has been applied to subcutaneous injectables for several therapeutic proteins, which are currently in human clinical trials, e.g. IFN-α against hepatitis C (phase II), a long acting basal insulin against diabetes type I and II (phase I), and IFN-β against multiple sclerosis (phase I) (http://www.flamel.com, May 2011).

OctoDEX® is a biodegradable hydrogel based on crosslinked dextran in the form of microspheres. The release of proteins can be tailored from days to months depending on the composition of the starting materials (Shi and Li 2005). OctoDex® has been shown good biocompatibility in human clinical trials (http://www.octoplus.nl, May 2011).

PolyActive® comprises of a series of poly(ether ester) multiblock copolymers, based on repeating blocks of hydrophilic poly(ethylene glycol) (PEG) and hydrophobic poly(butylene terephthalate) (PBT). The copolymer can be used to produce matrix systems such as microparticles or implants. The polymer matrix characteristics such as the rate of controlled release and degradation can be precisely controlled by the appropriate combination of the two copolymer segments. PolyActive® has been approved by the FDA for two implantable orthopedic medical devices (Bezemer, Radersma et al. 2000).

Locteron® comprising of IFN-α loaded PolyActive® has been developed for the treatment of hepatitis C**,** releasing the protein over two weeks. Clinical phase II studies have been successfully completed in 2011 (http://www.octoplus.nl, May 2011).

1.3 Poly(lactic/glycolic acid)

1.3.1 General

Based on the development of the first synthetic, absorbable suture made of poly(glycolic acid) (PGA) in the 1960s, PLA and PLGA have been investigated since the early 1970s as potential polymers for sustained parenteral delivery of drugs (Kulkarni, Moore et al. 1971; Brady, Cutright et al. 1973; Yolles and Sartori 1980). PLA and PLGA have become the most commonly used biodegradable polymers in research and commercialized products. The preference of PLA and PLGA over PGA may be explained by their more appropriate physico-chemical properties such as glass transition temperatures above the physiological body temperature (40-60 °C instead of 35-40 °C), better mechanical strength and superior solubilities in organic solvents (Arshady 1991; Tice 2004).

1.3.2 Synthesis

PLA, PGA and their copolymers can be synthesized in a wide range of molecular weights. Direct polycondensation of lactic and/or glycolic acid leads to low molecular weight products whereas high molecular weight polymers are generally synthesized via ring-opening polymerization of the cyclic dimers lactide and/or glycolide (Kiremitci-Guemuesderelioglu and Deniz 1999) (Figure 4). The polymer end group can be modified by varying the type of the polymerization initiator. Uncapped polymers, such as the PLGA Resomer® 502H, have free carboxyl end groups and are thus more acidic and hydrophilic. Capped polymers, such as Resomer® 503, exhibit ester linkages at the polymer terminus resulting in a more hydrophobic alkyl end (Tracy, Ward et al. 1999).

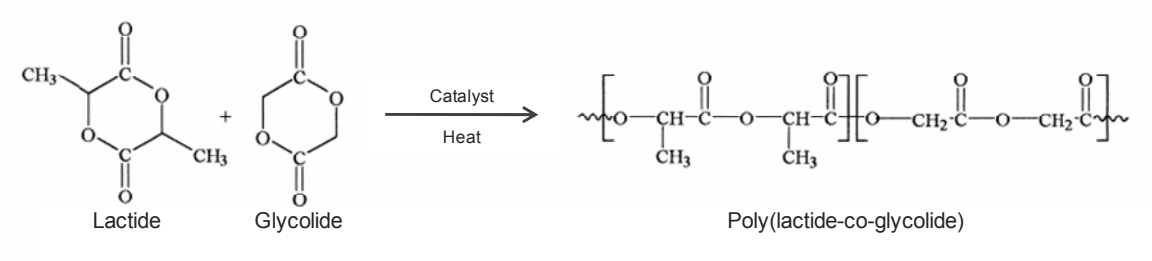

Figure 4 Synthesis of poly(lactide-co-glycolide) (PLGA) by ring-opening polymerization of the cyclic dimers lactide and glycolide.

1.3.3 Degradation behaviour

Lactic/glycolic acid based polyesters degrade via random cleavage of the ester bonds into biocompatible fragments through chemical hydrolysis. Erosion describes the mass loss of the polymer matrix and starts when the polymer degradation products become water soluble. PLGA, for instance, begins to dissolve in aqueous surroundings at a molecular weight of approximately 1000 Dalton (Körber 2010). The polymer degradation behaviour has been extensively characterized and reviewed (Göpferich 1996; Alexis 2005; Wischke and Schwendeman 2008). In summary, the degradation rate is affected by the polymers intrinsic properties like chemical structure, molecular weight, polydispersity index, end group functionality, crystallinity and water content, by release medium properties like pH, temperature, ionic strength, solvent and presence of biocatalysts or microorganisms, by formulation properties like particle size, presence of basic or acidic compounds, plasticizers or drug, and by sterilization.

Another factor influencing the degradation of polyester based matrices is known as the autocatalytic effect. As PLGA drug delivery systems undergo bulk erosion, acid degradation products are generated throughout the polymer matrix. The pH inside the matrix can decrease significantly if the diffusion of soluble fragments is hindered (e.g. by a dense, non-porous polymer shell). Ester bond cleavage can be catalyzed by protons. Thus, a decreased micro-pH will lead to accelerated polymer degradation in the matrix core (Klose, Siepmann et al. 2006).

The in vivo degradation of PGA, PLA and their copolymers is faster than in vitro (Tracy, Ward et al. 1999). This was attributed to a plasticizing effect of biological substances, immunological responses or an accelerated autocatalytic chain scission due to

an impeded outflow of acidic degradation products by surrounding tissue (Wischke and Schwendeman 2008).

1.3.4 Drug release from poly(lactic/glycolic acid) matrices

The explanation of drug release from poly(lactic/glycolic acid) based delivery systems is complex because of the interplay of many parameters. In general, incorporated drug is released via diffusion, osmotic pumping or bulk erosion of the polymer (Figure 5). Diffusion can occur through the polymer itself or through water-filled networks of pores or channels connected with the surface (Giteau, Venier-Julienne et al. 2008; Fredenberg, Wahlgren et al. 2011).

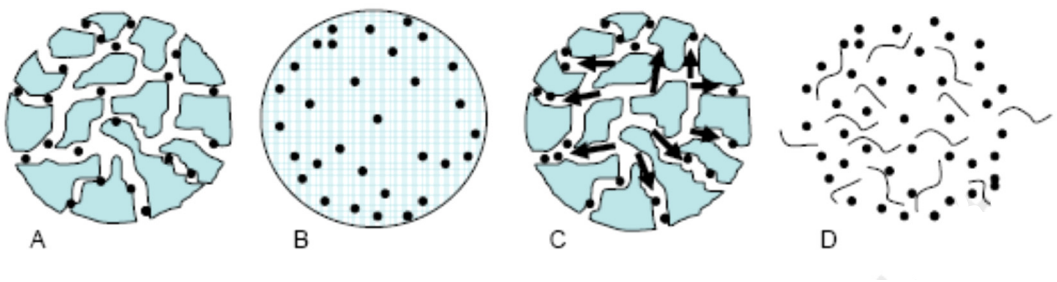

Figure 5 Release mechanisms from poly(lactic/glycolic acid) based matrices: **(A)** diffusion through water-filled pores, **(B)** diffusion through the polymer, **(C)** osmotic pumping and **(D)** erosion (Fredenberg, Wahlgren et al. 2011a).

In most cases drug release from polymer matrices can be divided into an initial diffusion controlled release and a subsequent degradation/erosion controlled release (D'Souza, Faraj et al. 2005). Some important parameters effecting the rate of drug diffusion and the polymer degradation/erosion kinetics are, for instance: 1) polymer properties such as composition, molecular weight, crystallinity and end group type (Alexis 2005), 2) polymer matrix characteristics such as geometry and porosity (Freiberg and Zhu 2004; Wischke and Schwendeman 2008), 3) environmental conditions such as composition of release media, temperature and *in vitro* vs. *in vivo* investigations (Giteau, Venier-Julienne et al. 2008), 4) water absorption (swelling) (Mochizuki, Niikawa et al. 2008; Desai, Olsen et al. 2010), 5) drug related effects such as aqueous solubility, dissolution rate, molecular weight, loading and drug-polymer interactions (Luan and Bodmeier 2006a;

Wischke and Schwendeman 2008), 6) addition of further additives such as basic salts (van de Weert, Hennink et al. 2000; Zhu, Mallery et al. 2000) and 7) pore opening and closure during the release period (Kang and Schwendeman 2007; Fredenberg, Wahlgren et al. 2011a).

Knowledge regarding the effects and the complex interplay of this large number of parameters is necessary to understand drug release in detail and to be able to control the release rate (Fredenberg, Wahlgren et al. 2011a). Fredenberg et al. developed an excellent illustrative picture, which shows the complexity of the factors influencing the drug release from PLGA matrices (Figure 6). Many of these processes affect the drug release in more than one way. For instance, hydrolysis of the polymer leads to erosion and pore formation which thus increases drug release. However, hydrolysis will also lead to a decrease of the polymer glass transition temperature (Tg), possible rearrangement of polymer chains and pore closure. This will potentially result in a decrease in drug release. Another example is the incorporation of basic salts to prevent autocatalytic effects by neutralizing acids which will decrease the drug release due to a slower erosion of the polymer matrix. On the other hand**,** the matrix porosity will increase due to an increased water uptake caused by the osmotic activity of dissolved salts and their diffusion out of the matrix (porogen). Therefore basic salts may also increase drug release (Fredenberg, Wahlgren et al. 2011a). More detailed information about processes involved in drug release from poly(lactic/glycolic acid) based delivery systems is provided in excellent reviews from Alexis (Alexis 2005), Giteau et al. (Giteau, Venier-Julienne et al. 2008), Mundargi et al. (Mundargi, Babu et al. 2008), Wischke et al. (Wischke and Schwendeman 2008) and Fredenberg et al. (Fredenberg, Wahlgren et al. 2011)

Although a zero-order release profile is mostly preferred, poly(lactic/glycolic acid) based drug delivery systems usually exhibit bi-phasic or more common tri-phasic release profiles (Figure 7). Especially macromolecules such as peptides and proteins show tri-phasic (also referred to as sigmoidal) release pattern. In general, phase I in the tri-phasic release profile is described as a burst release and has been attributed to diffusion of drug located on or close to the surface. Phase II describes a slow release phase, during which the drug diffuses slowly either through the relatively dense polymer or through few existing pores while polymer degradation proceeds. Phase III is usually described as a period of faster release, often attributed to the onset of erosion (Fredenberg, Wahlgren et al. 2011).

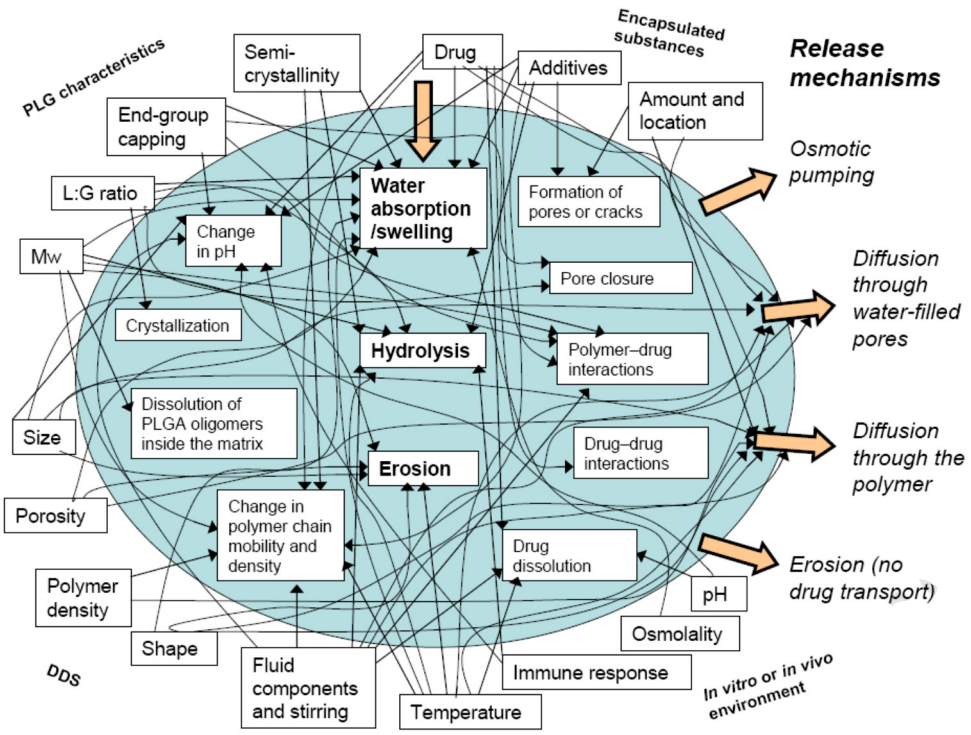

Figure 6 Schematic illustration of the complex interplay of different factors that influence drug release from poly(lactic/glycolic acid) based matrices (Fredenberg, Wahlgren et al. 2011).

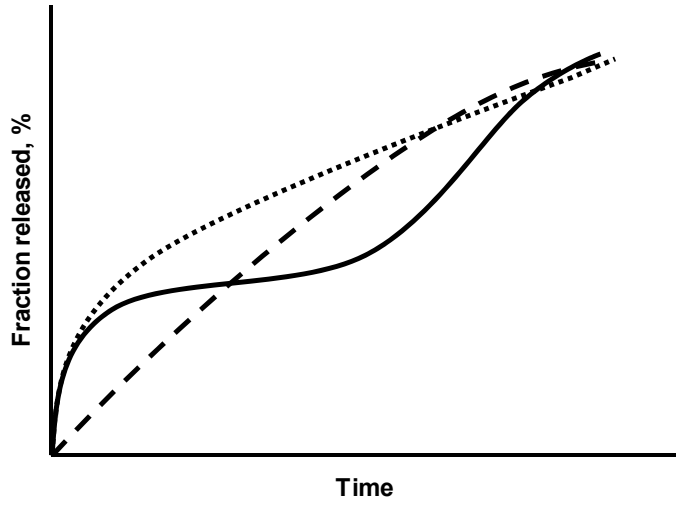

Figure 7 Illustrated examples of drug release profiles from, poly(lactic/glycolic acid) based drug delivery systems. Dashed line: zero-order release. Dotted line: bi-phasic release with a burst and zero order release. Continuous line: tri-phasic release with a burst, lag -and erosion controlled release phase.

1.4 Particular issues of protein delivery

1.4.1 General

In addition to the complex release mechanisms of proteins from poly(lactic-co-glycolic acid) based drug delivery systems the maintenance of the low structural stability and biological activity of proteins during manufacturing, storage and release is still very challenging (Ghalanbor, Körber et al. 2010). In this work only an overview about the special challenges of protein delivery can be given. More detailed information can be found in several excellent review articles (Wang 1999; Tamber, Johansen et al. 2005; Kerwin and Remmele 2007; Giteau, Venier-Julienne et al. 2008; Houchin and Topp 2008; Manning, Chou et al. 2010; Ye, Kim et al. 2010).

1.4.2 Protein structure

Proteins are high molecular weight polymers usually in the range of 10 to 500 kDa. The biological activity of proteins is based on the correct folding of the polymer chain into a unique three-dimensional structure which is actually a composite of many microstates (Hilser, Dowdy et al. 1998). However, over the past decade there has been a realization that some proteins can also function in an unfolded state (e.g. some acidic fibroblast growth factors). The current designation for these proteins is intrinsically denatured proteins (IDP's) (Uversky 2002; Manning, Chou et al. 2010).

The structure of folded proteins can be distinguished into four levels (Figure 8). The primary structure of proteins comprises of a unique sequence of 20 naturally occurring amino acids. The secondary structure refers to highly regular local sub structures such as α-helices, β-sheets and β-turns. The tertiary structure describes the packing of secondary structural units into an overall globular three-dimensional structure. In many proteins these globular units or individual domains are further packed into a larger assembly which is referred to as quaternary structure.

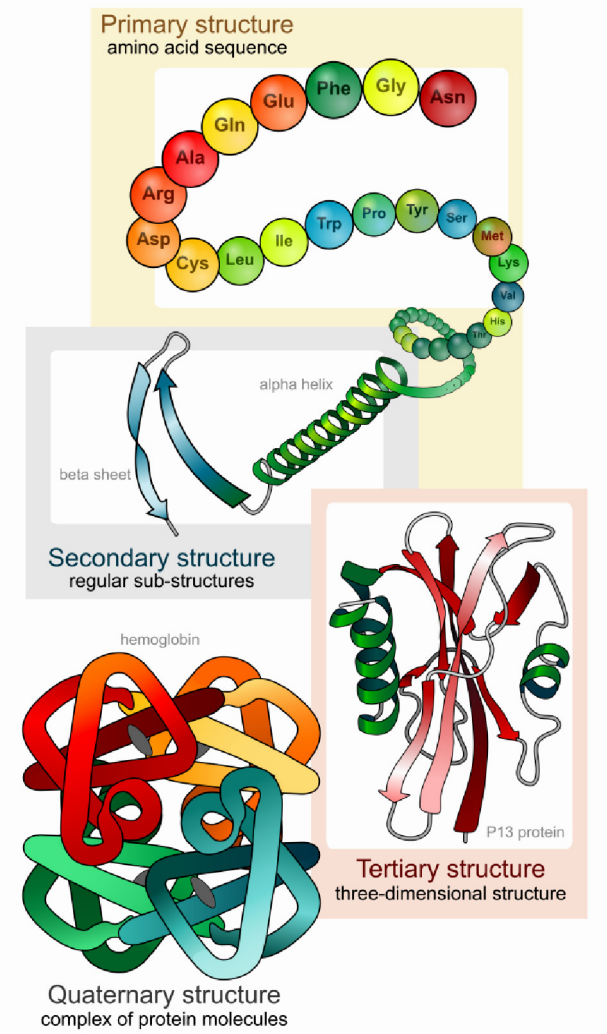

Figure 8 Illustration of main protein structures: from primary to quaternary structure (http://en.wikipedia.org/wiki/Protein_structure, June 2011).

1.4.3 Protein instabilities

Proteins are very labile macromolecules. Changes in the native protein structure often lead to a decrease or even loss of the biological activity or can induce unwanted immune responses in vivo (Hermeling, Crommelin et al. 2004).

Proteins are prone to undergo various chemical and physical degradation processes due to their variety of functional groups (e.g. acids, amines, alcohols, aromatics) and their complex globular structure. Table 2 summarizes chemical and physical instabilities which have been reported from therapeutic proteins.

Alterations in the protein conformation can facilitate the access to chemical labile or more reactive structures. Thus, chemical and physical instabilities are often interrelated. For instance, certain chemical degradation processes make a protein more prone to aggregation. Likewise, there are examples of denaturation increasing the chemical reactivity of a protein (Manning, Chou et al. 2010).

Table 2 Detected chemical and physical instabilities of therapeutic proteins.

Chemical	**Physical**
Deamidation (e.g. of Asn, Gln)	Denaturation
Isomerization (Asp)	Aggregation
Racemization (e.g. of Asp)	Precipitation
Proteolysis (e.g. Asp assisted)	Surface adsorption
β-elimination	
Oxidation (e.g. His, Met, Cys, Tyr, Trp, Phe)	
Metal catalyzed oxidation	
Photooxidation	
Free radical cascade oxidation	
Disulfide exchange	
Diketopiperazine (DKP) formation	
Condensation reactions (e.g. Maillard reaction)	
Pyroglutamic acid (pGlu) formation	
Tryptophan hydrolysis	

1.4.4 Protein analysis

The complex nature of proteins makes the analysis of proteins more challenging compared to small molecules. A single method is never sufficient to resolve and characterize proteins. The combination of several analytical methods is necessary to obtain a complete view of the protein and potential degradation products (Staub, Guillarme et al. 2011). In recent years, many new and sophisticated methods have been developed, e.g. miniaturized capillary electrophoresis (CE). This resulted in improved resolution, decreased detection limits and the possible use of smaller sample sizes. Table 3 gives an overview of currently used techniques to characterize protein stability. More detailed information is provided in

excellent reviews (Wang 1999; Liu, Andya et al. 2006; Tornvall 2010; Mach and Arvinte 2011; Staub, Guillarme et al. 2011).

Table 3 Examples of analytical techniques used for protein characterization and instability monitoring.

Chromatographic techniques
Affinity chromatography (AC), Hydrophilic interaction liquid chromatography (HILIC), Ion-exchange chromatography (IEC), Reversed-phase liquid chromatography (RPLC), Size exclusion chromatography (SEC)
Electrophoretic techniques
Capillary electrophoresis (CE), Isoelectric focusing (IEF), Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page)
Microscopic techniques
Atomic force microscopy (AFM), Nanoparticle tracking analysis (NTA), Transmission electron microscopy (TEM)
Spectroscopic techniques
Absorption, Circular dichroism (CD), Dynamic light scattering (DLS), Electron paramagnetic resonance (EPR), Fluorescence, Fourier transform infrared spectroscopy (FTIR), Nuclear magnetic resonance (NMR), X-ray
Others
Biological activity assays Differential scanning calorimetry (DSC) Field flow fractionation (FFF) Filtration Flow cytometry (FCM) Mass spectrometry (MS) Ultracentrifugation

1.4.5 Stabilization approaches

Proteins used as pharmaceuticals are exposed to a large number of stresses until they reach there final destination in the human body. For instance, shearing, contact to interfaces (e.g. air-solvent, solvent-solvent, solvent-vessel), pressure, temperature, changes in type, composition or pH of the medium and interactions with dosage form components

or degradation products can induce or accelerate instability processes (van de Weert, Hennink et al. 2000; Giteau, Venier-Julienne et al. 2008)..

Every protein is prone to specific instabilities due to the unique globular structure of each macromolecule. Thus, there exists no general stabilization method which can be applied to all proteins. Instead, the most appropriate method has to be evaluated for each protein separately. The exposure of proteins to as few stresses as possible, e.g. the use of easy preparation processes or the avoidance of higher temperatures**,** is regarded as a basic strategy. In many cases, stabilizing excipients have been added to the formulation to maintain the native state of the protein structure. Examples are polyols (e.g. trehalose, sucrose, mannitol or maltose) (Allison, Dong et al. 1996), amino acids (Valente, Verma et al. 2005), hydrophilic polymers such as polyethylene glycols (Randolph 1997) or basic salts (Zhu, Mallery et al. 2000). A further approach is to keep proteins in the solid state as long as possible where proteins exhibit higher conformational stability and chemical reactions proceed much slower (Ghalanbor, Körber et al. 2010). Many protein instabilities depend on an aqueous environment. Thus, also the use of water-free preparation processes could improve protein stability (Mok and Park 2008).

1.4.6 Lysozyme as model protein

In this work, hen egg white lysozyme was selected as a macromolecular model drug due to the broad existing knowledge with respect to structure, stability and analyzing techniques.

Lysozyme was first observed in hen egg white from Latschenko in 1909 (Laschtschenko 1909). Later in 1922, Flemming discovered lysozyme also in some human body fluids, e.g. in nasal secretion (Fleming 1922). It is part of our innate non-specific immune system. Lysozyme can damage cell walls of Gram-positive bacteria (e.g. Micrococcus lysodeikticus) by catalyzing the hydrolysis of glycosidic bonds which connect N-acetylmuramic acid and N-acetyl-D-glucosamine.

Hen egg white lysozyme is a basic (isoelectric point ~11), globular protein consisting of 129 amino acids and 4 disulfide bonds with a molecular weight of approximately 14 kDa (Figure 9). Lysozyme has been widely used as model protein for general studies due to its high stability and low price (Jiang, Woo et al. 2002; Al-Tahami, Meyer et al. 2006; Censi, Vermonden et al. 2008; Ghalanbor, Körber et al. 2010; Kokai, Tan et al. 2010).

However, when working with lysozyme, it should always be considered that most other proteins will not show the same stability but aggregate, oxidize or degrade in other ways much faster (Dickenhorst 2010).

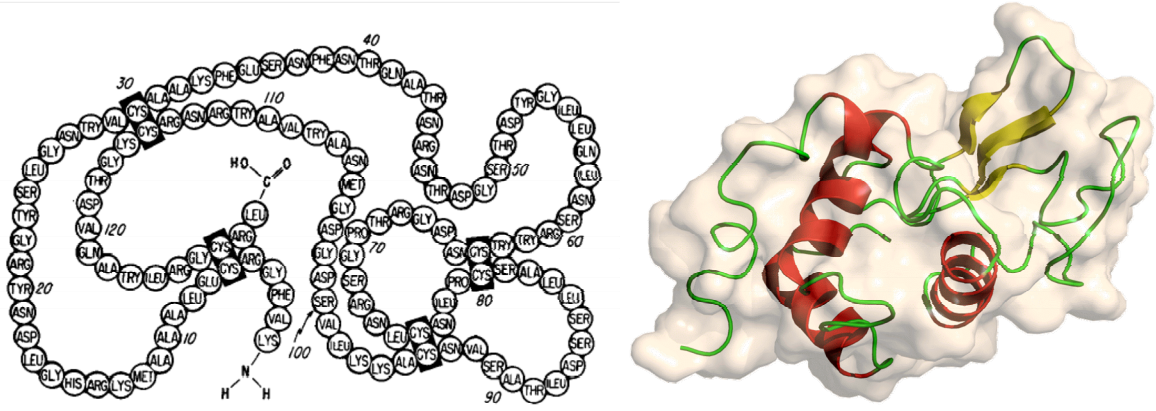

Figure 9 Primary (left) (Canfield and Liu 1965) and three-dimensional structure (right) (from http://www.pdb.org) of hen egg white lysozyme.

1.5 Poly(lactic/glycolic acid) based drug delivery systems

The most common biodegradable parenteral drug delivery systems based on poly(lactic/glycolic acid) are implants, microparticles and in situ forming systems. Other types include films (Klose, Siepmann et al. 2008), foams (Ong, Ranganath et al. 2009), nanoparticles (Sharma, Italia et al. 2007) and scaffolds (Xiong, Zeng et al. 2009).

1.5.1 Implants

Poly(lactic/glycolic acid) based implants are solid drug delivery systems which can extend the release of an active ingredient over weeks to several month. The shape of the implant (e.g. wafers, spheres or strands) and the surface area play a significant role in the rate of drug diffusion out of the implant as well as the rate of polymer degradation. The pre-formed shape makes the implant properties consistent and reproducible (Exner and Saidel 2008).

Mostly, implants have a cylindrical form with a length of 1-2 cm and a diameter of 1-2 mm. They can be manufactured easily and cost-effectively via solvent casting, freeze drying and in particular via solvent-free techniques such as melt compression, injection/compression molding or melt extrusion (Rothen-Weinhold, Besseghir et al. 1999; Jain 2000a). The major disadvantage of implants is the need of a surgery or a painful injection via large-bore needles (trocar) to place the delivery device into the body.

Based on the development of more patient friendly parenteral drug delivery systems such as microparticles or in situ forming systems, the interest in solid implants has been decreasing in recent years. However, implants may be a promising tool for the encapsulation of proteins. For instance, Ghalanbor et al. have successfully incorporated solid lysozyme in PLGA implants by melt extrusion which was released completely and remained 100 % active over approximately 60 to 80 days (Ghalanbor, Körber et al. 2010).

1.5.2 Microparticles

Biodegradable poly(lactic/glycolic acid) based microparticle drug delivery systems have been introduced in the 1980s. In recent years, there has been a growing interest in these systems especially for the controlled release of macromolecular drugs. In contrast to implants, microparticles are small spherical particles less than 250 µm in diameter. Thus, microparticles can be injected subcutaneously or intramuscularly as an aqueous dispersion trough smaller needles (Kissel, Brich et al. 1991). The use of smaller needles decreases the pain during injection and improves patient comfort. The more convenient administration of microparticles in comparison to implants makes them an attractive biodegradable delivery system. However, in many cases the fabrication of microparticles is more costly and complicated.

A large number of microparticle preparation methods have been developed. The choice of an appropriate technique depends on the polymer, the drug, the intended use and the duration of the therapy. Three basic and most widely used microparticle preparation techniques are: 1) solvent evaporation/extraction, including single emulsion processes such as *oil-in-water* (O/W) or *oil-in-oil* (O/O) emulsions and multiple emulsion processes such as *water-in-oil-in-water* (W/O/W) or *oil-in-oil-in-water* (O/O/W), 2) polymer phase separation and 3) spray drying (Tamber, Johansen et al. 2005). A schematic illustration of the preparation procedures is depicted in Figure 10.

Solvent evaporation/extraction processes are the most common microparticle preparation methods. Various modifications from the basic techniques have been developed, especially with the aim to produce preferably similar sized microparticles. Promising alterations are: 1) the use of supercritical fluids as phase separating agents, 2) interfacial phase separation via two ink-jet nozzles, 3) extrusion of the polymer solution through needles or micro-channels, 4) dripping of the polymer solution from a needle via electrostatic forces, ultrasonic atomization or acoustic excitation into the solvent extraction medium and 5) spray-freeze-drying processes. More detailed information about the large number of microparticle preparation methods is given in excellent reviews by Jain (Jain 2000a), Freitas et al. (Freitas, Merkle et al. 2005), Mundargi et al. (Mundargi, Babu et al. 2008), Shi et al. (Shi and Li 2005), Tamber et al. (Tamber, Johansen et al. 2005) and in scientific papers by Bock et al. (Bock, Woodruff et al. 2011), Porta et al. (Porta, Falco et al. 2011) and Ye et al. (Ye, Kim et al. 2010).

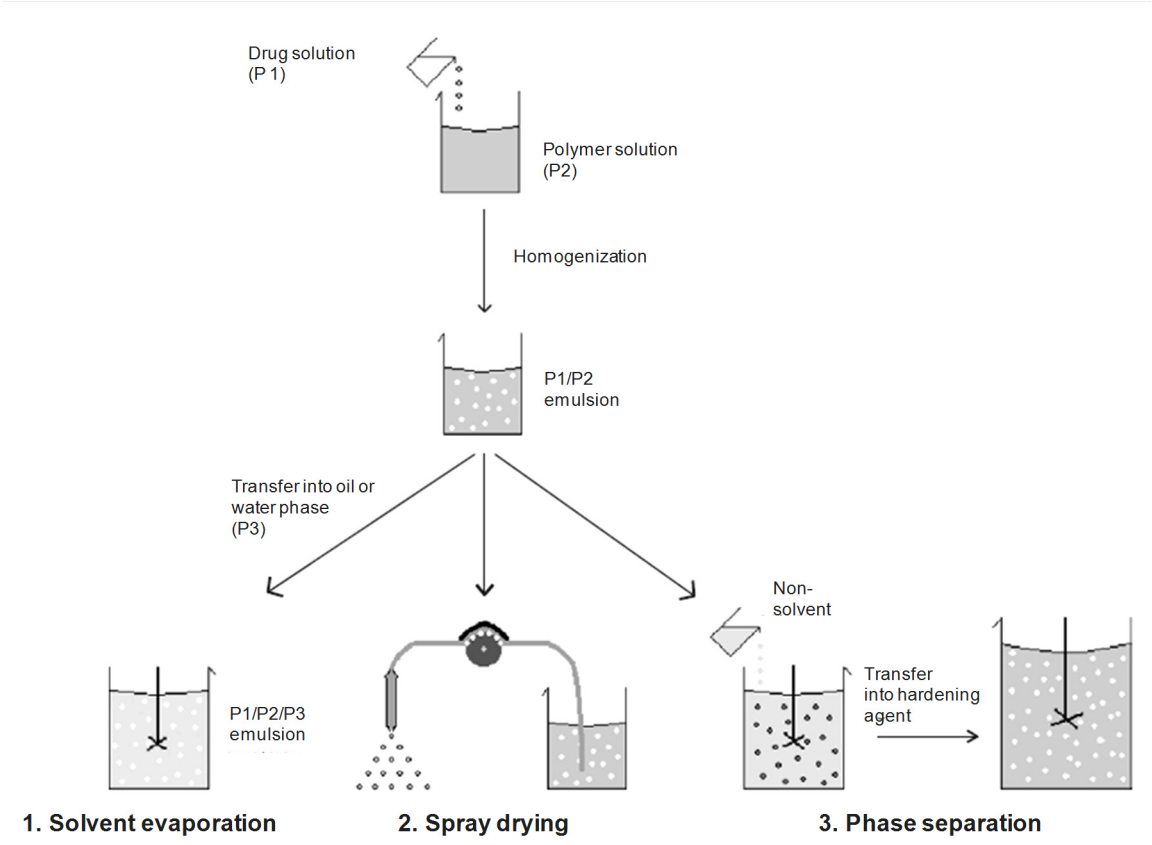

Figure 10 Schematic illustration of commonly used microencapsulation methods. A drug solution (Phase 1) is dispersed into an organic polymer solution (Phase 2) by ultrasonication or homogenisation (P1/P2 emulsion). The P1/P2 emulsion is further processed by the specific methods: 1) solvent evaporation, 2) spray drying, 3) polymer phase separation. Subsequently the microparticles are collected, washed and dried, e.g. through freeze drying (Tamber, Johansen et al. 2005).

Despite an increase in the number of marketed poly(lactic/glycolic acid) based microparticle products in recent years, only one protein containing microparticle formulation (Nutropin depot®) has launched the marked in 2000. However, the sale of Nutropin depot® containing recombinant human growth hormone was discontinued in 2004 due to high process costs (Shi and Li 2005). A complex cryogenic and non-aqueous process was developed to maintain the stability of the hormone during microparticle preparation. In this technique, a solid protein containing organic polymer solution was sprayed through a sonicating nozzle into a vessel containing frozen ethanol covered with liquid nitrogen. The vessel was then transferred to -80 °C where the ethanol melted and the microspheres hardened as the organic solvent was extracted by ethanol (Johnson, Jaworowicz et al. 1997).

1.5.3 In situ forming systems

Injectable in situ forming drug delivery systems have received increasing attention as an alternative to implants and microspheres due to less complicated fabrication, less stressful manufacturing conditions for sensitive drugs (e.g. proteins) and an easy administration (Packhaeuser, Schnieders et al. 2004). Upon injection into the body these systems become more viscous or solidify and thereby encapsulate drugs, which are then released over extended periods of time.

In situ forming systems can be classified according to their mechanisms of implant formation: 1) thermoplastic pastes, 2) in situ cross-linked implants, 3) thermally induced gelling systems, 4) pH induced gelling systems, 5) in situ forming cubosomes and 6) systems based on in situ precipitation (Hatefi and Amsden 2002; Matschke, Isele et al. 2002; Packhaeuser, Schnieders et al. 2004; Chitkara, Shikanov et al. 2006; Exner and Saidel 2008; Heller 2009).

The majority of in situ forming systems are based on non-PLGA polymers due to the utilization of specific polymer properties. Poly(lactic/glycolic acid) based in situ forming systems are in situ forming implants (ISI) and in situ forming microparticles (ISM).

1.5.3.1 In situ forming Implants

In situ forming implants are composed of a biodegradable polymer dissolved in a biocompatible organic solvent. Low molecular drugs, peptides or proteins can be either dissolved or dispersed in that solution. The earliest example of an in situ implant was patented in 1990 by Dunn and co-workers and consisted of PLGA dissolved in N-methyl pyrrolidone (NMP) (Dunn, English et al. 1990). This system has been developed as Atrigel® at Atrix Laboratories. To date two products have been marketed based on the Atrigel® technology, namely Eligard® and Atridox® (Table 1).

After subcutaneous or intramuscular injection of the polymer solution into the body the organic solvent dissipates into surrounding tissue while water penetrates in the polymer matrix. This leads to phase separation and precipitation of the polymer**,** forming a depot at the injection site (Al-Tahami, Meyer et al. 2006). The drug release pattern is strongly determined by the phase separation kinetics and the consequential morphological characteristics of the formed polymer depot. Thus, the phase separation process has been intensively studied (Brodbeck, DesNoyer et al. 1999; Graham, Brodbeck et al. 1999; Kempe, Metz et al. 2008; Solorio, Babin et al. 2010).

Drug release from systems exhibiting a fast phase separation is often characterized by a high initial burst followed by a much slower release (Brodbeck, DesNoyer et al. 1999). The fast solidification of the polymer matrix causes the formation of a highly interconnected network of solvent-water filled pores. Thus, drug molecules located in or near these interconnecting pores can diffuse out of the polymer depot rapidly. Studies of NMP based systems have shown that 75 % of the polymer was solidified in less than 30 min**,** taking place from the shell to the core (Kempe, Metz et al. 2008). In contrast, the water-solvent exchange in slow phase separating systems may take hours to days. The polymer matrix remains in a highly viscous state or forms a more or less uniformly**,** dense depot with few or no apparent pores resulting in a decreased initial drug release (Brodbeck, DesNoyer et al. 1999; McHugh 2005).

The rate of the phase separation process is affected by the polymer molecular weight, polymer concentration, incorporated drugs or additives, composition of the surrounding aqueous medium and in particular by the water miscibility of the organic solvent (DesNoyer and McHugh 2001; DesNoyer and McHugh 2003; Solorio, Babin et al. 2010).

A faster water-solvent exchange occurs when more hydrophilic, water-miscible solvents are used, for instance: NMP, dimethyl sulfoxide (DMSO) (Royals, Fujita et al. 1999), 2-pyrrolidone, propylene carbonate (Dunn, Tipton et al. 1992), ethyl acetate (Lambert and Peck 1995), glycerol formal (Chern and Zingerman 1999), glycofurol (Eliaz and Kost 2000) or low-molecular weight polyethylene glycol (Su, Ashton et al. 2005). Slower phase inverting systems are generally based on less hydrophilic solvents with lower water affinity such as benzyl benzoate, ethyl benzoate (Brodbeck, Gaynor-Duarte et al. 2000), triacetin, triethyl citrate (Shah, Railkar et al. 1993) or benzyl alcohol (Prabhu, Tran et al. 2005).

Beside the use of less water-miscible organic solvents to reduce a high initial release, increasing the polymer concentration could be an easier alternative (Lambert and Peck 1995). However, the high viscosity of such polymer solutions often makes the use of larger needle sizes (18-20 G) necessary. This may increase the injection pain to the patient and thus decrease patient compliance (Rungseevijitprapa and Bodmeier 2009). Another problem with in situ implants could be an unpredictable release pattern due to the formation of non-uniform implants exhibiting different surface areas (Jain, Rhodes et al. 2000b). Furthermore the use of organic solvents may cause local tissue irritations or intoxications although, in general, the administered amounts do not exceed the permitted daily doses (ICH guidelines: Q3C Impurities: Residual Solvents).

1.5.3.2 In situ forming microparticles

In situ forming microparticles (ISM) were developed to overcome problems associated with conventional microparticles and in situ forming implants (Bodmeier 1997; Luan and Bodmeier 2006b). The manufacture of conventional microparticles is often complex and costly. In contrast, ISM formulations can be prepared easily and cost-efficiently. Two fabrication approaches have been developed in different research facilities. Jain et al. proposed the preparation of injectable microglobules or pre-microspheres based on emulsification of two oil phases. A solution of PLGA, triacetin, a model drug (e.g. cytochrome c), PEG 400 and Tween 80 (oil phase 1) was added dropwise under continuous homogenization (10000 rpm) to Miglyol 812 containing Span 80 (oil phase 2). Thereby, phase separation (coacervation) of PLGA was induced and pre-microspheres, ready for injection, were formed. Upon contact with physiological fluid**,** the pre-microspheres hardened and formed solid microparticles**,** entrapping the drug. From these formulations cytochrome c was released in a continuous manner in vitro (Jain, Rhodes et al. 2000; Jain 2000a; Jain, Rhodes et al. 2000b).

Bodmeier invented a more convenient ISM preparation technique (Bodmeier 1997). Thereby a drug-containing organic polymer solution (ISI solution) is emulsified into an aqueous or non-aqueous oily continuous phase (e.g. sesame oil). Non-aqueous ISM emulsions are stabilized by the combination of a biocompatible emulsifier such as Span 80 or Pluronic F68 and a continuous phase viscosity enhancer such as aluminum monostearate (Luan and Bodmeier 2006b; Kranz and Bodmeier 2007; Rungseevijitprapa and Bodmeier 2009). Upon injection of the ISM emulsion into the body the polymer precipitates in form of microparticles due to the water-solvent exchange (Luan and Bodmeier 2006a). ISM emulsions can be prepared in a two-syringe/connector system directly prior to use by back-and-forth movement of the syringe plungers. This improved the practicability of such systems (Figure 11). Interactions between excipients and/or active ingredient during storage could be eliminated by a separate placement of drug, PLGA and solvents within the two syringes (Dong, Körber et al. 2006).

ISM formulations have shown advantages in comparison to the higher viscous ISI solutions. Easier injectability through smaller needle sizes (Rungseevijitprapa and Bodmeier 2009), improved muscle compatibility observed in Sprague Dawley rats (Kranz, Brazeau et al. 2001; Rungseevijitprapa, Brazeau et al. 2007) and decreased initial burst release of low molecular drugs and peptides in vitro and in vivo (Kranz and Bodmeier

2008; Kranz, Yilmaz et al. 2008a) were related to the additional lower viscous continuous phase.

The effects of various process and formulation parameters on the release of low molecular weight drugs and peptides from ISM systems have been investigated. For instance, a high initial release could be reduced by: 1) increasing the continuous phase:internal phase ratio of the emulsion, the viscosity of the continuous phase or the polymer concentration, 2) using less water miscible organic solvents (e.g. 2-pyrrolidone instead of DMSO), 3) replacing PLGA by PLA, or 4) by decreasing the drug loading (Luan and Bodmeier 2006a; Kranz and Bodmeier 2007; Kranz and Bodmeier 2008).

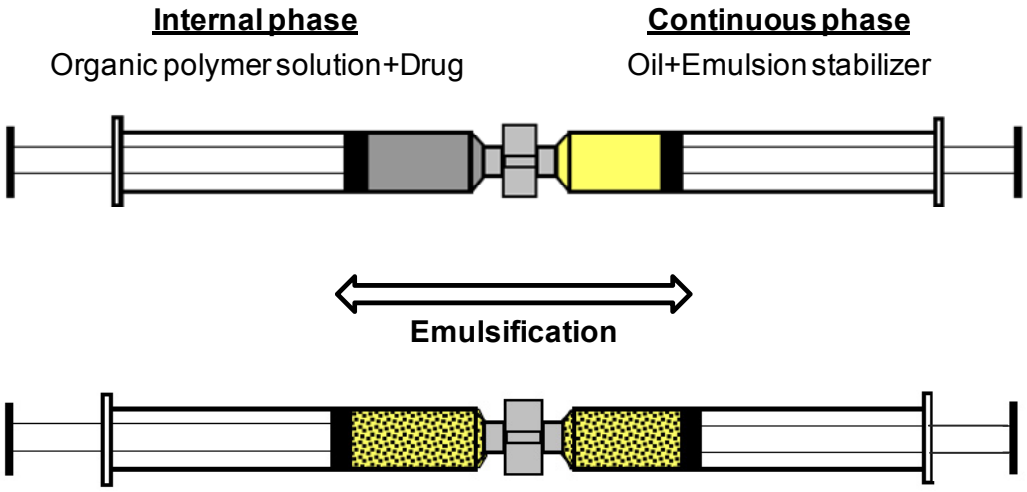

Figure 11 Preparation of an injectable ISM-emulsion prior administration in a two-syringe/connector system.

However, there is still the need for improvement. One challenge, especially associated with non-aqueous ISM systems, is the relatively low ISM emulsion stability. Koerber observed a beginning phase separation within minutes despite the utilization of various potential emulsion stabilizers (Koerber 2007). Coalescence of unstable viscous polymer solution droplets (lumps formation) prior administration may complicate an easy injection through thin needles. Furthermore**,** emulsion instability will result into the in situ formation of implant-like polymer lumps rather than microparticles. Differently shaped polymer matrices exhibit different surface areas which will likely affect the drug release pattern.

1.6 Objectives

Therefore, the first objective of this work was:

> to obtain physically stable non-aqueous ISM emulsions using parenteral approved substances.

A successful ISM emulsion stabilization consequently led to the development of following main objectives:

a. identification of underlying stabilization mechanisms

b. characterization of the stabilizing capacity of potential stabilizers with regard to changes in formulation and process parameters

c. evaluation of the effect of emulsion stabilization on the injectability of the resulting ISM formulations and

d. elucidation of the influences of emulsion stability and continuous phase composition of non-aqueous ISM systems on the release of low and high molecular model drugs.

2 Materials and methods

2.1 Materials

Model drugs

Ibuprofen, theophylline (BASF AG, Ludwigshafen, Germany); lysozyme (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); leuprolide acetate (Lipotec S.A., Barcelona, Spain); diltiazem hydrochloride (PCAS, Limay, France); carbamazepine, propranolol hydrochloride (K.-W. Pfannenschmidt GmbH, Hamburg, Germany); diprophylline (Selectchemie AG, Zürich, Switzerland); lidocaine hydrochloride (Sigma-Aldrich Inc., St. Louis, USA).

Oils

Castor oil (Caesar & Loretz GmbH, Hilden, Germany); peanut oil (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); medium chain triglycerides (MCT) (Fagron GmbH & Co. KG, Barsbüttel, Germany); soybean oil (Fluka Chemie AG, Buchs, Switzerland); sesame oil (Sigma–Aldrich Company, St. Louis, USA).

Organic solvents

Polyethylene glycol 400 (PEG 400) (Caesar & Loretz GmbH, Hilden, Germany); dimethylsulfoxide (DMSO), n-hexane, *N*-methyl-2-pyrrolidone (NMP), tetrahydrofuran (THF), triacetin (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); polyethylene glycol 400 (PEG 400) (Caesar & Loretz GmbH, Hilden, Germany); 2-pyrrolidone, benzyl benzoate (Merck, Darmstadt, Germany).

Polymers

Poly(d,l-lactide-*co*-glycolide) (capped - alkyl ester end groups - 50:50 PLGA: Resomer® RG 502, 502S, 503; uncapped - free carboxylic acid end - 50:50 PLGA: Resomer® RG 502H, 503H) (Boehringer Ingelheim, Ingelheim, Germany).

Surfactants

Poloxamer 188 (Pluronic F68), poloxamer 407 (Lutrol F127), polyethylene glycol 660 12-hydroxystearate (Solutol HS 15), polyoxyl 35 castor oil (Cremophor EL), polyoxyl 40 hydrogenated castor oil (Cremophor RH 40), polyoxyl 60 hydrogenated castor oil (Cremophor RH 60) (BASF AG, Ludwigshafen, Germany); chenodeoxy acid, cholesterol, cholic acid, polyoxyethylene 20 sorbitan monooleate (Tween 80), sodium dodecyl sulfate (Carl Roth GmbH&Co.KG, Karlsruhe, Germany); egg-phosphatide mixtures (Lipoid E80, EPC), soy-phosphatide mixtures (Lipoid S 100, S45) (Lipoid GmbH, Ludwigshafen, Germany); sorbitan monooleate (Span 80) (Merck, Darmstadt, Germany); glycerol monostearate (Imwitor 900 K) (Sasol Germany GmbH, Witten, Germany).

Other excipients

Acetic acid, potassium dihydrogen phosphate, potassium hydroxide, sodium azide, sodium hydroxide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany); aluminum monostearate (Fluka, Chemie AG, Buchs, Switzerland); ethanolamine (Merck, Darmstadt, Germany); methylene blue, micrococcus lysodeikticus (Sigma–Aldrich Company, St. Louis, USA); coomassie plus protein assay reagent (Thermo Fisher Scientific Inc., Rockford, USA).

2.2 Methods

2.2.1 ISM emulsion preparation

For example, the internal phase was prepared by dissolving 12 mg drug (4 %, w/w, based on polymer) in 700 mg organic solvent. Subsequently, 300 mg of PLGA (30 %, w/w, based on polymer and organic solvent) were added and dissolved under intermittent vortexing. The continuous phase was prepared by dissolving 50 mg stabilizer in 950 mg

vegetable oil (5 %, w/w, based on continuous phase). Insoluble stabilizers such as GMS were molten at 80 °C and subsequently cooled to room temperature. Both phases were stored in a desiccator for 24 h before further use if not mentioned differently.

ISM emulsions were prepared in a two-syringe/connector system, comprising the internal and continuous phases each in a 1 ml single-use syringe (B.Braun Melsungen AG, Melsungen, Germany). The syringes were coupled with a connector having a 1.4 mm orifice and the two phases were emulsified by back-and-forth movement of the syringe plungers.

Unless mentioned otherwise, ISM emulsions (0.4 g) were prepared under the following standard conditions: a DMSO solution containing 30 % PLGA Resomer® 502S was emulsified into 5 % stabilizer containing sesame oil with an internal phase:continuous phase ratio of 1:1.5 in the two-syringe/connector system (1.4 mm connector diameter) for 180 s and 1 mixing cycle per s.

2.2.2 Viscosity

The flow behaviour of continuous phases and the corresponding ISM emulsions were investigated with a software-assisted rheometer (Rheostress RS 100 with RheoWin Pro software, Haake Messtechnik GmbH, Karlsruhe, Germany) equipped with a plate-cone setup of 60 mm diameter (1° angle) at 23 ± 0.2 °C. Shear rate controlled ramps (CR) with increasing and decreasing segments (0 - 150 - 0 s^{-1}, 4 min per measurement) were used.

The viscosities of continuous and internal phases were studied under a fixed shear stress of 10 Pa for 120 s. The effect of cooling speed during continuous phase preparation on the viscosity was determined at a constant shear rate of 10 s^{-1} after the samples were treated with a shear rate of 100 s^{-1} for 1 min to mimic the shear stress during emulsification.

The yield point, indicating the gel-network strength of GMS in the continuous phase (sesame oil), was determined using controlled shear stress ramps (CS) with logarithmic distributed data points from 1 to 1000 Pa in 180 s and consecutively double logarithmic data plotting (shear rate against shear stress). The yield point was defined as the shear stress value where the slope of the resulting curve increased.

Prior to all measurements the samples were treated with a shear rate of 10 s^{-1} for 60 s and rested for further 60 s to standardize the sample history.

2.2.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to investigate the thermal characteristics of GMS in the continuous phase and in the corresponding ISM emulsions. 30 to 40 mg sample material were accurately weighed in closed 40 µl aluminum crucibles and analyzed with a DSC 821 (Mettler Toledo AG, Giessen, Germany) under nitrogen atmosphere. The samples were cooled to 15 °C and then heated to 80 °C at a constant rate of 10 °C/min. The resulting thermograms were standardized on a sample weight of 40 mg.

2.2.4 Hot-stage polarized light microscopy

A Zeiss Axioscope (Carl Zeiss Jena GmbH, Jena, Germany) equipped with a Mettler Toledo FP82HT hot stage (Mettler-Toledo GmbH, Giessen, Germany) and EasyMeasure analysis software (Inteq Informationstechnik GmbH, Berlin, Germany) were used to study 1) the morphology and the melting behaviour of GMS in continuous phases and in corresponding ISM emulsions, 2) emulsion droplet size distributions 15 min after preparation (n=3, each with 300 droplets), 3) the in situ formation of microparticles and 4) the morphology of in situ formed PLGA matrices such as microparticles.

2.2.5 ISM emulsion stability study

The stability of ISM emulsions stabilized with GMS was evaluated by changes in the particle size distribution (by polarized light microscopy) and oil separation (by eye) over 14 days from formulations stored vertically in 1 ml syringes in a desiccator under ambient conditions. The particle size distribution was characterized by the arithmetic mean diameter (d_{av}) and the particle size at 10 % (d_{10}) and 90 % (d_{90}) of the particle size distribution to visualize possible particle size growth (n=3).

2.2.6 Electron microscopy

2.2.6.1 Cryo - scanning electron microscopy (Cryo-SEM)

The emulsion droplet interface of freshly prepared non-aqueous ISM emulsions and the morphology of ISM microparticles were investigated with a Hitachi S-4800 scanning

electron microscope at -145 °C. Thereby, emulsions were rapidly frozen in liquid nitrogen slush, fractured at -190 °C and sputtered with platinum.

2.2.6.2 Freeze etching - transmission electron microscopy (FE-TEM)

Additionally, the droplet interface was investigated with a Zeiss EM-902 transmission electron microscope. The samples were pre-treated with the aid of a Balzers BAF 400T etching unit, i.e. emulsions were rapidly frozen in liquid propane, fractured at -100 °C and sputtered with platinum and carbon. Subsequently, the samples were dissolved in tetrahydrofuran. Transmission electron microscopy was then performed on the corresponding mirror images.

2.2.7 Injectability

The force-distance profile of a injected 35 % PLGA 502S containing DMSO (w/w) solution was compared with corresponding in situ microparticle emulsions using a texture analyzer (50 N load cell) equipped with Texture Expert Exceed software (TA.XTplus, Stable Micro Systems, Vienna Court, UK). The maximal force was set to 45 N. 0.275 mL of the PLGA solution or 0.550 mL of the corresponding ISM emulsions (sesame oil with or without 5% GMS; phase ratio 1:1, connector diameter 0.75 mm and 2 cycles per s for 180 s) were injected from 1 ml syringes (Injekt-F, B. Braun Melsungen AG, Melsungen, Germany) through hypodermic needles (24 G x 1.0 in) in order to compare the formulation at a similar polymer solution volume. The freshly prepared ISM emulsions were kept in horizontal position for 5 min (non-stabilized formulations) or 15 min (stabilized formulations) before injection. The injection speed was set to 5 mm/s, which corresponded to an injection of 1 mL per 10 s. The syringe friction force of 0.49 ± 0.16 N was not subtracted to present real injection forces. The injection energy was calculated from the area under the curve (AUC).

2.2.8 In vitro release of lysozyme

0.4 g of ISM emulsions containing 4 % lysozyme (w/w, based on polymer) were injected into screw cap sealed test tubes filled with 8 g 33 mM sodium acetate buffer containing 0.01 % sodium azide as preservative. The pH 5.0 of the release medium was

chosen according to the stability optimum of lysozyme (Claudy, Létoffé et al. 1992). The vials were incubated in vertical position in a horizontal shaker (Gemeinschaft für Labortechnik, Burgwedel, Germany) at 80 rpm and 37 °C (n = 3). The release medium was replaced with fresh medium at each sampling time point.

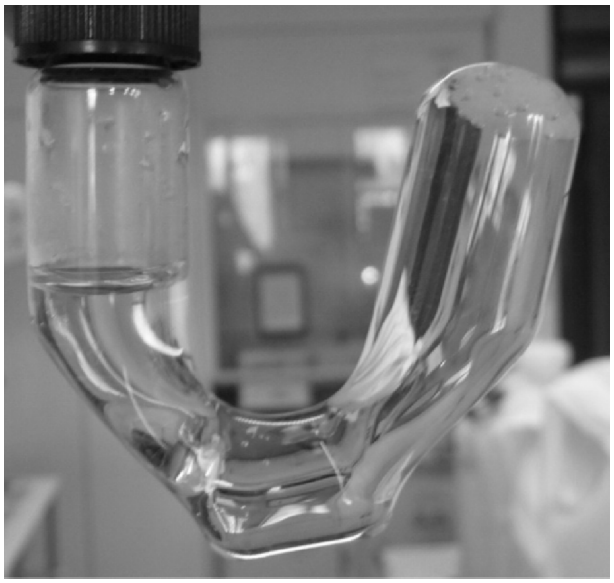

Figure 12 Lysozyme release from oils and corresponding polymer-free non-aqueous emulsions was performed in U-shaped glass test tubes.

1.92 mg lysozyme was also released from 0.16 g oils (1.2 % lysozyme, w/w, based on oil) and from corresponding 0.4 g polymer-free non-aqueous ISM emulsions (1.2 % lysozyme, w/w, based on internal phase) in U-shaped glass test tubes filled with 8 g of 33 mM pH 5 sodium acetate buffer containing 0.01 % sodium azide as preservative (Figure 12). The floating viscous formulations were placed in that part of the U-shaped tube which was completely filled with release medium so that the sampling could be carried out from the opened part of the test tube without sample damage or loss (n=3).

The concentration of released lysozyme was quantified by modified Coomassie Plus (Bradford) assays (1-28 µg/ml lysozyme, reagent to sample ratio 1:1 and 10-350 µg/ml lysozyme, reagent to sample ratio 1.2:10). The samples were allowed to stand for 10 to 15 min prior measurement of the UV absorption at 25 °C and 595 nm with a diode array UV-spectrophotometer equipped with a Peltier thermostatted cell holder (Agilent 8453, Agilent Technologies Inc., Palo Alto, USA).

2.2.9 In vitro release of low molecular weight drugs

0.4 g of ISM emulsions containing theophylline, diprophylline or carbamazepine (4 %, w/w, based on polymer) were injected into screw cap sealed test tubes filled with 10 g of 100 mM phosphate buffer solution and incubated in vertical position in a horizontal shaker at 80 rpm and 37 °C. 5 mL release medium was sampled and replaced at each sampling time point (n=3).

The concentration of released drug was determined with a UV-spectrophotometer (Agilent 8453, Agilent Technologies Inc., Palo Alto, USA) at λ_{max} for theophylline at 271 nm, for diprophylline at 274 nm and for carbamazepine at 285 nm.

2.2.10 Biological activity of lysozyme

The biological activity of released lysozyme was measured with a modified turbidimetric assay (Shugar 1952). The concentration of active enzyme was correlated with the linear rate of the absorbance decrease at 450 nm of a micrococcus lysodeikticus cell suspension in 66 mM phosphate buffer (pH 6.24) at 25.0 °C. The initial absorbance of the filtrated cell suspension was adjusted to values between 0.6 and 0.7. After the addition of 100 µl of aqueous lysozyme solution to 2.5 ml bacteria suspension, the turbidity was immediately measured for 2 min each 6 s. The slope of the linear portion was used for the quantification of the amount of active lysozyme in the sample based on a freshly prepared standard curve (0–30 µg/g). The samples were bracketed with standards after each fifth sample. A diode array UV-spectrophotometer with a Peltier thermostatted cell holder (Agilent 8453, Agilent Technologies Inc., Palo Alto, USA) equipped with a UV-Chemstation biochemical analysis software was used for data collection.

2.2.11 PLGA degradation

0.4 g of ISM emulsions containing 4% lysozyme (w/w, based on polymer) were injected into screw cap sealed test tubes filled with 8 g of 33 mM pH 5 sodium acetate buffer containing 0.01 % sodium azide and subsequently incubated in vertical position in a horizontal shaker at 80 rpm and 37 °C. The samples were similar treated as samples in the respective lysoyzme release studies.

At predetermined time points the ISM formulations were withdrawn from the test tubes, twice washed with n-hexane to remove the oil phase, frozen at -70 °C for 30 min, vacuum dried for 24 h and dissolved in THF to analyze the PLGA molecular weight distribution by GPC. A Shimadzu (Shimadzu, Tokyo, Japan) LD-10 liquid chromatograph equipped with degasser, pump, auto-injector and column oven in combination with a Viscotek triple detector (TDA-300, Viscotek, Malvern Instruments Ltd., Malvern, UK) was used. More detailed information can be found elsewhere (Körber 2010).

2.2.12 PLGA erosion

PLGA 502S degrades over weeks via random chain hydrolysis to soluble fragments (approx. 1000 Da) causing the erosion of the polymer (Körber 2010). The acidic soluble degradation products decreased the pH of the weak acetic acid buffer (pH 5.0, 33mM) of the medium. Thus, at each sampling point the pH of the removed release media was measured with a Sartorius PB-11 pH-meter (Sartorius AG, Göttingen, Germany) and converted into a cumulative pH shift which was shown to correspond to the erosion of PLGA (Ghalanbor 2011).

2.2.13 Miscibility studies

The maximal amount of DMSO or water which was miscible with vegetable oil was determined gravimetrically under stirring at room temperature. The solvents were dropwise added to 2.0 g oil until visible phase separation occurred. Miscibility was then expressed in % (w/w, based on vegetable oil).

3 Results and Discussion

3.1 Improved Stabilization and Injectability of Non-aqueous in situ PLGA Microparticle Forming Emulsions

3.1.1 Introduction

Injectable in situ forming biodegradable drug delivery systems have received increasing attention as an alternative to implants and microspheres due to simpler preparation, less stressful manufacturing conditions for sensitive drugs (e.g. proteins) and easy administration (Packhaeuser, Schnieders et al. 2004). Upon injection into the body these systems become more viscous or solidify and thereby encapsulate drugs, which are then released over extended periods of time.

Biodegradable in situ forming microparticle (ISM) systems are injectable emulsions where an internal phase, consisting of a drug-containing polymer solution (e.g. PLGA in DMSO), is emulsified into a stabilizer-containing aqueous or oily (e.g. sesame oil) continuous phase in a two syringe/connector system prior to administration (Luan and Bodmeier 2006a). These ISM emulsions showed advantages over the corresponding drug-containing polymer solutions (in situ implants; ISI), such as decreased myotoxicity (Kranz, Brazeau et al. 2001), more reproducible and burst-free drug release patterns (Kranz and Bodmeier 2007) and better injectability (Rungseevijitprapa and Bodmeier 2009). This, however, requires that the ISM emulsions are stable until their administration. The preparation of stable non-aqueous emulsions is not trivial and, in contrast to aqueous emulsions, kinetically stable non-aqueous emulsions have rarely been achieved (Suitthimeathegorn, Turton et al. 2007). The selection of suitable stabilizers is challenging due to a lack of general knowledge about the underlying stabilization mechanisms in non-

aqueous emulsion systems (Cameron and Sherrington 1996; Suitthimeathegorn, Jaitely et al. 2005; Payghan, Bhat et al. 2008). The hydrophilic-lipophilic balance (HLB) introduced by Griffin in 1949, for instance, does not apply (Petersen, Hamill et al. 1964). Stabilization has been achieved by either utilization of a suitable oil-immiscible polar liquid that can substantially replace water, e.g. formamide or by designing surfactants having two incompatible blocks, each of which is selectively soluble in one of the immiscible liquids (Imhof and Pine 1997). These substances, however, are not approved for parenteral applications.

The lower viscosity of ISM emulsions, due to the dispersion of the highly viscous polymer solution phase in a less viscous continuous phase, resulted in decreased injection forces compared to polymer solutions only (ISI) (Rungseevijitprapa and Bodmeier 2009). This viscosity-driven evaluation of the "injectability", however, neglected the injection volume to achieve similar drug doses. According to Hagen-Poisseuille's law, even highly viscous liquids (e.g. ISI) could be injected at low forces using for example large needles or low injection speeds (Ravivarapu, Moyer et al. 2000; Crawford, Sartor et al. 2006). Both means, however, would probably decrease the patient acceptance, considering that needle diameter is connected to pain level (Mitchell and Whitney 2001) as well as pain exposure time to patient discomfort.

Parameters of an "ideal" injection for both patients and health care professionals are therefore 1) short duration (≤ 10 s) (Dacre and Kopelman 2002), 2) small needle size (≥ 20 G) and 3) low maximal injection force (≤ 20 N) (Debra, Opincar et al. 2008; Schoenhammer, Petersen et al. 2009). The choice of the injection site will determine the limit of the injection volume, e.g. 1 mL for the deltoid site (Rodger and King 2000), and also set the ideal injection speed (e.g. 1 ml per 10 s). The evaluation of the "injectability" of an injectable depot formulation thus needs to include the aforementioned aspects.

The objectives of this study were therefore to obtain physically stable non-aqueous ISM emulsions, to elucidate the underlying stabilization mechanisms and to characterize the effect of the emulsion stabilization on the injectability of the resulting ISM formulations.

3.1.2 Results and Discussion

3.1.2.1 Stabilizer screening

The selection of stabilizing excipients for non-aqueous emulsions is not as straightforward as for aqueous emulsions. A number of parenterally approved potential stabilizers (Costarelli, Key et al. 2002; Rowe, Sheskey et al. 2005; Dassinger, Dootz et al. 2009) were screened for a stabilizing effect on non-aqueous ISM emulsions (Table 4). The excipients were added to the continuous phase (medium chain triglycerides (MCT) or sesame oil) and were then emulsified with a PLGA-containing DMSO solution (internal phase:continuous phase ratio 1:1) in a two-syringe connector system.

Table 4 Stabilizing effect of potential parenteral approved substances (5 %, based on continuous phase) on non-aqueous ISM emulsions containing MCT or sesame oil as continuous phase (internal phase:continuous phase ratio 1:1).

Emulsion stabilizers		**Emulsion stability**[1]	
Chemical name	**Trade name**	**MCT**	**Sesame oil**
Aluminum monostearate		-	-
Chenodeoxycholic acid		-	-
Cholesterol		-	-
Cholic acid		-	-
Egg-phosphatide mixtures	Lipoid E 80	-	-
Ethanolamine		-	+
Glycerol monostearate	Imwitor 900 K	+	+
PEG 660 12-hydroxystearate	Solutol HS 15	-	-
Poloxamer 188	Pluronic F 68	-	-
Poloxamer 407	Lutrol F 127	-	-
Polyoxyethylen sorbitane monooleat	Tween 80	-	-
Polyoxyl 35 Castor Oil	Cremophor EL	-	-
Polyoxyl 40 Hydrogenated Castor Oil	Cremophor RH 40	-	-
Polyoxyl 60 Hydrogenated Castor Oil	Cremophor RH 60	-	-
Sodium dodecyl sulfate		-	-
Sorbitan monooleate	Span 80	-	-
Soy-phosphatide mixtures	Lipoid S 100	+	-
	Lipoid S 45	±	-
	Lipoid EPC	-	-
α-tocopherol	Vitamin E	-	-

[1] ISM emulsions were macroscopically stable for: (-) 0-5 min, (±) 5-10 min and (+) more than 15 min

In most cases, two separate phases formed within 5 min. Only GMS, ethanolamine and Lipoid S 100 showed no phase separation over an observation period of 15 min which is an appropriate time frame to administer the ISM emulsion to the patient. The alkaline ethanolamine formed the emulsifying substances, hydroxyethylammonium carboxylates, in situ via saponification of vegetable oils (McMahon, Hamill et al. 1963). In general, the use of saponifying agents might be a promising tool to stabilize non-aqueous emulsions. However, ethanolamine can also potentially hydrolyze PLGA in ISM emulsion systems and was thus not studied further.

Emulsions containing Lipoid S100 in MCT showed phase separation after about 20 min. However, emulsions stabilized with GMS showed no signs of phase separation over more than 1 hour independent of whether MCT or sesame oil was used as continuous phase. Stabilization with GMS was thus more effective compared to the previously used stabilizer Span 80, Pluronic F68 and aluminum monostearate (Jain, Rhodes et al. 2000; Luan and Bodmeier 2006b; Kranz and Bodmeier 2007).

3.1.2.2 Stability of GMS-stabilized emulsions

Increasing the internal phase:continuous phase ratio from 1:1 to 1:1.5 further improved the ISM emulsion stability against droplet coalescence. No sign of instability was observed within 12 h of storage in a horizontally stored 1 mL syringe at room temperature in a desiccator (Table 5). The average particle size (d_{av}) increased from 19.1 µm after 12 h to 21.9 µm at day 1 and steadily further increased to 29.6 µm at day 14. The particle size where 90 % of the emulsion droplets have a smaller diameter (d_{90}) increased slightly faster during storage compared to the particle size where 10 % of the droplets have a smaller diameter (d_{10}). This phenomenon was attributed to Ostwald ripening (Imhof and Pine 1997). Separation of the continuous phase was observed after 1 d, where 5 % of the oil could be decanted. This amount increased to about 20 % within 14 d. The formation of a separate PLGA solution phase was not observed. The PLGA-containing internal phase thus remained finely dispersed, which indicated that microparticle formation could be still achievable.

Table 5 Stability of GMS containing ISM emulsions (5 %, based on continuous phase) during storage in horizontally stored 1 mL syringes.

Storage time	Droplet size distribution (µm)			Oil separation
(days)	d_{av}[1]	d_{10}	d_{90}	(%)
0	19.3 ± 3.4	15.8	22.6	0.0
0.5	19.1 ± 3.2	15.4	23.0	0.0
1	21.9 ± 4.1	17.4	25.5	4.9 ± 0.1
2	25.0 ± 5.2	19.9	29.8	9.7 ± 0.3
5	26.0 ± 5.9	20.4	32.6	14.3 ± 0.9
9	28.2 ± 6.5	20.7	35.7	19.2 ± 0.8
14	29.6 ± 6.8	22.4	38.4	19.2 ± 0.8

[1] d_{av} is the arithmetic mean diameter, d_{10} and d_{90} are the droplet diameters at 10 % and 90 % of the particle size distribution

3.1.2.3 Stabilization mechanisms

Polarized light microscopy revealed that GMS formed a fine matrix of amorphous-like and rod-like GMS crystals embedded in the oily continuous phase (Figure 13A). After emulsification, a birefringent layer instead of the rod-like crystals was located at the interface between the oil phase and the PLGA solution droplets (Figure 13B).

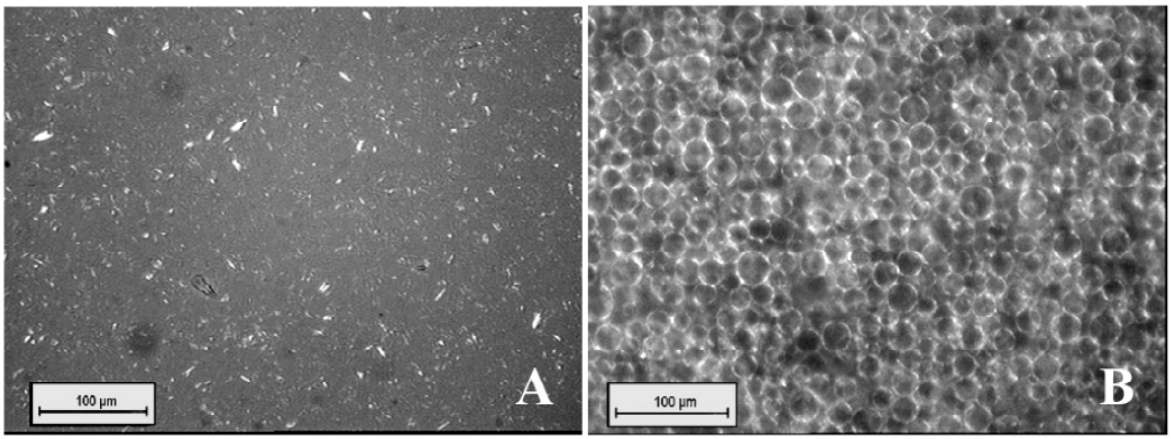

Figure 13 Polarized light microscopy of **(A)** the continuous phase (5 % GMS in sesame oil) stored 1 day at room temperature and **(B)** the corresponding ISM emulsion 15 min after preparation.

The transformation of rod-like GMS crystals into the birefringent layer at the phase boundary was accompanied by a polymorphic phase transition. DSC chromatograms revealed a multiphasic melting behaviour for the GMS crystals in the continuous phase but only one melting peak for the corresponding emulsion (Figure 14). A similar transition was attributed to the formation of a liquid crystalline GMS phase previously (Mele, Khan et al. 2002). The amphiphilic structure of GMS and the high polarity of the PLGA solvent DMSO (relative permittivity: 80 for water and 48 for DMSO at 25°C) appeared to facilitate the arrangement of GMS molecules in lamellar bilayers at the droplet interface. Thermal microscopy showed that the melting points of the remaining amorphous-like and the liquid crystalline GMS were similar, which explained the single melting event seen with DSC.

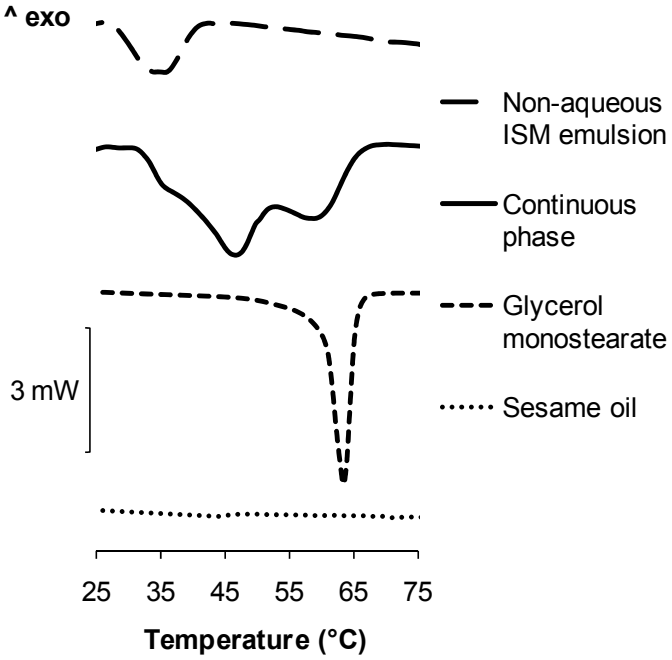

Figure 14 DSC thermograms of sesame oil, pure glycerol monostearate, continuous phase (5% GMS in sesame oil) stored 1 day at room temperature after melting and the corresponding non-aqueous ISM emulsion.

Cryo-SEM and freeze etching-TEM confirmed the presence of liquid GMS crystals at the interface (Figure 15). Lamellar, platelet-like structures of GMS were observed on the droplet surface by Cryo-SEM (Figure 15A) and similarly by freeze etching-TEM in the corresponding mirror images of the droplet interface (Figure 15B). In agreement with

studies on aqueous systems (Macierzanka, Szelag et al. 2009), interface stabilization by GMS was identified as one stabilization principle for non-aqueous ISM emulsions.

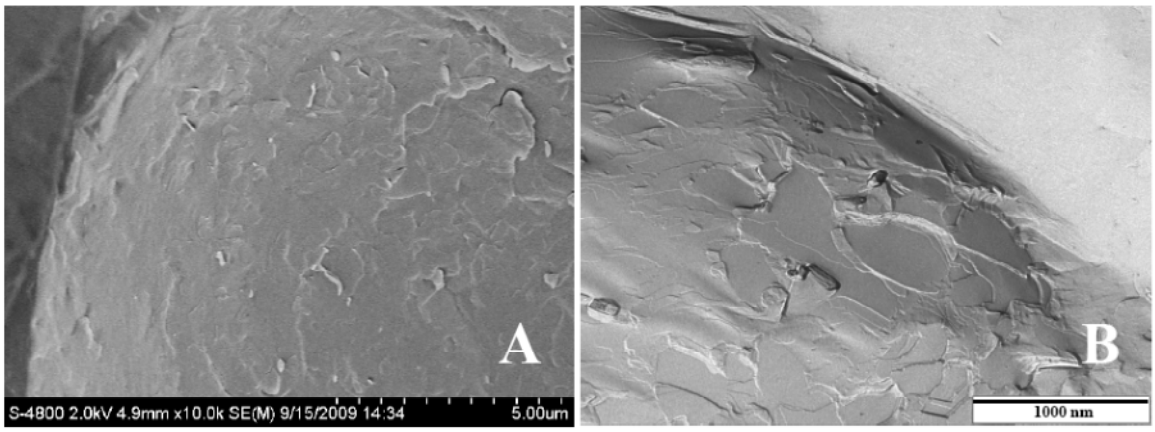

Figure 15 Cryo - SEM (surface) **(A)** and freeze etching - TEM (interface) **(B)** images of GMS stabilized ISM emulsion droplets.

Monoglycerides such as GMS are able to gel vegetable oils (Chen and Terentjev 2009), which is known to decrease droplet coalescence in W/O emulsions (Hodge and Rousseau 2005). The strength of the gel network and thus the stabilization potential can be expressed by the yield point (T0), which is defined as the minimal shear force required to break down the network structure and hence initiate plastic flow (Lee, Moturi et al. 2009). The presence of a yield point at GMS concentrations ≥ 2.5 % (Figure 16) indicated GMS network formation in sesame oil. The yield point and hence the gel strength and viscosity increased with the GMS concentration, being 0.5, 2.0 and 8.0 Pa at the 2.5, 4 and 5 % GMS concentration, respectively. The occurrence of a network structure at 2.5 % GMS concentration correlated well with the minimal amount of GMS necessary to form stable ISM emulsions (Table 6), indicating its importance for the stabilization of the emulsion.

A yield point of 6 Pa in the non-aqueous ISM emulsion containing 5 % GMS indicated that the GMS network remained intact upon emulsification. The gel strength, however, was decreased compared to the 5 % GMS-containing sesame oil (8 Pa), which was attributed to the partitioning of a portion of GMS from the continuous phase into liquid crystalline phase at the interface.

The gel network forming and viscosity enhancing effect of GMS was probably the dominant emulsion stabilization mechanism in the present study, since liquid crystalline

layers were already seen at 2 % GMS, whereas a stable emulsion required more than 2.5 % GMS content in the continuous phase. Other potential stabilizing effects of GMS, e.g. a reduction of the density difference of dispersed and continuous phase (Derkach 2009) or a decrease of the surface tension (both 4.5 dynes/cm) could be excluded.

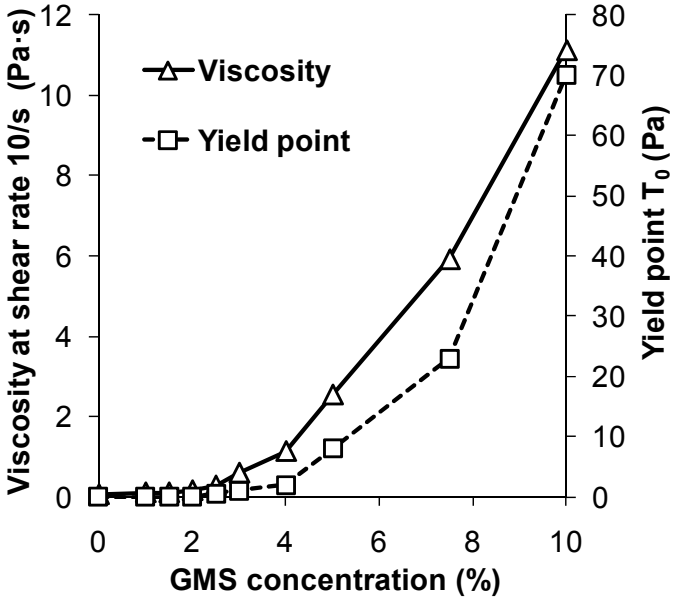

Figure 16 Effect of GMS concentration in the continuous phase on yield point (network formation) and the viscosity at a shear rate of 10 s^{-1}.

Table 6 Effect of GMS concentration on ISM emulsion droplet size.

GMS concentration (%)	Mean emulsion droplet size (µm)
0	-
2	-
2.5	45.8 ± 11.0
3	32.3 ± 10.4
5	18.9 ± 2.9
7.5	17.9 ± 3.1

3.1.2.4 Flow behaviour and injectability

GMS-containing sesame oil showed a shear rate dependent decrease of the viscosity, opposite to the Newtonian flow behaviour of pure sesame oil (Figure 17). The shear thinning was reversible with decreasing shear rate. The hysteresis loop indicated a slightly thixotropic flow behaviour, which is typically seen for fluids containing gel networks (Lee, Moturi et al. 2009)

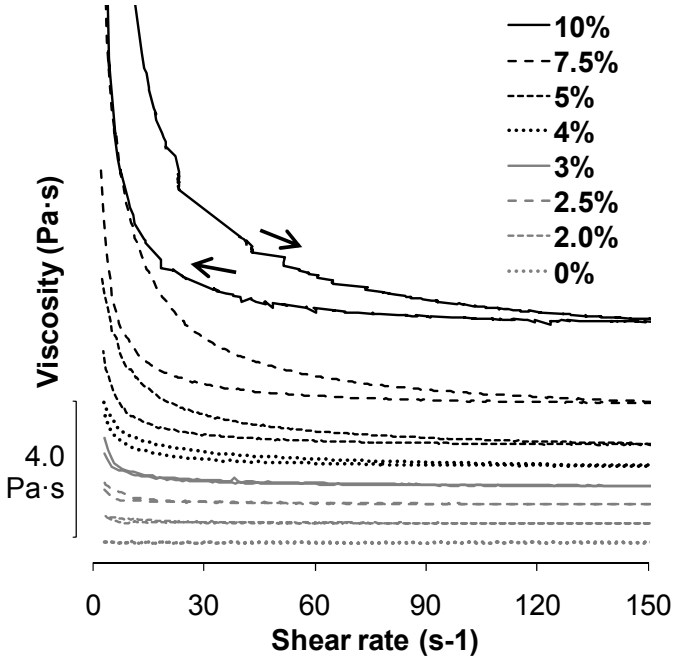

Figure 17 Flow behaviour of sesame oil as a function of GMS concentration under increasing (→) and decreasing (←) shear rates.

ISM emulsions showed similar flow properties to the continuous phase (Figure 18). Applying a high shear rate (100 s^{-1}) to the continuous phase and the corresponding ISM emulsion to mimic the emulsification step resulted in viscosities as low as 0.46 Pas and 0.34 Pas, respectively. The viscosity increased rapidly when the shear rate was decreased from 100 to 5 s^{-1}. Values of 2.47 Pas for the continuous phase and 1.87 Pas for the corresponding ISM emulsion corresponded to a more than a 5-fold viscosity increase in both cases. The handling of the ISM emulsion could benefit from shear thinning, if the stability-enhancing viscosity increase (low shear) does not compromise the injection of the emulsions (high shear).

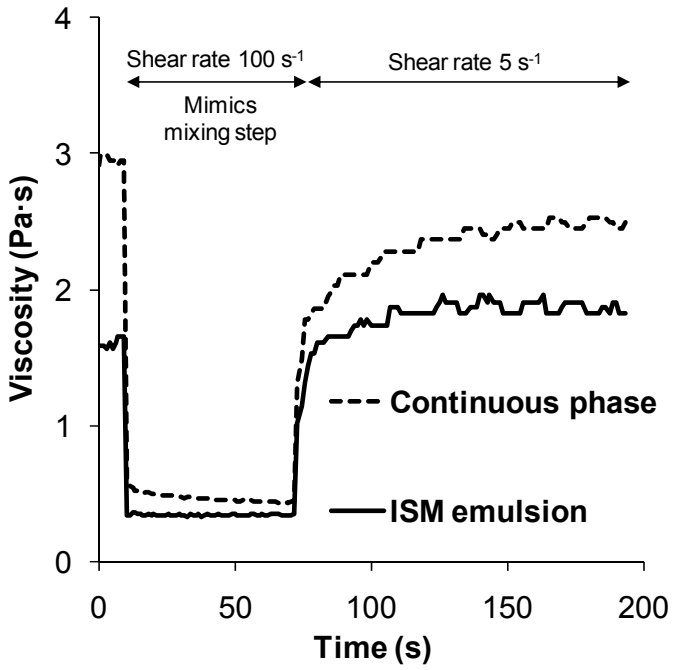

Figure 18 Flow behaviour of the continuous phase (5 % GMS in sesame oil) and the corresponding ISM emulsion (internal phase:continuous phase ratio 1:1.5) treated with different shear rates on a rheometer to mimic the emulsification via the two syringe system. During the first 10 s the samples were exposed to a shear rate of 5 s^{-1}.

The injectability of ISM emulsions and the corresponding PLGA solution was therefore evaluated using force-displacement profiles of the formulations ejected through thin hypodermic needles (24 G x 1.0 in). The ejection speed was set to 0.1 ml/s in order to have an acceptable injection time of 10 s (Dacre and Kopelman 2002) for the maximum dose (1 ml) administrable to the deltoid (Rodger and King 2000). GMS-stabilized ISM emulsions could be injected at a constant force of 10.2 ± 0.29 N (Figure 19 and Table 7). The force necessary to inject 5 % GMS containing sesame oil was very similar to the emulsion (9.45 ± 0.64 N), which highlighted the viscosity-determining role of the continuous phase in an emulsion containing droplets of the highly viscous PLGA solution, which can pass the needle. As a result of rapid droplet coalescence in unstabilized emulsions, peak forces approaching the injection force of the PLGA solution (31.08 ± 1.40 N) were detected. Such high forces, however, exceeded the upper limit for an acceptable injection of 20 N (Schoenhammer, Petersen et al. 2009).

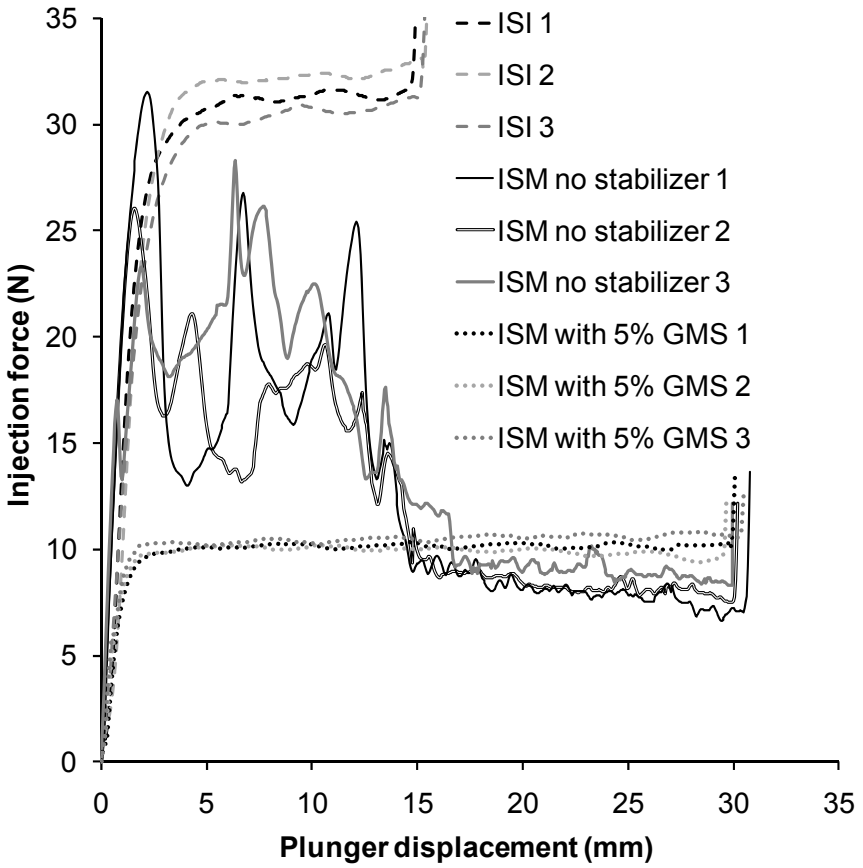

Figure 19 Required force to inject ISI (0.275 mL of 35 % PLGA 502S dissolved in DMSO) and the corresponding ISM emulsions (0.550 mL) stabilized with 5 % GMS (after 15 min of vertical storage) or without stabilizer (after 5 min of vertical storage) from 1 mL syringes equipped with 24 G x 1.0 inch hypodermic needles.

The high peak forces, however, were not apparent from the average force of the complete injection (12.84 ± 5.49 N) due to the low force necessary to inject the separated oil portion of the emulsion. Besides this disadvantage, comparing the injectability on the basis of average injection forces ignores, that the injection volume required to administer a similar drug dose is lower for the polymer solution (in situ implant) compared to the ISM emulsion, which leads to either a shorter injection time (Figure 19) or allows for a lower injection speed and hence a lower injection force. In contrast to the average force, an evaluation on the basis of the dose-normalized area under the force-displacement curve (injection energy) overcomes these shortcomings. A 25-30 % lower injection energy of the GMS-stabilized ISM emulsion compared to the corresponding PLGA solution and the unstabilized ISM emulsion (Table 7) showed the superior injectability of the stabilized

ISM emulsion. Thus, injection times could be shortened by about 30 % and handling issues due to peak injection forces would be eliminated.

Table 7 Required force and energy to inject in situ forming implant (ISI) solutions, the corresponding ISM emulsions and the continuous phases of the emulsions through 24 G hypodermic needles.

	Injection force (N)	**Injection energy (mJ)**
ISI	31.08 ± 1.40	2901 ± 39
ISM no stabilizer	12.84 ± 5.49	2753 ± 180
ISM with 5% GMS	10.20 ± 0.29	2051 ± 60
Sesame oil	5.15 ± 0.23	518 ± 20
5% GMS in sesame oil	9.45 ± 0.64	901 ± 52

3.1.3 Conclusions

The stability of non-aqueous in situ forming microparticle (ISM) emulsions was significantly improved from a few minutes to more than 12 h through the addition of glycerol monostearate (GMS) to the continuous oil phase, due to a viscosity increase of the continuous phase and the formation of a liquid crystalline GMS layer at the interface.

Homogeneous injection profiles with low injection forces were obtained with GMS-stabilized emulsions. The injectability of stabilized ISM emulsions was improved by 30 % compared to the corresponding highly viscous PLGA solution, allowing for a faster administration and hence a reduction of the pain exposure time for patients or the use of smaller more acceptable needles.

3.2 Potential of Glycerol monostearate to Stabilize Non-aqueous Emulsions Forming Biodegradable PLGA Microparticles in situ

3.2.1 Introduction

In a large number of marketed parenteral drug depot formulations poly(lactic-co-glycolic acid) (PLGA) has been used as depot former, due to its appropriate degradation rates from weeks to months and its good biocompatibility, evaluated in over more than 40 years of use (Giteau, Venier-Julienne et al. 2008; Körber 2010). PLGA based drug delivery systems (DDS) can be mainly divided into solid systems, like implants and microparticles, and in situ forming systems. In situ forming systems have been developed as an alternative to solid formulations due to an easier and more cost-effective preparation and decreased pain during administration and thus improved patient-friendliness (Heller 2009). The first approach of such formulations was the in situ forming implant (ISI) system developed by Dunn et. al (Dunn, English et al. 1990; Dunn, Tipton et al. 1994). ISI systems are composed of a viscous drug-containing organic PLGA solution. Upon injection into the body, the depot becomes more viscous or solidifies due to dissipation of the organic solvent into the surrounding tissue and water influx (Brodbeck, DesNoyer et al. 1999).

The use of potentially toxic solvents might be one major disadvantage of such systems. However, only small amounts are being administered within longer time periods (e.g. monthly), which do not exceed the permitted daily doses as defined in the ICH guidelines. Furthermore, organic solvents are also being used in other approved parenteral dosage forms (Strickley 2004). Currently two ISI products are on the market: Atridox® (U.S. approval in 1999) containing doxycycline and Eligard® (U.S. approval in 2002) containing leuprolide acetate.

At the end of the 1990s Bodmeier et al. invented the non-aqueous in situ forming microparticle (ISM) system as an alternative to the ISI approach (Bodmeier 1997). It can be prepared in a two-syringe/connector system prior to use and is based on an aqueous or non-aqueous emulsion. Non-aqueous ISM emulsions consist of a drug-containing organic

polymer solution which is emulsified into a stabilizer-containing oily phase. Upon injection the polymer solidifies and microparticles are formed (Luan and Bodmeier 2006a; Kranz and Bodmeier 2007). The additional low viscous continuous phase in ISM systems was advantageous in comparison to the higher viscous ISI solutions: 1) easier injectability through smaller needle sizes (Rungseevijitprapa and Bodmeier 2009), 2) improved muscle compatibility (Kranz, Brazeau et al. 2001; Rungseevijitprapa, Brazeau et al. 2007) and 3) decreased initial burst release of low molecular weight drugs and peptides in vitro and in vivo (Kranz and Bodmeier 2008; Kranz, Yilmaz et al. 2008a).

Recently the stability of non-aqueous ISM emulsions was increased from only a few minutes to more than 12 h using glycerol monostearate (GMS) as emulsion stabilizer instead of Span 80, Pluronic F68 and aluminum monostearate (Section 3.1). It was observed that GMS stabilized non-aqueous emulsions by a more than 5-fold viscosity increase in the continuous phase after emulsification and the formation of a liquid crystalline GMS layer around the polymer solution droplets.

However, changes in formulation parameters (e.g. different drugs, PLGA content, type of oil or organic solvent) and process parameters (e.g. preparation time and temperature, connector diameter) may affect the ISM emulsion stability or even impede emulsion formation. In addition, monoacylglycerols such as GMS exhibit monotropic polymorphism and appear in at least 3 polymorphic forms (Sato 2001; Ojijo, Neeman et al. 2004b; Himawan, Starov et al. 2006). Alterations in the polymorphic composition or crystal growth due to storage or different preparation methods can cause changes in the physical properties of GMS und can thus also affect emulsion formation and stability (Sutananta, Craig et al. 1994).

Improper emulsion formation or potential droplet coalescence of viscous polymer solution droplets prior to administration will complicate an easy and smooth injection through small hypodermic needles (e.g. 24 G). Moreover, inadequate stability of ISM emulsions will result more likely in the formation of large microparticles, lumps or even implant-like polymer matrices in the body due to coalescence of the emulsion droplets prior to polymer solidification. Changes in geometry and size of the formed polymer depot and thus in its surface area will also presumably affect the drug release pattern.

The objective of the present study was therefore to study the effects of formulation and process parameters on GMS stabilized non-aqueous ISM emulsions, the formation of

microparticles and drug release and thus to evaluate the practicability of GMS as a stabilizer for non-aqueous ISM systems.

3.2.2 Results and discussion

3.2.2.1 Stabilizing-capacity of GMS for non-aqueous ISM emulsions

3.2.2.1.1 Effect of formulation parameters

In order to evaluate the practicability of GMS as an ISM emulsion stabilizer, ISM formulations were prepared with varying parameters and the affinity to droplet coalescence was determined under a microscopic cover glass 15 min after preparation (Table 8).

Table 8 Effect of formulation parameters and phase viscosity on the ability to form ISM emulsions and their affinity to droplet coalescence under a microscopic cover glass 15 min after preparation.

	Phase viscosity (mPa·s)	Emulsion stability[1]		Phase viscosity (mPa·s)	Emulsion stability[1]
Internal phase:continuous phase ratio			Organic solvent type		
1:0.5		▪	DMSO	178.8	●
1:1		○	NMP	200.3	▪
1:1.5		●	2-Pyrrolidone	2482.4	▪
1:2		●	Benzyl bezoate	7247.3	▪
1:4		●	Triacetin	9830.4	▪
			PEG 400	10339.2	▪
Vegetable oil type			PLGA type		
MCT	251.4	○	capped 502	262.3	●
Soybean oil	456.8	●	502S	178.8	●
Sesame oil	603.7	●	503	1749.8	▪
Peanut oil	589,3	●	uncappec 502H	153.4	▪
Castor oil	1960.4	●	503H	530.4	▪
PLGA concentration (capped 502S) (%)[2]			GMS concentration (%)[2]		
0	1.3	○	0	57,8	▪
10	7.1	○	2	145.3	▪
20	33.1	●	2,5	248.9	○
30	178.8	●	3	324,8	○
35	355.4	●	5	603,7	●
40	793.2	▪	7,5	934,2	●

[1] ▪ no emulsion formation ○ partial coalescence ● no coalescence

[2] w/w, based on internal phase; [3] w/w, based on continuous phase

An internal phase:continuous phase ratio of at least 1:1 was necessary to form emulsions. Thereby the stability increased with increasing continuous phase content due to a decreased probability of coalescence by larger distances between the droplets. Various oils could be used as continuous phase to form stable ISM emulsions. The lower viscosity of continuous phases containing MCT (251.4 mPas) instead of sesame oil (603.7 mPas) decreased the stabilizing potential of the continuous phase and thus led to an increased susceptibility for coalescence.

ISM emulsions could be prepared with PLGA 502S concentrations up to 35 % (w/w, based on internal phase). The emulsions stability was increased with polymer concentrations of 20 to 35 % exhibiting higher internal phase viscosities. This could explain the decreased susceptibility against droplet coalescence (Yao, Maris et al. 2005).

However, ISM emulsions could not be formed when the internal phase viscosity exceeded the viscosity of the continuous phase (603.7 mPas) which occurred e.g. at high polymer concentrations (40 % PLGA 502S, 793.2 mPas), with the use of other solvents like PEG 400 (10339.2 mPas) or with other types of polymer such as 30 % PLGA 503 (1749.8 mPas). This could be attributed to an increased hindrance of droplet break-up during emulsification with a higher internal phase viscosity in comparison to the continuous phase viscosity (Grace 1982).

The sensitivity of GMS stabilized ISM emulsions to changes in the internal phase could also be explained by changes in the polarity and hydrogen bonding capability of the internal phase. This could hinder the formation of the stabilizing liquid crystal layer at the droplet interface and thus decrease the emulsion stability (Imhof and Pine 1997). For instance stable ISM emulsions containing the more hydrophilic polymer PLGA 502H, having free carboxylic end groups instead of alkyl esters (502S), could not be prepared.

The main influence on ISM emulsion stability was observed with increased GMS concentrations. GMS concentrations $\geq$ 2.5 % led to the formation of an increasingly stronger stabilizing GMS gel network in the continuous phase (Section 3.1). Thus, higher GMS concentrations in comparison to the ISM standard formulation (5 %) could be used to increase the range of stable ISM emulsion formation. For example, 7.5 % GMS were appropriate to stabilize ISM emulsions containing the more hydrophilic polymer 502H instead of 502S. However, the increase of the GMS concentration is limited because the ISM emulsion viscosity also increases with increasing GMS content which could complicate the injection through thin needles.

3.2.2.1.2 Effect of drug incorporation

The incorporation of active ingredients into emulsions can negatively affect emulsion stability (Washington 1996). Thus drugs differing in their molecular weight (from 180 to 14388 Da) and loading (5 to 100 %, based on polymer) were added to the internal phase and their influence on the formation of standard ISM emulsions was evaluated (Table 9).

Table 9 Effect of drug incorporation on ISM emulsion droplet size 15 min after preparation.

Model drug	Molecular weight (Da)	Drug loading (%)	Emulsion droplet size
-	-	-	18.9 ± 2.9
Lysozyme[1]	14388	5	23.6 ± 3.5
Leuprolide	1209	5	16.6 ± 3.9
Diltiazem HCL	451	5	28.8 ± 5.6
		20	25.6 ± 6.0
Propranolol HCL	295	5	26.2 ± 4.7
Lidocaine HCL	271	5	28.1 ± 6.7
		10	26.4 ± 5.4
		25	24.1 ± 5.5
		50	23.0 ± 4.4
		75	24.7 ± 4.7
		100	22.0 ± 5.2
Diprophylline	254	5	24.0 ± 4.8
Carbamazepine	236	5	20.8 ± 4.3
Ibuprofen	206	5	22.9 ± 4.2
Theophylline	180	5	22.1 ± 4.4

[1]Lysozyme precipitated in the PLGA solution, wheras all other model drugs remained dissolved

Stable ISM emulsions with similar emulsion particle sizes could be formed with all tested drugs and loading. All drug containing ISM emulsions remained stable for more than 12 h.

3.2.2.1.3 Effect of process parameters

ISM standard emulsions could be formed using various connector diameters from 0.75 to 2.00 mm and in the temperature range investigated from 15 to 33 °C (Table 10). The slightly increased susceptibility to droplet coalescence observed at 33 °C could be explained with decreased continuous phase viscosity with increasing temperature.

Table 10 Effect of process parameters on the ability to form ISM emulsions and the affinity to droplet coalescence under a microscopic cover glass 15 min after preparation.

	Emulsion stability[1]		Emulsion stability[1]
Temperature (°C)		Preparation time (s)	
14	●	60	-
23	●	120	-
33	O	180	●
Connector diameter (mm)		Cycles per s	
0.75	●	0.5	-
1.4	●	1.0	●
2.0	●	2.0	●

[1] - no emulsion formation O partial coalescence ● no coalescence

At least 180 s with a mixing speed of 1 cycle / s were required to form ISM emulsions in the two-syringe/connector system. However, increased shear forces during preparation should increase droplet break-up and hence the ability to form emulsions (Riess, Cheymol et al. 2004). Therefore, the use of smaller connector diameters (e.g. 0.75 instead of 1.40 mm) and a faster mixing speed (e.g. 2 instead of 1 cycle / s) resulted in the formation of stable ISM emulsions containing, for instance 40 % PLGA 502S. In addition the increased shear forces during emulsification also led to a faster formation of ISM emulsions. The preparation time could be thus decreased from 180 s to less than 30 s which makes the preparation prior to use more comfortable.

3.2.2.1.4 In situ formation of microparticles

Upon injection into aqueous media, unstabilized ISM emulsions formed implant-like polymer matrices due to complete phase separation of the oily and internal phase prior to

polymer precipitation (Figure 20 A). The stabilization of ISM emulsions with 5 % GMS (w/w, based on continuous phase) resulted in the formation of microparticles often larger than 100 µm in size and to some extend in the formation of polymer lumps (>1 mm) (Figure 20 B). ISM emulsions containing 7.5 % GMS showed sufficient stability against droplet coalescence, which resulted in the formation of small microparticles in the size of approximately 15 to 30 µm. (Figure 20 C).

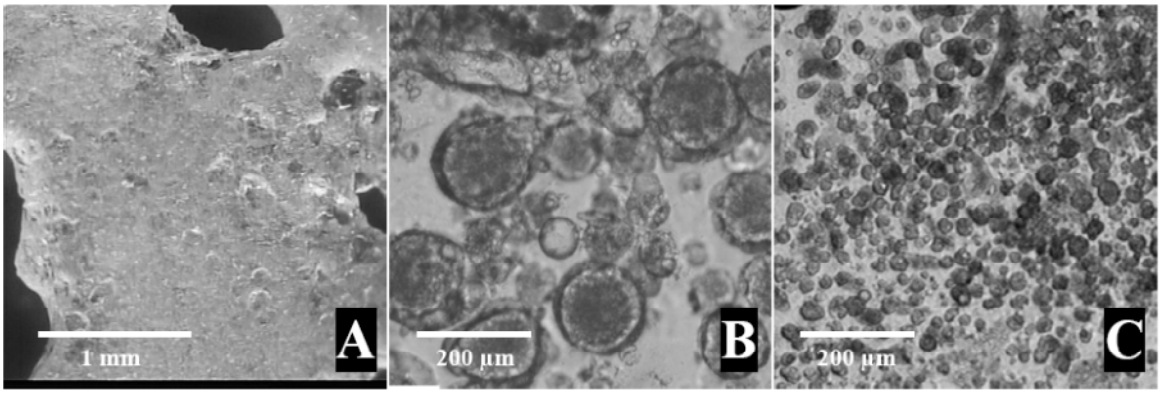

Figure 20 (A) Cross section of an implant-like matrix formed from an unstabilized ISM emulsion upon exposure to aqueous media. **(B)** Microparticles formed from an ISM emulsion stabilized with 5% GMS (w/w, based on polymer) and **(C)** with 7.5% GMS, respectively.

3.2.2.1.5 Drug release

The ISM emulsion stability and hence the subsequent formation of different sized polymer depots affected the drug release (Figure 21). During the first 24 hours the release of the model drug diprophylline from ISM formulations was increased from formulations containing higher amounts of GMS (0, 5.0 and 7.5%, respectively). This could be explained with the larger surface area of the drug containing polymer depots with increasing GMS concentrations due less emulsion droplet coalescence based on the increased ISM emulsion stability.

However, beyond 1 day the diprophylline release increased from unstabilized ISM formulations. A more than 100 % swelling of the implant-like polymer matrices after day 1 suggested an increase in the porosity of the polymer matrix causing a faster drug diffusion

out of the depot (Fredenberg, Wahlgren et al. 2011). ISM formulations containing GMS showed no visible particle size growth.

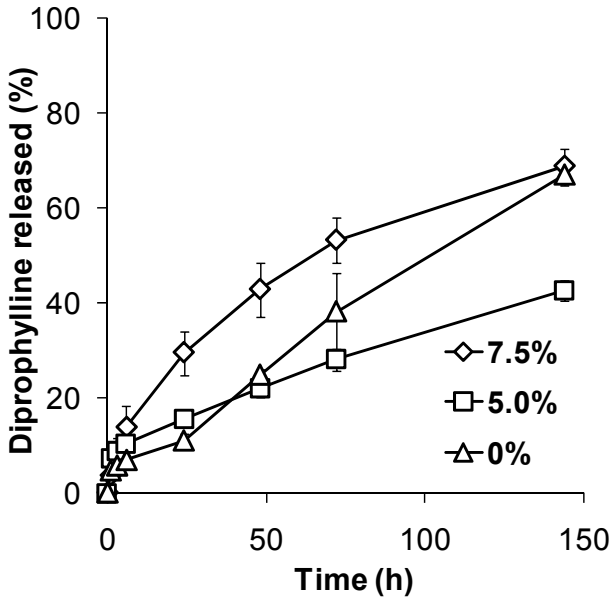

Figure 21 Effect of GMS concentration on diprophylline release from ISM standard formulations.

In addition, the influence of the drug type on the release from GMS stabilized ISM emulsions was investigated. The model drugs theophylline, diprophylline and carbamazepine were chosen due to their similar molecular size but different solubilities in the release media as well as in the liquids of the ISM formulation (Table 11).

The drug release was in the order theophylline > carbamazepine > diprophylline which was inversely proportional to the solubility of the drugs in the organic solvent DMSO of the internal phase (Figure 22 and Table 11). This indicated an increased drug affinity to the organic solvent (Larsen, Parshad et al. 2002). The higher DMSO affinity of diprophylline in comparison to carbamazepine and theophylline may have led to decreased drug diffusion out of the internal phase before the polymer precipitated. Simultaneously, the diffusion of DMSO into the surrounding medium may also decrease with increasing drug affinity. This could result in the formation of less porous microparticles, resulting in a decreased diffusion controlled drug release from the polymer depot (Brodbeck, DesNoyer et al. 1999). A more detailed study is required to verify this hypothesis.

However, it was shown that drug release from ISM formulations could be more affected by properties of the drug than by the stability of the ISM emulsion.

Table 11 Solubilities (S, mg/mL) of low molecular weight drugs used in release studies at 37 °C.

Drug	S_{DMSO}	$S_{sesame\ oil}$	$S_{PBS\ buffer\ pH\ 6.8}$ [1]
Theophylline	42.7 ± 2.2	0.20 ± 0.02	13.1
Carbamazepine	155.0 ± 3.6	1.60 ± 0.23	0.24
Diprophylline	419.8 ± 8.9	0.08 ± 0.01	173.8

[1] Data taken from (Steiner 2011)

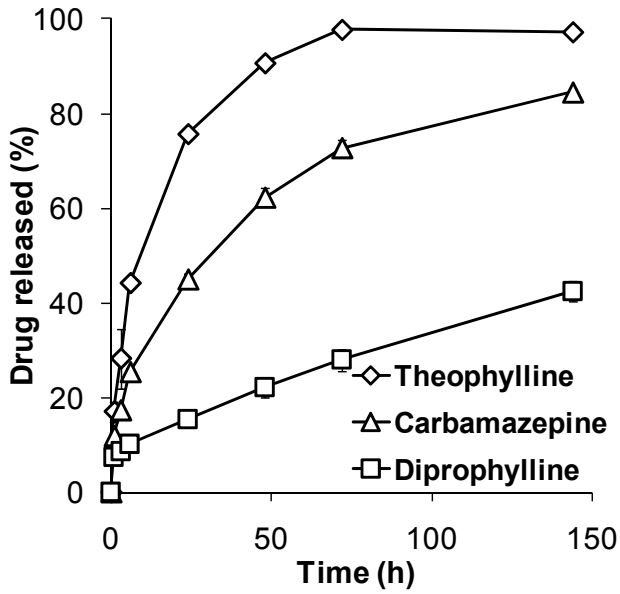

Figure 22 Release of low molecular weight drugs from ISM standard formulations (7.5 % GMS, w/w, based on continuous phase).

3.2.2.2 Effect of GMS polymorphism and crystal growth on ISM emulsion formation

DSC and hot-stage polarized optical light microscopy revealed that continuous phase (sesame oil) containing GMS occurred in parallel in 3 polymorphic forms exhibiting different crystal morphologies: 1) the α-form, consisting of fine amorphous-like particles melting between 27 °C to 40 °C, 2) the β'-form, consisting of tiny, bulky particles or small

plates melting between 40 °C to 54 °C and 3) the β-form, consisting of bulky particles or needles melting between 54 °C to 68 °C. The formation of the emulsion stabilizing, viscous GMS gel network could be primarily attributed to the amorphous-like, fine dispersed α-polymorph, whereas the β' -and β crystals completely transformed into the droplet-interface-stabilizing liquid crystals during ISM emulsion preparation (Section 3.1).

However, the polymorphs can transform from α to β' to β over time which may decrease the amount of the α-polymorph. β' or β crystal can grow in size (Schubert, Schicke et al. 2005). Both changes could lead to decreased ISM emulsion stability. Therefore, the GMS polymorphic composition and possible crystal growth upon variations of the continuous phase preparation method or during long-term storage of the continuous phase were investigated and their effects on ISM emulsion formation evaluated.

Continuous phases were prepared by melting of GMS in oil and subsequent cooling. The use of different cooling speeds, e.g. slow cooling at room temperature or rapid cooling in liquid nitrogen at -196 °C, resulted in the formation of different GMS polymorphic compositions in the oil phase. DSC and polarized optical light microscopy showed that more amorphous-like, non-crystalline α-polymorphic structures were formed through rapid liquid nitrogen cooling. During 24 h of storage at room temperature the GMS in samples from both cooling methods transformed into a similar polymorphic composition (data not shown). However, these variations in the polymorphic composition and hence also in the viscosity (0.6 - 4.4 Pa·s) had no effect on the ability to form stable ISM emulsions (Table 12).

Table 12 Effect of cooling speed during continuous phase (cp) preparation on viscosity and the affinity to droplet coalescence under a microscopic cover glass 15 min after preparation.

	Liquid nitrogen (-196 °C)		Air (25°C)	
Storage time	**Cp viscosity (Pa·s)**	**Emulsion stability[1]**	**Cp viscosity (Pa·s)**	**Emulsion stability[1]**
10 min	1.3	●	0.6	●
1h	4.4	●	1.0	●
24h	2.4	●	2.4	●
168h	2.5	●	2.5	●

[1] - no emulsion formation ○ partial coalescence ● no coalescence

A prolonged storage of the continuous phase over 374 days at different temperatures (4 °C, 25 °C and 37 °C) revealed further changes in the polymorphic composition over time (Figure 23 A). Significant crystal growth of β'- and β-polymorphs up to approximately 100 µm after ≥ 30 days was observed with samples stored at 37 °C (Figure 24). The β'- and β-crystals completely transformed into liquid crystals during emulsification. This was indicated by the appearance of only one GMS melting peak after ISM emulsion formation instead of the multi-phasic melting peak observed in the continuous phase (Figure 23 A and B).

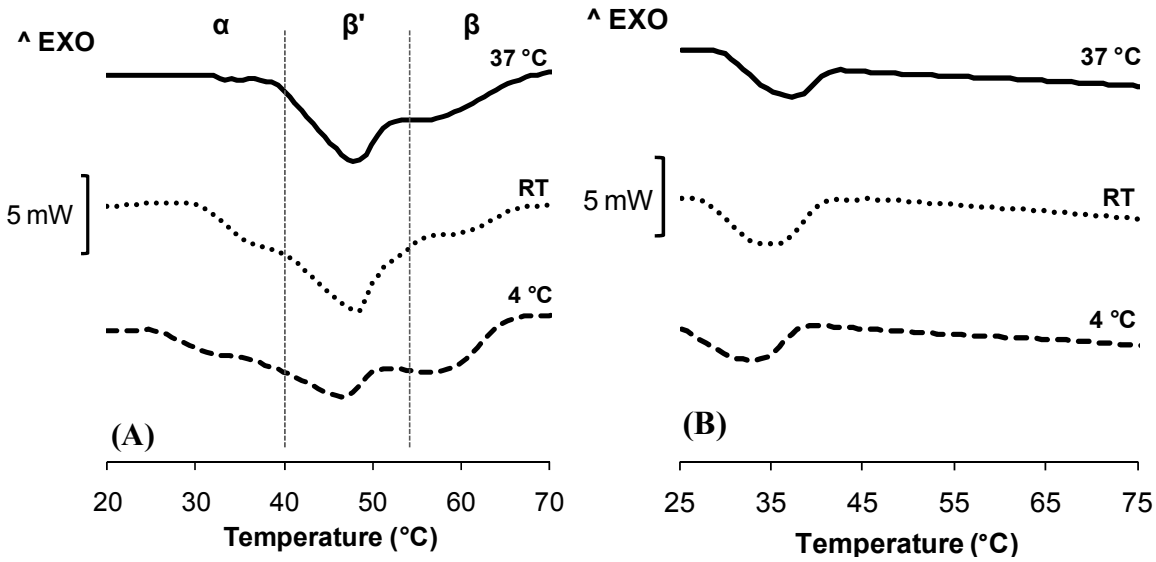

Figure 23 Standardized DSC thermograms: **(A)** of 5 % GMS containing continuous phases (sesame oil) stored for 30 days at different temperatures; α, β' and β are the melting ranges of the polymorphic forms of GMS and **(B)** corresponding ISM emulsion.

Continuous phases could be stored at 4 °C for more than 1 year or at room temperature till 136 days without compromising the stability of the respective ISM emulsion (Figure 25). However, ISM emulsions formed with continuous phases stored at 37 °C and at room temperature for 374 days showed increased susceptibility to droplet coalescence. This resulted in increased emulsion droplet sizes and could be explained with the formation of a less pronounced stabilizing GMS gel network due to a decreased amount of the α-polymorph in the continuous phase at higher storage temperatures (Figure 23 A). However, more likely, rancidification of oil containing glycerol fatty acid esters with increasing storage temperature and time may have caused the reduced ISM emulsion

stability (Cosio, Ballabio et al. 2007). The addition of antioxidants such as α-tocopherol could probably avoid such potential rancidification. A more detailed study is required to verify this hypothesis.

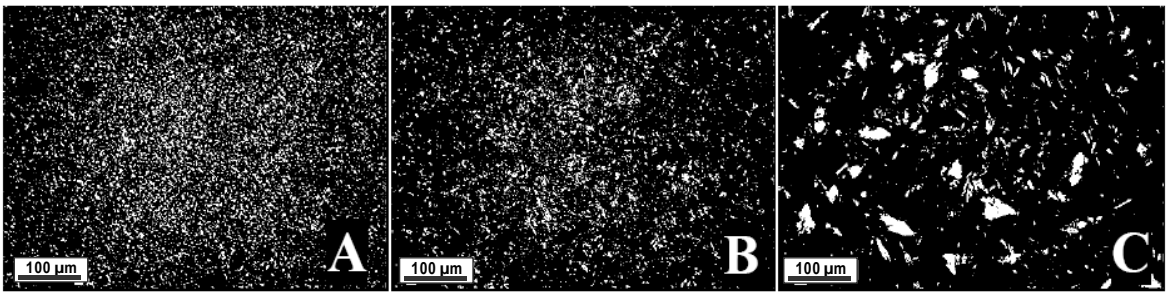

Figure 24 Polarized optical light microscope images (100 % contrast) showing the β' and β polymorphic forms of sesame oil containing 5 % GMS stored for 30 days **(A)** at 4 °C, **(B)** at room temperature and **(C)** at 37 °C. The amorphous-like α-form could not be captured in a distinguishable resolution.

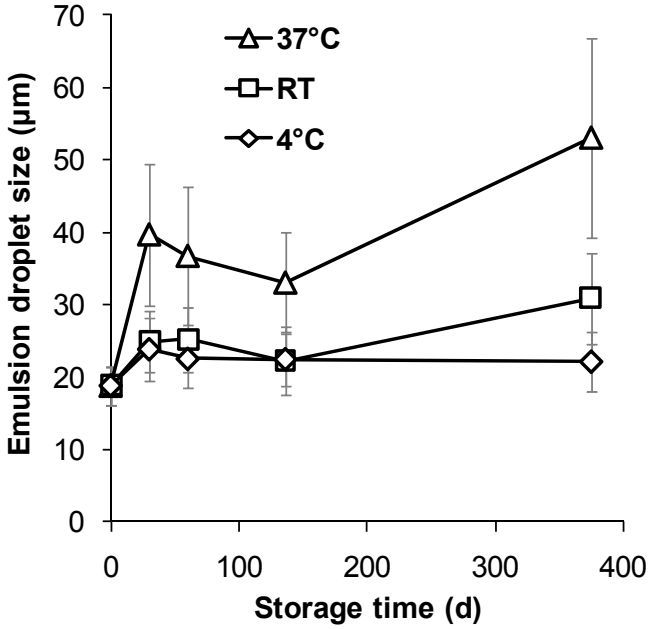

Figure 25 Effect of continuous phase (5 % GMS in sesame oil) storage at different temperatures over time on the droplet size of the respective ISM emulsions.

3.2.2.3 Drug release

Changes in the continuous phase during long-term storage could potentially affect the drug release pattern. Therefore the release of the model protein lysozyme from ISM emulsions containing continuous phases stored for 1 day at room temperature (standard ISM emulsion) and for 374 days at room temperature or at 4 °C, respectively, was compared.

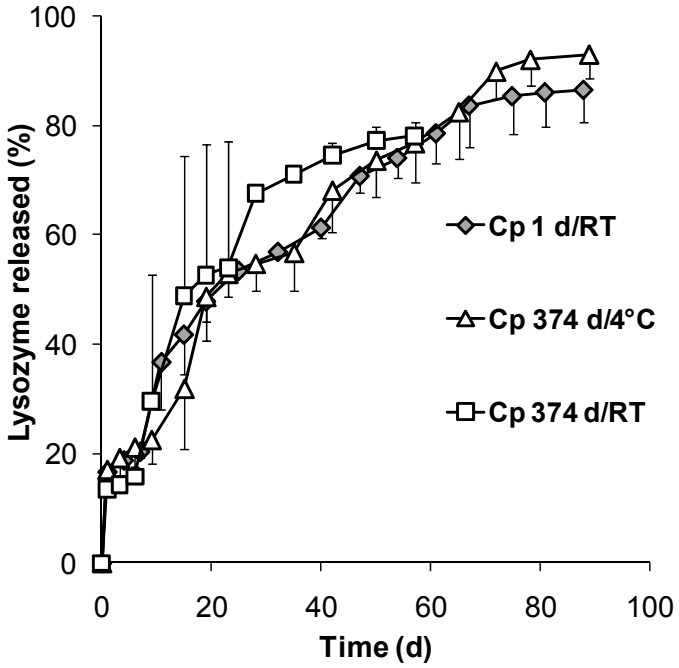

Figure 26 Effect of continuous phase (cp) storage at 4 °C and room temperature (RT) on the release of lysozyme (4 %, based on polymer) from ISM formulations stabilized with 5 % GMS (based on continuous phase).

ISM emulsions containing continuous phases stored at 4 °C for 374 days showed similar lysozyme release profiles in comparison to standard ISM emulsions (Figure 26). However, the use of continuous phases stored in ISM emulsions at room temperature for 374 days significantly changed the lysozyme release pattern. The more discontinuous and incomplete release could be explained with the decreased ISM emulsion stability and/or with an ongoing rancidification of the continuous phase. The formation of free radicals during the oxidative rancidification process, for instance, could initiate lysozyme degradation processes.

3.2.3 Conclusions

Glycerol monostearate showed great potential as a stabilizer for non-aqueous ISM emulsions which can be prepared in a two-syringe/connector system within 30 s prior to use. Stable ISM emulsions could be prepared in a broad range of formulation and process parameters. Drug incorporation as well as changes in the polymorphic composition of GMS or crystal growth did not affect the ability to form emulsions. Notwithstanding, during long-term storage, GMS containing continuous phases should be kept at 4 °C to prevent potential oil rancidification and to ensure similar drug release profiles.

The morphology and the size of the in situ formed polymer depots were controlled by the stability of the ISM emulsion. Microparticles were formed with ≥ 5 % GMS. The release of low molecular weight drugs was increased with decreasing drug solubility in the organic solvent and with decreased particle size of the in situ formed drug reservoir. Further studies are being carried out to evaluate the underlying drug release mechanisms in detail.

3.3 Influence of the Continuous Phase Composition on the Release of Lysozyme from Non-aqueous in situ Forming Microparticles

3.3.1 Introduction

In particular the growing marked of biopharmaceuticals has pushed the development of injectable biodegradable poly(lactic acid-co-glycolic acid) (PLGA) based depot formulations such as implants, microparticles and in situ forming systems in recent years (Packhaeuser, Schnieders et al. 2004; Giteau, Venier-Julienne et al. 2008; Tsuji and Tsutani 2008; Ye, Kim et al. 2010). A large number of new biopharmaceuticals are proteins. Besides the maintenance of conformational stability and activity of the protein, often a suitable continuous release remains a big challenge (Giteau, Venier-Julienne et al. 2008). High initial protein release rates followed by a slow incomplete release or three-phasic, sigmoidal shaped release pattern have been reported (Wang, Wang et al. 2002; Luan and Bodmeier 2006a; Ye, Kim et al. 2010). A three-phasic protein release can be divided into an initial burst release of protein located on or close to the surface, a slow release phase (lag phase) of protein diffusing slowly through few existing pores while polymer degradation proceeds and a faster protein release period often attributed to the onset of polymer erosion (D'Souza, Faraj et al. 2005; Giteau, Venier-Julienne et al. 2008; Fredenberg, Wahlgren et al. 2011).

Biodegradable non-aqueous in-situ forming microparticle (ISM) systems have been developed as an alternative injectable PLGA depot system to microparticles and in situ forming implants (ISI) (Bodmeier 1997; Luan and Bodmeier 2006b). Non-aqueous ISM systems are based on an emulsion consisting of a drug-containing organic polymer solution (ISI) which is emulsified into a stabilizer-containing oily continuous phase in a two syringe/connector system prior to administration (Luan and Bodmeier 2006a). Upon injection into the body microparticles are formed in situ due to the precipitation of the water insoluble polymer (Kranz and Bodmeier 2007).

Recently, the stability of non-aqueous ISM emulsions has been significantly improved (Section 3.1). The use of glycerol monostearate (GMS) instead of Span 80, Pluronic F68 and aluminum monostearate, respectively, prolonged the ISM emulsion stability from a few minutes to more than 12 h. These ISM formulations showed increased emulsion stability with increased amounts of GMS (≥ 2.5 %, w/w, based on continuous phase). ISM emulsions were stabilized against droplet coalescence due to the formation of a viscous GMS gel network in the continuous phase and a liquid crystalline GMS layer around the polymer solution droplets.

ISM systems showed good muscle compatibility in Sprague-Dawley rats (Kranz, Brazeau et al. 2001; Rungseevijitprapa, Brazeau et al. 2007) and an easier injectability in comparison to the higher viscous ISI systems (Rungseevijitprapa and Bodmeier 2009). Formulation parameters affecting the drug release from ISM formulations have also been studied but were mainly focused on the composition of the microparticle-forming internal phase (Luan and Bodmeier 2006a; Kranz and Bodmeier 2007; Kranz and Bodmeier 2008). Effects of the continuous phase composition especially on the release of proteins have not received much attention yet. However, using different amounts of GMS to stabilize the ISM emulsions or changing the oil type are expected to affect protein release. For instance, the increased susceptibility against droplet coalescence of less stable ISM emulsions due to lower GMS contents will result in the formation of more likely implant-like structures instead of microparticles. This will increase the surface area or could change the porosity of the polymer matrix which may thus also affect the release.

The continuous oily phase surrounding the microparticles may impede water inflow which could potentially slow down the polymer degradation rate (Dong, Körber et al. 2006). Furthermore, the oil layer may also act as an additional protein release barrier which could be differently pronounced with the type of oil used.

Hence, the objective of this study was to elucidate the effects of the continuous phase composition (GMS concentration and oil type) of non-aqueous ISM systems on the release and activity of the model protein lysozyme.

3.3.2 Results and discussion

3.3.2.1 Effect of GMS concentration on lysozyme release

The stability of lysozyme containing ISM emulsions increased with increasing GMS content which resulted in the formation of different shaped polymer depots upon injection into aqueous media. Implant-like matrices were formed from ISM systems stabilized with 0 and 2.5 % GMS (w/w, based on the continuous phase) due to complete phase separation prior to polymer precipitation (Figure 27 and Table 13). Optical light microscopy showed that decreased droplet coalescence in formulations containing 5 % GMS resulted in the formation of polymer lumps (> 1 mm) and microparticles mainly larger than 100 µm. The increased stability of ISM emulsions containing 7.5 % GMS led to the formation of small microparticles from approximately 15 to 30 µm.

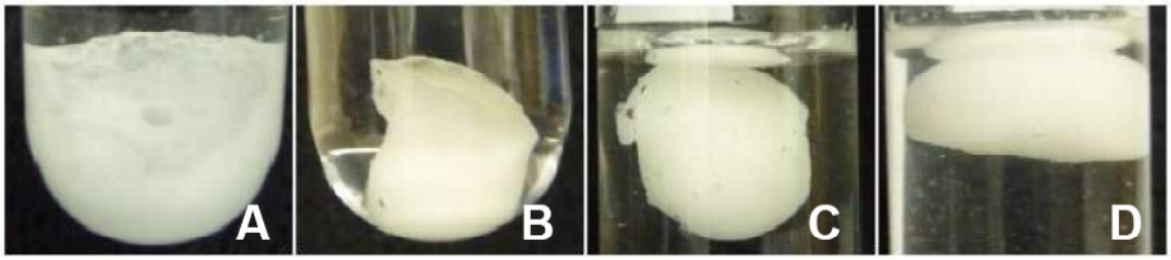

Figure 27 Biodegradeable polymer depots formed from ISM emulsions containing **(A)** 0 %, **(B)** 2.5 % (located at the bottom of the tube) and **(C)** 5.0 %, **(D)** 7.5 % (swimming) GMS in the continuous phase in glass test tubes filled with 8 mL acetic acid buffer (pH 5.0) 11 days after incorporation.

The release of lysozyme from ISM formulations was sensitive to the GMS concentration in the oil phase (Figure 28). Although a change in the initial relase was suggested by the formation of different sized polymer matrices exhibiting different surface areas, a nearly similar lysozyme release at day 1 between 14 to 19 % was observed. 80 % of the total recovered amount of lysozyme was released after 19, 15, 45 and 65 days from ISM formulations stabilized with 0, 2.5, 5.0 and 7.5 % GMS, respectivley. ISM formulations stabilized with 2.5 % GMS showed continuous release over approximately 20 days while a lag phase prior to the main part of the lysozyme release (three-phasic release pattern) was observed for the other formulations. The lag time was more than doubled to approximately 25 days with the use of 7.5 % GMS.

Table 13 Effect of GMS concentration (%, w/w, based on continuous phase) on the macroscopic appearance of ISM formulations during release.

		GMS concentration in sesame oil (%)			
	Day	**0**	**2.5**	**5.0**	**7.5**
Morphology	3	Implant-like	Implant-like	Lumps / large microparticles	Microparticles
Oil separation					
	1	+	+	-	-
	7		+	-	-
	11		+	-	-
	15			-	-
Swelling					
	1	↑	-	-	-
	7	↑↑	-	-	-
	11	↑↑	-	-	-
	15	↑	-	-	-

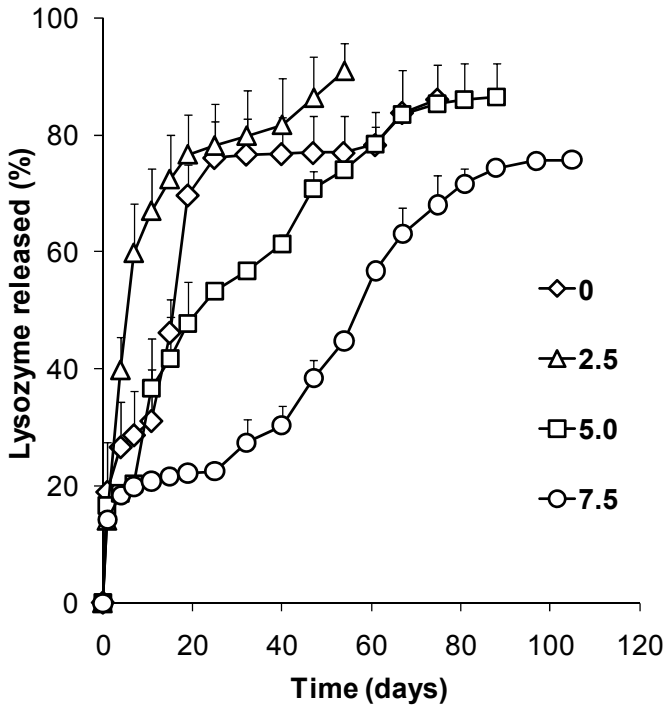

Figure 28 Effect of GMS concentration (%, w/w, based on continuous phase) on the lysozyme release from non-aquoeus ISM systems.

These significant variations in the drug release profiles as a function of GMS concentration were expected to be due to changes in the main drug release mechanisms (diffusion vs. erosion controlled). Gel permeation chromatography revealed that the PLGA degradation rate was not effected by the GMS concentration (Figure 29). Erosion is defined as the wheight loss of the polymer matrix due to the dissolution of acidic degradation products (≤ 1000 Da) resulting in a decreased pH of the low buffered media (pH 5) (Ghalanbor 2011). Thus similar shifts of the cumulative pH changes in the release medium between day 35 and 75 confirmed a likewise identical PLGA erosion (Figure 30).

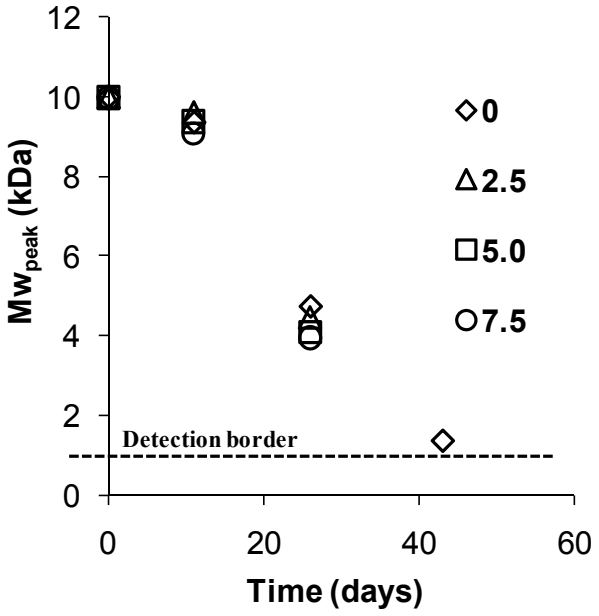

Figure 29 Effect of GMS concentration (%, w/w, based on continuous phase) on the PLGA 502S degradation from ISM formulations upon injection into acetic acid buffer (pH 5.0, 33 mM) at 37 °C.

The PLGA erosion time frame from day 35 to 75 matched with the main lysozyme release phase from ISM formulations stabilized with 7.5 % of GMS as well as with an increase in the release from all other formulations. Thus, lysozyme relased beyond 35 days could be primarly releated to an erosion controlled release. Accordingly, lysozyme released before day 35 was attributed to diffusion through a porous network. This was in agreement with findings from Jiang et al. who observed similar lysozyme release pattern from conventional microspheres (Jiang, Woo et al. 2002).

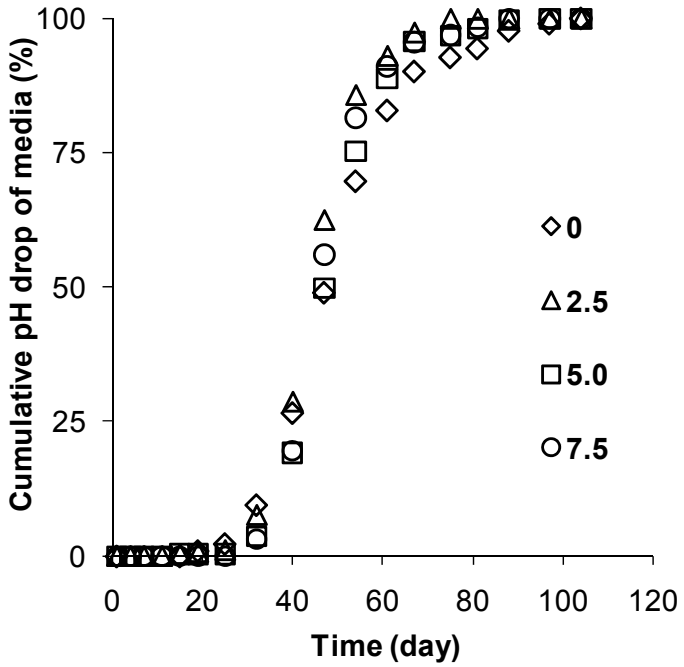

Figure 30 Cumulative pH drop of the release media due to degradation of PLGA 502S to water-soluble acidic oligomers representing the erosion behaviour as a function of GMS concentration in the continuous phase (Ghalanbor 2011).

The differences in the diffusion controlled lysozyme release pattern (≤ 35 days) of ISM formulations stabilized with 0, 2.5 and 5 % GMS, respectivly (Figure 28) may be explained with a varying degree of interconnected voids or channels with access to the surface (Graham, Brodbeck et al. 1999; Allison 2008). Light microscopy and cryo-SEM revealed changes in the porous structure with increasing GMS content (Figure 31 and 32). ISM systems containing 0 % GMS formed a sponge-like swollen polymer matrix in situ exhibiting rather non-connected voides which could explain the lag time of 10 days after the initial release. This was in agreement with findings from Fredenberg et al. (Fredenberg, Wahlgren et al. 2011a). Subsequent to the lag time approximately 50 % of lysozyme were released within 14 days due to a collapse of the polymer matrix (Table 13). The high degree of a sponge-like structure and a decrease in the mechanical strength of the polymer matrix caused by proceeding PLGA degradation could explain the increased release, as new surfaces and pores may be created and fragments of the polymer matrix may fall off (Park 1995; Friess and Schlapp 2002).

ISM formulations containing 2.5 % GMS exhibited large interconnected or channel-like pores with access to the surface in a non-swollen implant-like matrix (Figure 31 and

32). This allowed lysozyme to diffuse out of the PLGA matrix in a continuous manner within the first 20 days. Brodbeck et al. found a similar relationship between lysozyme release and porosity in in situ forming implant systems (Brodbeck, DesNoyer et al. 1999). Opening of new interconnecting voids or pores during the release with proceeding PLGA degradation further supported the lysozyme diffusion out of the matrix (Kang and Schwendeman 2007; Fredenberg, Wahlgren et al. 2011a).

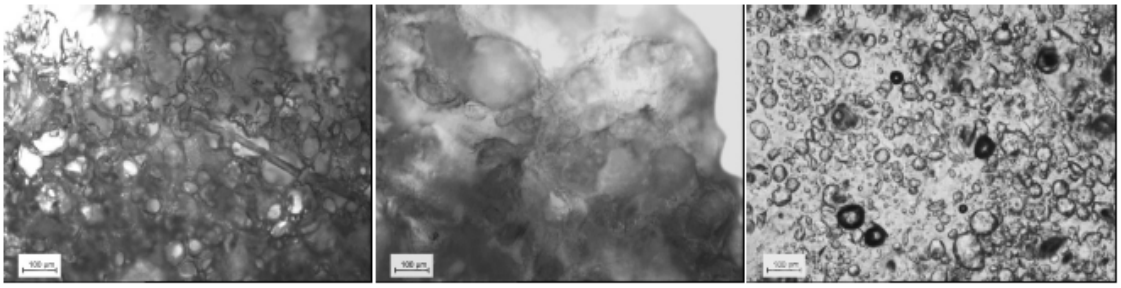

Figure 31 Microspcopic images of polymer matrices formed from ISM emulsions containing **(left)** 0 %, **(middle)** 2.5 % and **(right)** 7.5 % GMS, repectivley, in the continuous phase 11 days after incubation in acetic acid buffer pH 5.0.

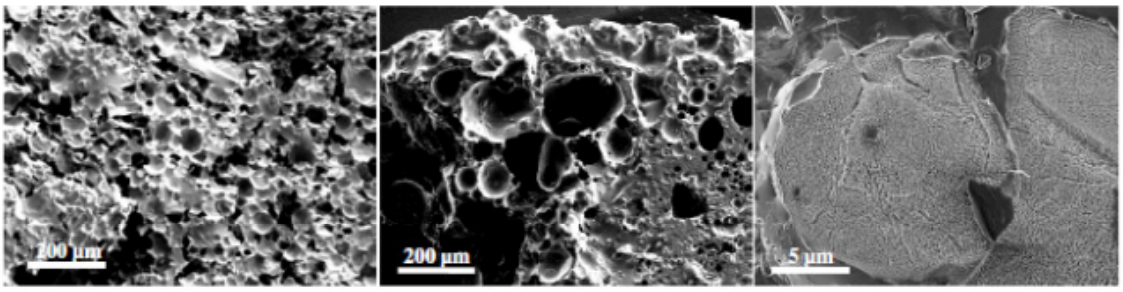

Figure 32 Cryo-SEM images of polymer matrices formed from ISM emulsions containing **(left)** 0 %, **(middle)** 2.5 % and **(right)** 7.5 % GMS, respectivley, in the continuous phase 6 days after incubation in acetic acid buffer pH 5.0.

Microparticles formed from ISM emulsions stabilized with 7.5 % GMS exhibited a fine nano-porous structure which impeded lysozyme diffusion through pores. This could explain the long lag time of 24 days prior to the erosion controlled release phase.

ISM emulsions stabilized with 5 % GMS formed an intermediate in situ consisting of polymer implant-like lumps and microparticles. The lumps showed a similar porosity like ISM formulations stabilized with 2.5 % GMS, while the microparticles exhibited a nano-porous structure similar to formulations stabilized with 7.5 % GMS (data not shown). Thus, the lysozyme release pattern (Figure 27) was probably composed of a diffusion controlled release mainly from polymer lumps and a subsequent erosion controlled release which could be more related to the nano-porous microparticles.

3.3.2.2 Effect of oil type on lysozyme release

MCT, sesame oil and castor oil were chosen to study the effect of the oil type on the release of lysozyme due to different physical properties such as in the viscosity and polymer solvent miscibility (Table 14).

Table 14 Physical properties of investigated oils used in ISM formulations.

	Viscosity (mPa·s)	Viscosity (+5 % GMS) (mPa·s)	DMSO* miscibility (%)
MCT	25.3	251.4	12
Sesame oil	57.8	603.7	5
Castor oil	82.2	1960.4	100

* contains < 0.05% water

The lysozyme release pattern significantly changed with the type of oil used (Figure 33). ISM formulations containing MCT instead of sesame oil released lysozyme in a more continuous manner until approximately 85 % of total recovered lysozyme was released. The corresponding ISM emulsions were less stable in comparison to sesame oil containing emulsions due to a decreased viscosity of the continuous phase (Table 14). Upon injection into aqueous media non-swollen porous implant-like matrices were formed, which showed a similar morphology as polymer matrices formed from ISM formulations stabilized with 2.5 % GMS (Section 3.3.2.1). Thus, lysozyme could be continuously released by diffusion out of the matrix.

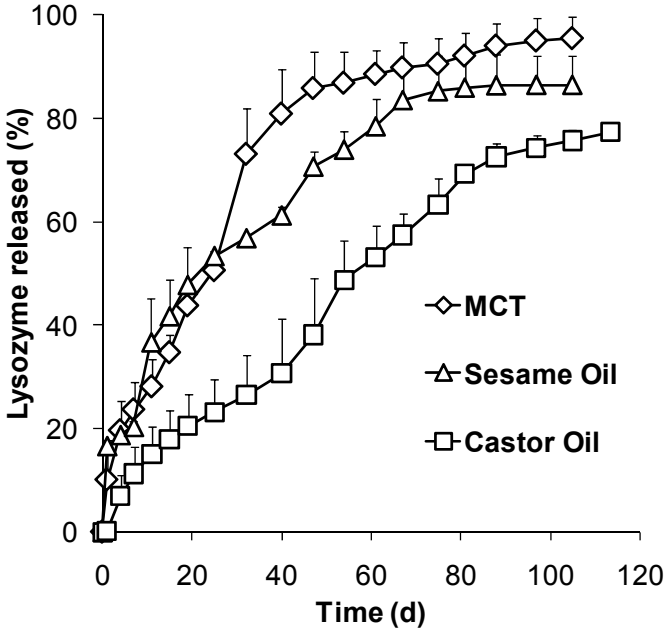

Figure 33 Effect of the continuous phase oil type of non-aquoeus ISM formulations on the release of lysozyme.

Almost no initial release at day 1 (0.3 %) was observed from ISM formulations containing castor oil. The abcence of an initial release was surprising, because many researchers have shown a significant initial drug release from in situ forming systems like ISI systems (Brodbeck, DesNoyer et al. 1999; Al-Tahami, Meyer et al. 2006) and, although less pronounced, also from ISM systems (Jain, Rhodes et al. 2000; Luan and Bodmeier 2006b). The nonappearance of an initial release could be explained by two mechanisms. Firstly, the internal phase solvent DMSO could diffuse into the oil phase during emulsification due to a full miscibility of the two phases (Table 14). As a result the viscosity of the internal phase increased. Upon injection, the polymer precipitation from a concentrated solution was faster leading to the formation of less porous microparticles. This resulted in a decreased initial drug release (Graham, Brodbeck et al. 1999; Kranz and Bodmeier 2007).

Secondly and probably of more importance, the highly viscous castor oil phase surrounding the microparticles acted as an additional lysozyme diffusion barrier. This could be verified by investigating lysozyme release from oils and the corresponding polymer-free ISM emulsions. More than 75 % of lysozyme were released within 1 day from MCT and sesame oil containing formulations (Figure 34 A). However, only 35 % and

14 % of lysozyme were released from castor oil and the corresponding polymer-free emulsions in the same time period, respectivley (Figure 34 B). The release retarding effect of castor oil was further confirmed by the observation of gel-like lysozyme particles dispersed in the remaining castor oil/GMS matrix from polymer-free ISM formulations at day 4 (Figure 35). The highly viscous castor oil hindered lysozyme particles to reach the oil / water interface to be dissolved in the release media (Larsen, Frost et al. 2008).

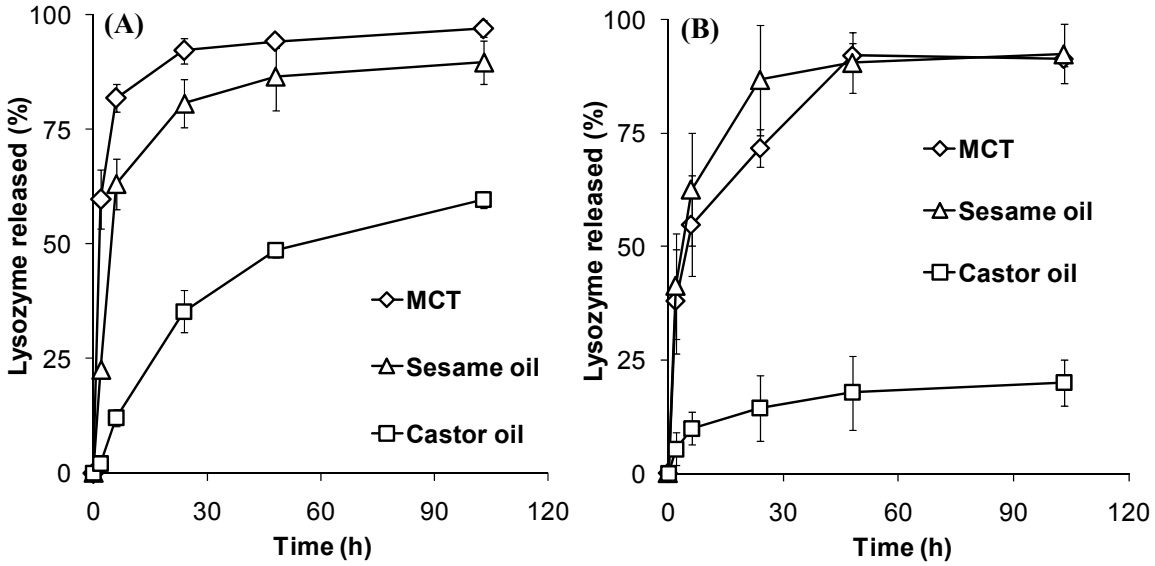

Figure 34 Lysozyme release from **(A)** vegetable oils (lysozyme dispersed) and **(B)** from the corresponding polymer-free ISM emulsions (lysozyme dissolvend in internal phase) performed in U-shaped test tubes filled with 8 mL acetic acid buffer pH 5.

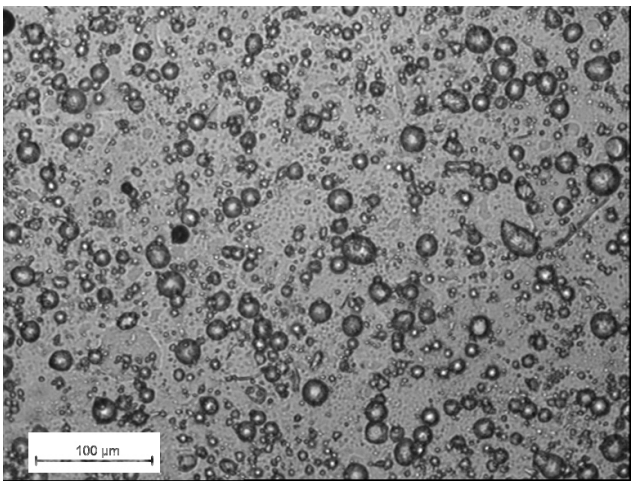

Figure 35 Gel-like lysozyme particles within the remaining castor oil / GMS matrix formed from polymer-free ISM emulsions 4 days after incubation into release media.

Furthermore, the overall more linear and extended lysozyme release from castor oil and polymer containing ISM formulations (Figure 33) could be also attributed to the release retarding effect of the additional oil phase.

3.3.2.3 Effect of continuous phase composition on enzymatic activity of lysozyme

Lysozyme released from ISM formulations exhibited full enzymatic activity over more than 40 days irrespective of which amount of GMS or oil type was used (Figure 36).

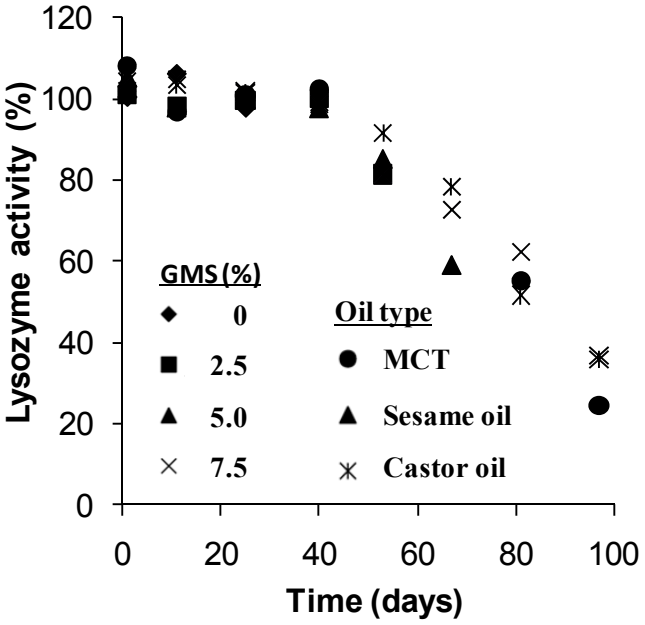

Figure 36 Effect of the continuous phase composition (GMS content, type of oil) of ISM formulations on the enzymatic activity of lysozyme during the release. Activity could not be measured, if less than 3 µg/g lysozyme were released at each sampling point.

However, ISM formulations which released lysozyme beyond 40 days showed a continuous decrease in the enzymatic activity to approximately 39 ± 4 % at day 97, while lysozyme in a control solution still showed full activity.

The decrease in the enzymatic activity was coincided with the formation of an increasing amount of soluble acidic PLGA degradation products which could be derived by the increasing pH drop in the media beyond day 35 (Figure 37). Potential interactions between lysozyme and acidic, soluble PLGA degradation products and/or the consequential decreased aqueous micro pH could explain the decreased enzymatic activity.

This was also often observed from others (van de Weert, Hennink et al. 2000; Giteau, Venier-Julienne et al. 2008).

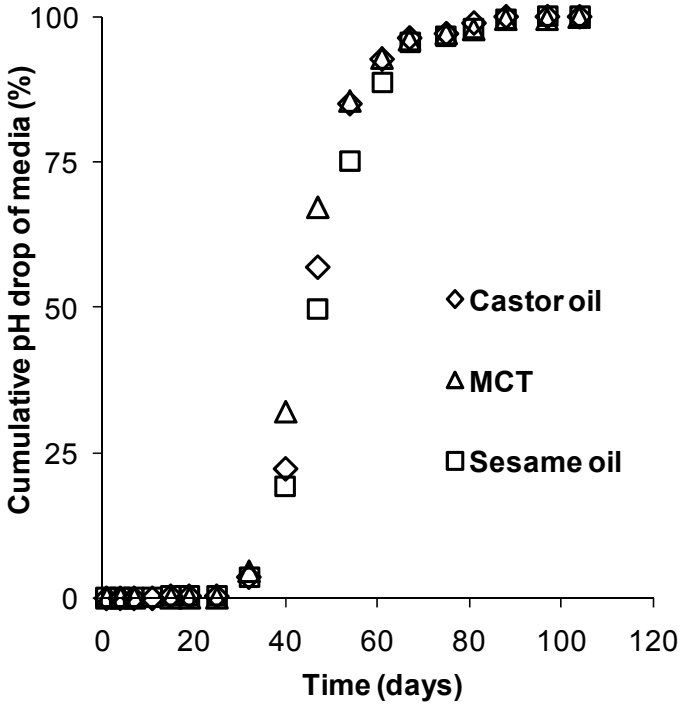

Figure 37 Cumulative pH drop of the release media due to PLGA 502S degradation to water acidic soluble oligomers representing the erosion behaviour as a function of the continuous phase oil type.

A complete release of fully active lysozyme could be obtained by releasing the total amount of lysozyme before the onset of the main PLGA erosion phase. Porous polymer matrix forming ISM formulations could be potential systems to release lysozyme only via diffusion prior to the PLGA erosion phase. However, further studies are required to optimize the porosity to completely prevent erosion controlled release.

3.3.3 Conclusion

The lysozyme release pattern from non-aquouos ISM emulsion systems was significantly affected by variations in the continuous phase composition (GMS concentration and type of oil) which could be attributed to alterations in the ISM emulsion

stability. Enhancement of the ISM emulsion stability with at least 5 % GMS in sesame or castor oil resulted in the formation of nano-porous microparticles instead of micro-porous implant-like polymer depots due to decreased droplet coalescence prior to polymer precipitation. Thereby the main lysozyme release mechanism changed from diffusion to an erosion controlled extended release from the microparticles. The additional oil layer surrounding the in situ formed microparticles did not affect the PLGA degradation rate. Nevertheless, continuous phases containing highly viscous castor oil impeded an initial burst release and extended the total release pattern. The lysozyme activity was not affected by the continuous phase composition but was sensitive to increased PLGA erosion. Hence, ISM emulsion systems forming high porous polymer matrices may be a potential way to completely release fully active lysozyme via diffusion prior to the onset of the polymer erosion phase.

4 Summary

The extended and controlled drug delivery from biodegradable, poly(lactic-co-glycolic acid) (PLGA) based parenteral depot systems represents an attractive way in the long-term treatment of many diseases. In the late 1990s non-aqueous *in situ* forming microparticle (ISM) systems have been developed as an alternative to implants, microparticles and *in situ* forming implants (ISI). Non-aqueous ISM systems are injectable emulsions where a drug-containing organic polymer solution (ISI) is emulsified into a stabilizer-containing oily continuous phase. ISM emulsions can be prepared in a two-syringe/connector system prior to administration by back and forth movements of the syringe plungers. Upon injection into the body the polymer solidifies forming microparticles *in situ*. The encapsulated drug is then released over extended periods of time from days to months.

However, non-aqueous ISM emulsions showed a relatively low emulsion stability of only a few minutes. Coalescence of the highly viscous polymer-containing internal phase may complicate the injection through small needles and may result in the formation of implant-like polymer matrices instead of microparticles which could change the release pattern of incorporated drugs.

In order to improve the stability of non-aqueous ISM emulsions and due to a lack of general knowledge about the stabilization of non-aqueous emulsions, a variety of parenteral approved excipients were screened for a stability-enhancing effect. Ethanolamine, the phospholipid Lipoid® S 100 and glycerol monostearate (GMS) were found to stabilize non-aqueous ISM emulsions for more than 15 minutes which is an appropriate time frame to administer the ISM emulsion to the patient. Interestingly, GMS stabilized emulsions showed superior stability over more than 12 hours.

The objective of this work was therefore to investigate the stabilizing potential of GMS in more detail. Hence, the underlying stabilization mechanisms were identified, the effect of emulsion stabilization on the injectability of the resulting ISM formulation evaluated, the stabilizing capacity of GMS with regard to changes in formulation and

process parameters characterized and finally the influences of GMS stabilized ISM formulations on the release of different drugs elucidated.

Flow behaviour analysis, differential scanning calorimetry, hot-stage polarized light- and cryo electron microscopy revealed that the ISM emulsions were stabilized trough the formation of a liquid crystalline GMS layer surrounding the polymer solution droplets and a more than 5-fold viscosity increase in the continuous phase immediately after emulsification due to the formation of a viscous, shear thinning GMS gel network.

GMS stabilized ISM emulsions could be easily and smoothly injected through hypodermic needles, while high injection peak forces were observed from unstable ISM emulsions due to coalescence of the highly viscous PLGA solution droplets. Moreover, ISM emulsions stabilized with GMS showed an improved injectability of about 30 % in comparison to the corresponding ISI solutions. The injectability improvement allows a faster administration or enables the use of thinner needles and hence reduced patient discomfort.

Stable ISM emulsions could be formed with at least 5 % GMS (based on continuous phase) under various changes in formulation and process parameters such as with a PLGA 502 S content up to 40 %, a continuous phase:internal phase ratio of at least one, various oil types and in a temperature range from 15 to 33 °C. Low and high molecular weight drugs could be incorporated without a visible destabilizing effect. However, GMS stabilized ISM emulsions were sensitive to changes in the internal phase as the organic solvent and the polymer type. This was explained with an increased internal phase viscosity decreasing droplet break-up during emulsification and/or changes in the polarity impeding the formation of the droplet interface stabilizing liquid crystals. The preparation time of GMS containing ISM formulations in the two-syringe/connector system could be reduced from 180 s to less than 30 s by increasing the shear force during emulsification using smaller connector diameters (0.75 mm) and a fast mixing speed (2 cycles / s). This simplified the handling of the system which can be prepared prior to administration by the patient himself.

While evaluating the ISM stabilization mechanisms, pronounced polymorphism of GMS in the continuous oil phase was identified. This may also have an effect on the ISM emulsion stability. Therefore, the polymorphic characteristics of GMS were further investigated. Three GMS modifications (α, β’ and β) were identified in the continuous oily phase. The formation of the stabilizing GMS gel network in the oil phase could be attributed to the fine amorphous-like α-modification. β’ and β crystals grew up to

approximately 100 µm in size throughout continuous phase storage, but were completely transformed into liquid crystals during ISM emulsion preparation. Hence, variations in the polymorphic composition of GMS did not affect its ability to stabilize ISM emulsions.

The drug release characteristics from GMS stabilized non-aqueous ISM formulations were investigated with low molecular weight model drugs (theophylline, diprophylline and carbamazepine) and the high molecular weight model protein lysozyme. Low molecular weight drug release was dependent on drug properties like the solubility in the organic solvent of the internal phase as well as on formulation properties such as the size and shape of the *in situ* formed polymer matrix. Droplets from less stable ISM emulsions coalesced prior to polymer precipitation forming implant-like matrices, while microparticles were formed with at least 5 % GMS in the continuous phase. Microparticles showed a faster drug release in comparison to the implant-like matrices which could be related to the overall increased surface area.

Polarized light microscopy and cryo-scanning electron microscopy revealed that the ISM emulsion stability did not only influence the external morphology of *in situ* formed polymer depots but also affected their porosity. The *in situ* formed microparticles exhibited a nano-porous structure in comparison to a micro-porous structure observed in implant-like matrices. This significantly changed the release pattern of the model protein lysozyme. The underlying main release mechanism was shifted from diffusion through the porous network within the implant-like depots to a more extended erosion controlled release from microparticles after approximately 5 weeks.

In addition, the impact of the continuous phase oil type (MCT, castor and sesame oil) surrounding the polymer depots on PLGA degradation and on lysozyme release was investigated. Surprisingly, gel permeation chromatography showed no effect of the continuous phase on the PLGA degradation rate. However, the higher viscous castor oil was able to prevent an initial burst release of lysozyme.

Furthermore, also the lysozyme activity was not influenced by the continuous phase composition. It was found that the activity was sensitive to the increased polymer erosion rate after approximately 5 weeks. Thus, active lysozyme may be completely released via diffusion within 1 month from ISM formulations forming high porous polymer matrices.

Finally, a long-term stability study revealed that continuous phases could be stored at 4 °C for more than 1 year without compromising the ISM emulsion stability and the release pattern of the model protein lysozyme.

In conclusion, glycerol monostearate showed high potential as a stabilizer for non-aqueous ISM emulsion systems feasible in a broad range of formulation and process parameters. Stable ISM emulsions showed improved injectability in comparison to the corresponding ISI systems. The drug release pattern was affected by alterations in the stability of the ISM emulsion due to the formation of differently shaped and dense polymer depots.

5 Zusammenfassung

Die verlängerte und kontrollierte Arzneistofffreisetzung aus bioabbaubaren, auf Polylactidglykolid (PLGA) basierenden, parenteral applizierbaren Depotsystemen stellt eine attraktive Möglichkeit der Langzeitbehandlung vieler Erkrankungen dar. Als Alternative zu bekannten Depotsystemen, wie Implantaten, Mikropartikeln und *in situ* bildenden Implantaten (ISI) wurde gegen Ende der 1990er Jahre ein System entwickelt, das auf der Bildung von *in situ* Mikropartikeln (ISM) aus wasserfreien Emulsionen beruht. Diese Emulsionen bestehen aus einer organischen PLGA Lösung, die in eine Stabilisator enthaltende äußere Ölphase emulgiert ist. Mit Hilfe eines 2-Spritzensystems können ISM-Emulsionen direkt vor der Anwendung hergestellt werden. Dabei werden die zwei Phasen durch hin- und herbewegen der zwei Spritzenkolben miteinander vermischt. Nach erfolgter subkutaner oder intramuskulärer Injektion präzipitiert das Polymer in der Form von Mikropartikeln. Darin eingeschlossener Arzneistoff kann dann über Tage bis hin zu Monaten kontinuierlich freigesetzt werden.

Trotz mehrerer Vorteile gegenüber den anderen Depotsystemen könnte die relativ niedrige Emulsionsstabilität der bisherigen wasserfreien ISM-Systeme von nur ein paar Minuten problematisch sein. Ein Zusammenfließen der hochviskosen Polymer enthaltenden Emulsionströpfchen würde die Injektion durch dünne patientenfreundliche Subkutannadeln erschweren oder sogar unmöglich machen. Des Weiteren würden sich eher implantatartige Polymerdepots anstatt Mikropartikel *in situ* bilden, was demzufolge auch das Arzneistofffreisetzungsverhalten beeinflussen würde.

Daher wurde in einem ersten Experiment versucht, die Stabilität der wasserfreien ISM-Emulsionen zu erhöhen. Aufgrund eines generellen Mangels an grundlegendem Wissen über die Stabilisierung von nichtwässrigen Emulsionssystemen wurde eine größere Anzahl von parenteral zugelassenen Hilfsstoffen auf einen emulsionsstabilisierenden Effekt hin untersucht. Dabei wurde festgestellt, dass Ethanolamin, das Phospholipid Lipoid® S100 und Glycerolmonstearat (GMS) wasserfreie ISM-Emulsionen für mehr als 15 Minuten

gegen Tröpfchenkoaleszenz stabilisieren konnten. Ein Zeitraum von 15 Minuten wurde gewählt, da dieser ausreichen sollte, um die Formulierung Patienten zu injizieren. Interessanterweise zeigten GMS enthaltende Emulsionen eine herausragende Stabilität von mehr als 12 Stunden.

Die Zielsetzung dieser Arbeit war es daher, das Potenzial von GMS detaillierter zu untersuchen. Dafür wurden als erstes die stabilisierenden Mechanismen von GMS in wasserfreien ISM-Emulsionen aufgeklärt und anschließend der Einfluss der Emulsionsstabilisierung auf die Injektionsfähigkeit der ISM-Formulierungen evaluiert. Des Weiteren wurde die Stabilisierungskapazität von GMS im Hinblick auf Veränderungen von Formulierungs- und Prozessparametern charakterisiert und schließlich das Freisetzungsverhalten von verschiedenen Arzneistoffen aus wasserfreien GMS-stabilisierten ISM-Formulierungen untersucht.

Durch eine Analyse des Fließverhaltens, Dynamische-Differenz-Kalorimetrie, Heiztischpolarisations- und Kryorasterelektronenmikroskopie wurde herausgefunden, dass die wasserfreien ISM-Emulsionen zum einen durch flüssigkristalline GMS-Filme stabilisiert wurden, welche sich während des Emulgierungsvorganges um die Polymerlösungströpfchen geformt hatten. Zum anderen wurden die Emulsionen durch eine mehr als fünffache Viskositätserhöhung der äußeren Phase nach dem Emulgierungsvorgang stabilisiert. Dies konnte auf die Bildung eines scher-verdünnenden GMS-Gelnetzwerkes in der äußeren Phase zurückgeführt werden.

GMS-stabilisierte ISM-Emulsionen konnten leicht und gleichmäßig durch Subkutannadeln injiziert werden. Nicht stabilisierte ISM-Formulierungen hingegen zeigten hohe Injektionskraftspitzen aufgrund von Koaleszenz der hochviskosen Polymerlösungströpfchen. Darüber hinaus zeigten stabilisierte ISM-Emulsionen eine um etwa 30 % verbesserte Injektionsfähigkeit gegenüber den entsprechenden ISI-Systemen (einfache Polymerlösung). Die verbesserte Injektionsfähigkeit erlaubt entweder eine schnellere Injektion oder die Benutzung von dünneren Nadeln. Beide Möglichkeiten würden zu einer Verbesserung der Patientenfreundlichkeit führen.

Ein Gehalt von mindestens 5 % GMS in der äußeren Phase (m/m) war nötig, um stabile ISM-Emulsionen in einem großen Bereich von Formulierungs- und Prozessparametern herstellen zu können, wie zum Beispiel mit PLGA 502S Konzentrationen bis 40 %, mit einem Verhältnis der Äußeren zur Inneren Phase von mindestens eins, mit verschiedenen Ölsorten, sowie in einem Temperaturbereich von 15 bis 33 °C. Verschiedene nieder- und hochmolekulare Arzneistoffe konnten ohne einen

sichtbaren emulsionsdestabilisierenden Einfluss in die ISM-Formulierungen eingebracht werden. Jedoch zeigten GMS-stabilisierte ISM-Emulsionen eine Sensibilität gegen Veränderungen in der Inneren Phase (organisches Lösungsmittel, Polymer). Dies konnte mit einer erschwerenden Tröpfchenzerkleinerung mit ansteigender Viskosität der inneren Phase und/oder einer Verhinderung der Bildung von flüssigkristallinen Strukturen von GMS an der Tropfengrenzfläche erklärt werden. Durch eine Erhöhung der Scherkräfte während des Emulgiervorganges im 2-Spritzensystem konnte mit der Benutzung von dünneren Konnektoren mit einem Durchmesser von 0,75 mm und einer hohen Mischgeschwindigkeit von zwei Mischzyklen pro Sekunde die Herstellungszeit von GMS-stabilisierten ISM-Emulsionen von 180 zu weniger als 30 Sekunden reduziert werden. Dadurch wurde die Handhabung des Systems vereinfacht, das direkt vor der Applikation vom Patienten selbst hergestellt werden kann.

Während der Ermittlung der Stabilisierungsmechanismen wurde ein ausgeprägter Polymorphismus von GMS in der äußeren Phase festgestellt. Da dies unter anderem auch die Emulsionsstabilität beeinflussen könnte, wurden die Charakteristika der GMS-Modifikationen eingehender erforscht. Drei GMS-Modifikationen (α, β' and β) konnten in der äußeren Ölphase nachgewiesen werden. Die Bildung des stabilisierenden GMS-Gelnetzwerkes konnte auf die feine amorph-ähnliche α-Modifikation zurückgeführt werden. Während der Lagerung der äußeren Phase wuchsen β'- und β-Kristalle bis zu einer Größe von etwa 100 µm. Interessanterweise beeinträchtigte dies nicht die Fähigkeit, ISM-Emulsionen zu stabilisieren. Während der Emulgierung transformierten die Kristalle vollständig zu flüssigkristallinen Strukturen in der Phasengrenzfläche.

Das Freisetzungsverhalten von Arzneistoffen aus GMS-stabilisierten wasserfreien ISM-Formulierungen wurde an niedermolekularen Modellarzneistoffen (Theophyllin, Diprophyllin und Carbamazepin) und dem hochmolekularen Modellprotein Lysozym untersucht. Die Freisetzung von niedermolekularen Substanzen war abhängig von Arzneistoffeigenschaften, wie deren Löslichkeit im organischen Lösungsmittel der inneren Phase, und von Formulierungsparametern, wie die Größe und Form der *in situ* gebildeten Polymerdepots. Große implantatähnliche Depots wurden aufgrund des Zusammenfließens von Emulsionströpfchen vor der Polymerpräzipitation von zu gering stabilisierten ISM-Emulsionen geformt. Mikropartikel konnten von ISM-Formulierungen geformt werden, die mit mindestens 5 % GMS (basierend auf der äußeren Phase) stabilisiert waren. Niedermolekulare Arzneistoffe wurden im Vergleich zu den implantatähnlichen Depots

von Mikropartikeln schneller freigesetzt, was mit der höheren Oberfläche von Mikropartikeln begründet werden könnte.

Mit Hilfe der Polarisations- und Kryorasterelektronenmikroskopie wurde herausgefunden, dass die Emulsionsstabilität nicht nur die äußere Morphologie der *in situ* geformten Polymerdepots beeinflusste, sondern auch das Ausmaß der Porosität. *In situ* geformte Mikropartikel zeigten nano-poröse Strukturen. Eher mikro-poröse Strukturen wurden in den implantatähnlichen Gebilden gefunden. Dies veränderte signifikant das Freisetzungsverhalten vom Modellprotein Lysozym. Der zugrundeliegende Hauptfreisetzungsmechanismus verlagerte sich von Diffusion durch Poren aus den implantatähnlichen Depots zu einer verlängerten, erosionskontrollierten Freisetzung aus den nano-porösen Mikropartikeln. Diese setzte etwa nach 5 Wochen ein.

Anschließend wurde der Einfluss der Öl-Sorte der äußeren Phase (MCT, Sesamöl und Rizinusöl) auf den PLGA-Abbau und die Lysozym-Freisetzung untersucht. Mit Gelpermeationschromatographie konnte gezeigt werden, dass der Abbau des Polymers nicht von der äußeren Phase beeinflusst wurde. Somit konnte ein verändertes Freisetzungsverhalten erneut auf die Bildung von unterschiedlich porösen Polymerdepots zurückgeführt werden. Interessanterweise zeigte sich jedoch, dass durch die Nutzung von hochviskosem Rizinusöl ein initialer Burst komplett vermieden werden konnte.

In einem weiteren Experiment konnte gezeigt werden, dass die Zusammensetzung der äußeren Phase auch nicht die Aktivität von Lysozym beeinflusste. Diese reagierte jedoch empfindlich auf eine nach etwa 5 Wochen ansteigende Polymererosionsrate. Daher könnten ISM-Formulierungen, die die Lysozymbeladung innerhalb eines Monats durch Diffusionsprozesse freigeben, genutzt werden, um ausschließlich aktives Lysozym freizusetzen.

Eine abschließende Langzeitstabilitätsstudie zeigte, dass die äußere Phase für mehr als ein Jahr bei 4 °C gelagert werden kann, ohne einen Einfluss auf die ISM-Emulsionsstabilität und das Freisetzungsverhalten von Lysozym aufzuweisen.

Fazit: Glycerolmonostearat zeigte ein hohes Potenzial, wasserfreie ISM-Emulsionen in einem breiten Bereich variierender Formulierungs- und Prozessparameter zu stabilisieren. Stabile ISM-Emulsionen wiesen eine verbesserte Injizierbarkeit im Vergleich zu ISI-Systemen auf. Das Arzneistofffreisetzungsverhalten von ISM-Systemen wird unter anderem von Veränderungen in der Emulsionsstabilität - aufgrund der Bildung von verschieden geformten Polymerdepots unterschiedlicher Dichte - beeinflusst.

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7 Publications and Presentations Resulting from this Work

Journal publications

M. Voigt, M. Koerber, R. Bodmeier, *Improved Stabilization and Injectability of Non-aqueous in situ PLGA Microparticle Forming Emulsions*, (In preparation).

M. Voigt, R. Bodmeier, *Potential of Glycerol monostearate to Stabilize Non-aqueous Emulsions Forming Biodegradable PLGA Microparticles in situ*, (In preparation).

M. Voigt, M. Koerber, R. Bodmeier, *Influence of the Continuous Phase Composition on the Release of Lysozyme from Non-aqueous in situ Forming Microparticles*, (In preparation).

Poster presentation

M. Voigt, M. Koerber, R. Bodmeier, *Stabilization of Non-Aqueous Emulsions to Form Biodegradable Microparticles in situ*, "The 36th Annual Meeting and Exposition of The Controlled Release Society", 2009, Copenhagen, Denmark.

8 Curriculum Vitae

The Curriculum Vitae is not included in the online version due to reasons of data protection.