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SPRAY CONGEALED SOLID LIPID MICROPARTICLES FOR THE SUSTAINED RELEASE OF PEPTIDES IN VETERINARY USE

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CRS Annual Meeting and Exposition, Seattle, WA, USA, July 17-20, 2016

Oral presentations

K. Hoffmann, W. Zaremba, J. Kauffold, W. Frieß „Release and Long-Term-Stability of Gonadorelin [6-D-Phe]-loaded lipid microparticles produced by spray-congealing

PBP Worldmeeting, Glasgow, United Kingdom, April 4-7, 2016

K. Hoffmann, W. Zaremba, J. Kauffold, W. Frieß „Influence of Monoglycerides on Release of Gonadorelin [6-D-Phe] from Spray-Congealed Solid Lipid Microparticles

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Patents

W. Zaremba, J. Kauffold, W. Frieß, Y. Yordanova, K. Hoffmann „Veterinary pharmaceutical composition and use thereof "

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AE	Adverse events
ASP	Aspartame
β-hCG	Beta human Chorionic Gonadotropin
BSA	Bovine Serum Albumin
BST, rBGH	Bovine somatotropin, recombinant Bovine Growth Hormone
CMC	Carboxymethylcellulose
CSA	Cetylstearyl alcohol
D112, D114, D116, D118, D120	Dynasan112, Dynasan114, Dynasan116, Dynasan118, Dynasan120
DDS	Drug Delivery System
DLG	Deutsche Landwirtschaftliche Gesellschaft
DSC	Differential Scanning Calorimetry
eCG	Equine Chorionic Gonadotropin
EE	Encapsulation Efficiency
EPO	Erythropoietin
FDA	Food and Drug Administration
FIV	Feline Immunodeficiency Virus
FSH	Follicle stimulating hormone
G [6-D-Phe]	Gonadorelin [6-D-Phe]
GLP-1	Glucagon-like peptide-1
GMS	Glycerol monostearate
GnRH	Gonadotropin releasing hormone
eCG	Equine Chorionic Gonadotropin
HBsAg	Hepatitis B surface Antigen
HCl	Hydrochloride
IFN-α	Interferone alpha
i.m.	intra muscular
LAF	Laminar Air Flow

LH	Luteinizing hormone
O/W	Oil-in-Water
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PEG-24-GS	Polyethylene glycol-24-glyceryl stearate
PGF-2α	Prostaglandin F-2 alpha
PLA	Poly lactic acid
PLGA	Poly (lactic-co-glycolic acid)
PMSG	Pregnant mare serum gonadotropin
POD	Polycystic ovarian degeneration
PS	Polysorbate
PVP	Polyvinylpyrrolidone
rhEPO	Recombinant human erythropoietin
RP-HPLC	Reversed-phase high-performance liquid chromatography
s.c.	subcutaneous
SEM	Scanning electron microscopy
SLI	Solid lipid implants
SLM	Solid lipid microparticles
SLN	Solid lipid nanoparticles
TG	Triglyceride
TP-4	Thymocartin
t-PA	Tissue-type Plasminogen Activator
TRP	Tryptophan
TSPG	Thermosensitive poloxamer gels
VEGF	Vascular endothelial growth factor
WPN	Wide pneumatic nozzle
XRPD	X-Ray powder diffraction

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1 GENERAL INTRODUCTION

1.1 Introduction

Biopharmaceutical drugs have gained more and more importance in the field of pharmaceutical research [1] [2]. Being able to cure severe and rare diseases, the development of pharmaceutical products for the delivery of peptides and proteins became an important task in recent years [3] [4]. Challenges within the development, manufacturing, production and storage of these products are, that especially proteins are sensitive to a variety of external factors, like shear stress, temperature and pH [5] [6]. Due to poor oral absorption, peptide and protein drugs are typically administered parenterally. Furthermore a short half-life in the body causes a high frequency of administration via the parenteral route, which negatively impacts the patient compliance [2]. For this reason, the development of sustained release delivery systems is of high importance to achieve sustained blood levels with reduced side effects and improved patient acceptance [7].

In the field of veterinary medicine, the focus is set on facilitating therapy for owners and veterinarians and to reduce distress and potential side effects for the treated animal [8]. For livestock breeding, the application of biopharmaceutical drugs opens new doors with respect to wait-times and residuals in animal products. With the application of biopharmaceutical drugs in a sustained release delivery system, their main drawback, the short half-life can be overcome [9]. As strict rules for wait times concerning milk and meat exist for small molecules due to accumulation, this problem is not given for peptides, e.g. G [6-D-Phe] in Gonvet®Veyx (Veyx Pharma GmbH) [10]. Consequently, sustained release formulations entered the marketplace in veterinary medicine. ProHeart® 6 by Zoetis, an injectable sustained delivery system for protection against the canine heartworm disease, based on lipid microparticles is FDA approved [11]–[13]. Another example is the application of the GnRH-agonist deslorelin via the subcutaneously injected lipid implant Suprelorin® by Virbac for the chemical castration in male, mature dogs [14] [15]. Suprelorin® was recently also investigated for taint removal of boars and for suppression of sexual function in male cats [16] [17]. Bovine somatotropin (recombinant bovine growth hormone, BST, rBGH) is the active ingredient formulated in an oily zinc suspension in Posilac® (Elanco Animal Health). The controlled release induces an improved lactation in dairy cows [8] [18] [19]. For livestock animals, the requirements are to provide an acceptable and effective therapy with low costs, facilitating the already existing treatment.

1.2 Drug Delivery Systems for the Sustained Release of Peptides and Proteins

A variety of drug delivery systems can be utilized to sustain the release of peptides and proteins.

1.2.1 Implants

Implants usually have a cylindrical shape and can be manufactured by compression, molding or extrusion [20]–[22]. A distinction between biodegradable and non-biodegradable implants is commonly made in literature. Advantages of biodegradable implants are the lack of surgical removal and the high biocompatibility [2] [23] [24]. Release is mainly controlled by diffusion, which takes place in both degradable and non-degradable implants. Furthermore, for biodegradable implants also matrix erosion, swelling and osmotic effects need to be taken into consideration [25] [26].

Poly (lactide-co-glycolide) (PLGA) derived implants

The delivery of proteins via PLGA-based implants was extensively studied, revealing outstanding biocompatibility and degradation properties [27]–[29]. A disadvantage concerning implants based on PLGA is the formation of an acidic microenvironment within the implant, leading to an enhanced degradation of the protein and potentially covalently modified peptide and protein [27] [30] [31]. Nevertheless, PLGA-based implants delivering the peptide drugs goserelin (Zoladex®, Astra Zeneca) [32] and buserelin (Profact® Depot, Sanofi Aventis Deutschland GmbH) [33], as well as an in-situ forming implant containing leuprolide (Eligard®, Astellas) [34] are approved for breast and prostate cancer treatment, whereas Zoladex® is further approved for the treatment of severe endometriosis [32] [35].

Solid lipid implants

The manufacturing of biodegradable implants made from solid lipids, like triglycerides, also in combination with monoglycerides and cholesterol for the delivery of therapeutic proteins is of high interest in the field of controlled delivery [22] [26] [36] [37]. An advantageous production system is the twin-screw extrusion process, allowing to treat the lipid excipient slightly below the melting point which helps to avoid polymorphic changes [22] [37] [38]. Drug release can be managed in time frames from weeks to months [39] [40]. The addition of additives like polyethylene glycol (PEG) can enhance and complete the release, due to the formation of an interconnected pore network [26] [40] [41].

1.2.2 Suspensions

Oily suspensions are suitable delivery systems for peptides and proteins intended for controlled release [2]. The sustained delivery is due to the formation of a depot after injection in the tissue, which slowly releases the drug [8]. In human as well as veterinary medicine oily suspensions are of high interest. In veterinary medicine, e.g. the products Excede® and Excenel® RTU by Zoetis containing ceftiofur and its hydrochloride, a cephalosporin against infectious diseases in cattle, is applied via an oily suspension [8] [42] [43] .

1.2.3 Nanoparticles

Nanoparticles ranging from 10 to 1000 nm can e.g. be produced by spray drying and emulsion-based preparation methods, like hot or cold homogenization techniques [44]–[47]. Natural or synthetic polymers, like gelatin, PLGA, or triglycerides can be used for their preparation [46] [48]–[51]. Triglycerides, like tristearin, trimyristin or composed lipids like Compritol® 888 ATO are commonly used as lipid bases upon manufacturing [52]–[54]. Regarding biopharmaceutical drugs, proteins like BSA and peptides like calcitonin and gonadorelin have been entrapped successfully into nanoparticles [55]–[58].

1.2.4 Microparticles

Microparticles from natural and synthetic polymers, for example solid lipids [1] [59] [60], PLGA [61]–[63] and poly lactic acid (PLA) [64], are suitable for parenteral drug delivery. Various microparticulate sustained release systems are described for biopharmaceuticals [1] [65]–[68].

Poly lactic acid (PLA) and Poly lactic co-glycolic acid (PLGA) microparticles

PLGA is described as suitable polymer for the preparation of microparticles, also for protein and peptide delivery [24]. Recombinant human erythropoietin (rhEPO), vascular endothelial growth factor (VEGF) and the peptide somatostatin could be successfully entrapped into PLA and PLGA-based particles [3] [69] [70]. Problems arising from PLGA are changes in the microclimate which afford a special stabilization of the encapsulated proteins [24] [71] and the high costs which explain the only few available veterinary products. The drug delivery system SMARTshot® B_{12} Prime Lamb by Virbac delivers vitamin B_{12} in a PLGA suspension in an oily vehicle for suckling lambs [8] [72]. For diabetes treatment, the PLGA-based microparticle system Bydureon® by AstraZeneca is approved delivering exenatide [73].

Solid lipid microparticles

Solid lipid microparticles are of particular interest for the delivery of biopharmaceuticals due to several advantages compared to other polymers like PLGA [1] [67] [74]–[76].The solid lipids are highly biocompatible and degraded by endogenous lipases [5] [23] [77]. A pH shift, which may destabilize peptides and proteins, cannot be observed [78]. The peptides somatostatin, insulin, thymocartin, desmopressin and the GnRH antagonist antide have been loaded into solid lipid microparticles [1] [68] [74]–[76]. The sustained delivery of proteins like BSA has also been realized with incorporation into lipid microparticles [79] [80].

1.3 Preparation Techniques for Lipid-Based Microparticles

Lipid-based microparticles are often produced by emulsion-based methods [81]–[83]. Furthermore, preparation techniques with presence of organic solvents are common [75] [76]. Ideally, the manufacturing technique works without an additional water phase, which may induce degradation or aggregation processes at the interface, and without organic solvents to avoid the problem of solvent residuals in lipid microparticles.

1.3.1 Hot emulsion

This method is preferably applied for preparation of small molecule-loaded lipid microparticles. Lipid excipients are melted together and mixed with a hot aqueous phase containing emulsifier to obtain a O/W emulsion, potentially via phase inversion. Upon cooling, the lipid droplets solidify which leads to the formation of microparticles [84] [85].

1.3.2 Double emulsion

In the double emulsion method, the drug is dissolved in an aqueous phase, which is afterwards emulsified into a lipid melt. Often stabilizers such as gelatin or poloxamers are used. Subsequently, this primary emulsion is transferred into a second aqueous phase. Upon stirring and cooling, the microparticles solidify and can be removed by filtration [81] [86].

1.3.3 Solvent evaporation

The solvent evaporation method was also reported as suitable for the encapsulation of peptides into microparticles [68] [74]. The underlying principle is, that the solid triglyceride is dissolved in methylene chloride or another organic solvent and the grinded peptide afterwards added. The suspension is subsequently added to an aqueous solution, which might be cooled, and stirred to allow the evaporation of the organic solvent leading to the formation of the microparticles [74]. Another technique deriving from this process is the so called solvent stripping method used for the entrapment of the GnRH antagonist antide [76]. Other researchers also successfully

incorporated hepatitis B surface antigen (HbsAg) and somatostatin via a modified emulsion-solvent evaporation method into lipid microparticles [67] [75].

1.3.4 Spray drying

Spray drying is an efficient drying process in a preheated airstream. Although the contact of the liquid phase with the hot air is quite short, this drying technique leads to a considerably higher thermal stress than freeze-drying [87]–[89]. For this reason, the co-current preparation method should be favored compared to the counter current method, as the air of the lowest temperature is getting in contact with the drug solution, e.g. as described for recombinant methionyl human growth hormone (hGH) and tissue-type plasminogen activator (t-PA) [87] [88]. To achieve a sustained release, atomization can either occur out of an organic solution, an emulsion or a suspension, whereas the removal of one phase commonly leads to the formation of microparticles [3] [90]–[92]. Used lipids for the spray drying process are usually triglycerides also in combination with emulsifiers like lecithin [91]. But also the use of other polymers like PLGA [90] [61], PLA [64], cellulose derivatives [93] or chitosan [94] is described in literature. Thus, this preparation method is used for the preparation of microparticles loaded with biopharmaceutical drugs, like erythropoietin and insulin [3] [95].

1.3.5 Spray congealing

Spray congealing is related from spray drying using a similar apparatus setup with installation of additional parts (*Figure 1-1)* [1]. The lipid melt with suspended peptide or protein particles, is sprayed into an air stream, which is either kept at room temperature or cooled down [1] [96]–[99]. Atomization typically occurs with the use of a two-fluid nozzle whereas compressed air is commonly used to disperse the melt [100] [101]. Furthermore, the use of atomization via ultrasound [102]–[105] and rotating discs or wheels [96] [106] [107] is described. The contact with the air of lower temperature leads to solidification of the produced droplets. The separation in different size fractions occurs in analogy to the spray drying process [108].

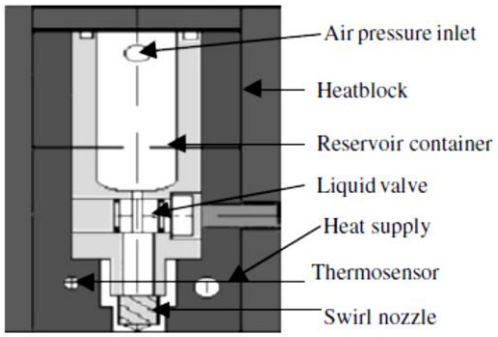

Figure 1-1: Schematic image of the spray congealing equipment based on the Büchi B-290 Mini spray dryer [1]

In literature, the successful incorporation of a variety of small molecule drugs, like indomethacin [102], verapamil HCl [97] and aminolevulinic acid [101] is described. The encapsulation of proteins via spray congealing may be critical due to thermal stress affecting the ternary and quaternary structure. Nevertheless the peptides insulin [1], somatostatin [75] and thymocartin [74] as well as proteins like BSA were successfully incorporated into spray congealed lipid microparticles [79] [109]. Major benefits of the spray congealing process are, that no further aqueous phase is needed which helps to avoid peptide or protein aggregation [5] [110].

1.4 Estrus Synchronization

The term estrus synchronization describes the simultaneous achievement of the fertile state in female animals by the help of pharmaceuticals [111]–[113]. It is a suitable tool to reduce costs and efforts for veterinarians and farmers, due to termination of separation of the female animals, as well as littering and weaning [114] [115]. Species treated to achieve estrus synchronization are mainly swine, but protocols do also exist for cattle, sheep, goats and mares [115]–[123]. Over decades, the application of synthetic gestagens turned out to be highly efficient in synchronizing the estrus in swine, whereas the treatment is successful for both gilts and sows [111] [113] [117]. Moreover, gestagen treatment leads to absence of non-responders and no severe side effects [113]. These advantages made this therapy to the gold-standard in estrus synchronization in recent years. Drawbacks of this therapy arise from the environmental entry of the gestagens by the produced manure [124] [125]. Analysis of the surrounding runoff to large agricultural holdings evidenced the presence of gestagens in levels above non-harmful concentrations [126] [127]. Gestagens exhibit toxicity for water organisms by leading to reproductive disorders or even infertility, further discussed in *1.5* [128] [129].

1.4.1 Hormonal regulation mechanisms

To understand the mode of action underlying estrus synchronization, the hormonal regulation of sex hormones in female mammalians is briefly discussed. Under control of luteinizing hormone (LH) and follicle stimulating hormone (FSH) progesterone and estrogen are released. LH and FSH release again is controlled by the decapeptide gonadotropin-releasing hormone (GnRH), also referred to as luteinizing hormone-releasing hormone (LHRH), with the sequence p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 [130]–[133]. A single or pulsatile application of GnRH or its analogues results in an increase in LH and FSH levels. If applied continuously, these hormones are down regulated due to a negative feedback mechanism taking place in the gonadal-pituitary-hypothalamic axis and a following receptor down regulation [132] [133].

1.4.2 Therapeutic regimen and pharmaceutical products

Estrogens and gestagens

Altrenogest, also referred to as allyl trenbolone, is the gestagen mainly used for estrus synchronization in swine. It is usually fed in amounts of 15-20 mg per day over a time period of 14-18 d [111] [113] [117]. Besides, also norgestomet, a gestagen used in goats for estrus synchronization [121]. Melengestrol acetate is used in postpartum beef cattle and heifers in combination with prostaglandin derivatives to synchronize the estrus [134] [135].

GnRH and its analogues

GnRH has a half-life of approximately 1 h in the blood plasma and is released physiologically in a pulsatile manner [136]. GnRH analogues, or so called "superagonists" with a higher affinity to GnRH receptors and a higher stability in vivo were developed [130] [137]. GnRH analogues are widely applied in both human and veterinary medicine for a variety of indications (*Table 1-1*). Gonadorelin [6-D-Phe] is one analogue, reported to have a higher affinity to GnRH receptors [130]. Other related substances are buserelin, goserelin and triptorelin, which are indicated in human medicine for different cancer types, endometriosis and infertility [35]. Recently, goserelin was also tested as add-on to conventional medication to protect the ovarian function against aggressive chemotherapy in breast cancer in pre-menopausal females [138] [139].

Table 1-1: Structural changes in amino acid sequence of GnRH analogues compared to GnRH

Substance	Amino acid sequence	
GnRH	p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	[133]
G [6-D-Phe]	p-Glu-His-Trp-Ser-Tyr-**D-Phe**-Leu-Arg-Pro-Gly-NH_2	[10]
Triptorelin	p-Glu-His-Trp-Ser-Tyr-**D-Trp**-Leu-Arg-Pro-Gly-NH_2	[136]
Buserelin	p-Glu-His-Trp-Ser-Tyr-**D-Ser (tBu)**-Leu-Arg-**Pro**(**NHEt**)	[35]
Goserelin	p-Glu-His-Trp-Ser-Tyr-**D-Ser (tBu)**-Leu-Arg-Pro(NEt)-**AzaGly**-NH_2	[140]
Peforelin	pGlu-His-Trp-Ser-**His-Asp-Trp-Lys**-Pro-Gly-NH_2	[141]
Leuprolide	p-Glu-His-Trp-Ser-Tyr-**D-Leu**-Leu-Arg-**Pro(NHEt)**	[142]

In veterinary medicine, G [6-D-Phe] (Gonavet® Veyx, Veyx Pharma GmbH), goserelin (Fertilan®, Bremer Pharma) and peforelin (Maprelin®, Veyx Pharma GmbH) formulated in immediate releasing formulations are commonly used for a successful induction of ovulation in sows and gilts [123] [143] [144]. According to the professional information of Gonavet® Veyx, G [6-D-Phe] should be administered approx. 80 h respectively after the administration of PMSG or peforelin to gilts [10]. In sows, the new estrus should be induced with the application of PMSG 24 h after weaning. Depending on the duration of the suckling period, Gonavet®Veyx should be administered 56 to 80 h after peforelin or PMSG. Artificial insemination should be performed twice, 24-26 and 40-42 h after application of G [6-D-Phe] [10]. Furthermore, the application of GnRH analogues in a sustained release delivery system was successfully introduced into the market with the implant Suprelorin® (Virbac) leading to a chemical castration in male mature dogs as alternative to surgical castration [14] [145].

Beta-human chorionic gonadotropin (β-hCG) and equine chorionic gonadotrophin (eCG)/ pregnant mare serum gonadotropin (PMSG)

β-hCG, another gonadotropin, can usually be found in blood plasma of pregnant females, responsible for the sustaining of pregnancy [146]. ECG, also called PMSG, is obtained from blood of pregnant mares. It is the active compound in Fertipig® (Ceva Santé Animale) and Suigonan® (MSD Animal Health) in combination with hCG [147] [148]. Intergonan® (MSD Animal Health) contains only eCG [149]. For induction of estrus, hCG is used successfully for treatment of weaned sows [143]. In 1982, von Kaufmann and Holtz already reported the successful treatment of prepubertal gilts coming into estrus by a single injection of hCG and PMSG [150].

Prostaglandin F2-α (PGF2-α)

PGF2-α, a prostaglandin deriving from the metabolism of arachidonic acid, is reported to have positive effects on artificial insemination in swine [151] [152]. Due to local effects on the myometrium, they are able to enhance the success of artificial insemination (AI) by induction of local uterus contractions with positive effects on synchronizing the ovulation [122] [153].

1.5 Problems arising from Estrus Synchronization

Meat and milk consumption is very high in western countries and is continuously increasing in developing countries which makes an effective livestock breeding necessary [154]. Estrus synchronization by gestagens is one element to achieve this. Orlando et al. reviewed problems going hand in hand with high doses of gestagens getting into the ground water, as side products of large agricultural facilities [126]. Collecting samples from a cattle runoff in the US revealed high amounts of gestagens and their metabolites in the manure and dry feedlot surface soil. The authors assume, that excretes of large agricultural holdings play an important role in the environmental entry of steroid hormones reaching levels over the predicted no-effect concentration [127]. High steroid concentrations of up to 375 ng/l were found in the melt water in the neighborhood of large agricultural holdings in Wisconsin [124]. Possible harmful effects on reproduction are reported for the fathead minnow and zebrafish, as well as for alligators and frogs [124] [128] [155]–[158].

1.6 Aim of the Thesis

The present thesis was performed to develop an environmentally friendly alternative for estrus synchronization in swine. Treatment with gestagens is the current gold standard for estrus synchronization. But substantial entry into the environment has been shown and it is likely that gestagens negatively influence reproduction of water organisms [126] [128] [129]. This work should focus on the development of a delivery system for the decapeptide G [6-D-Phe], which is able to guarantee a sustained release over two weeks. The delivery of the peptide via triglyceride microparticles seems to be the best choice for our purpose based on costs and tolerability. The system should guarantee a drug release in an effective dose over a time period of 15 days, followed by a more or less immediate drop to allow the simultaneous onset of a new estrus in all treated animals for a fixed-time insemination program.

Chapter Two describes the first tests to establish a spray congealing process focusing on the impact of melt viscosities and apparatus settings on particle size and yield. Furthermore, lipid microparticles are evaluated for polymorphism.

In ***Chapter Three*** the incorporation of model substances, like the amino acid tryptophan and the dipeptide aspartame with focus on yield and drug content is studied. First release experiments from pure triglyceride matrices and in combination with emulsifiers and sugars were performed.

Chapter Four focuses on the optimization of the spray congealing process with respect to yield and encapsulation efficiency**.** Material distribution during the spray congealing process and evaluation of possible sources for high material loss are studied. Possibilities for the optimization of the process are discussed.

In ***Chapter Five*** G [6-D-Phe]-loaded microparticles obtained via the spray congealing process were tested. Factors influencing the release behavior, like surface morphology, water uptake upon incubation and polymorphic behavior are investigated. The wettability of lipid matrices is determined, also in combination with surfactants, by contact angle measurements using the sessile-drop method. The addition of different emulsifier types at various concentrations is screened to achieve a release over two weeks.

Chapter Six shows the results of a particle and drug distribution study in carcasses after application in the lateral neck muscles.

Chapter Seven summarizes the results of the two performed pre-clinical studies. The first study focuses on the dose finding of a future delivery system by application of two different G [6-D-Phe] doses via two different formulations. The second study evaluates four different formulations using only the low dose of the first study. Treated swine are investigated concerning biocompatibility of the tested formulations and possible adverse reactions. Most important is the effect on cycle blockage and onset of follicular growth, as well as determination of ovulation. Furthermore, the side effect of ovulatory cysts is discussed.

Chapter Eight describes the results of a long-term stability study performed over a 12-month time at 2-8, 25 and 40 °C. Microparticles are investigated concerning drug content, peptide stability, particle morphology and size, suspension in the reconstitution medium and release profile. Possible instabilities arising from the storage are explained.

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2 DEVELOPMENT OF A SPRAY CONGEALING PROCESS FOR TRIGLYCERIDE MICROPARTICLES

2.1 Abstract

A spray congealing process for the manufacturing of solid triglyceride microparticles was to be developed. The instrument settings, like product flow, spray pressure and spray flow were evaluated with respect to their effect on particle size and yield. Spray flow was identified as the most important factor with a moderate effect on yield and mean particle size. An improvement of yield and size reduction was obtained with higher spray flow. Particles of approximately 50 µm were obtained at 20 to 50 % yield. Upon spray congealing, the triglycerides crystallized in the metastable α-polymorph. Transition into the thermodynamically stable β-form could be achieved by an additional tempering step.

2.2 Introduction

Microparticles are widely used delivery systems for both small molecules and biopharmaceutic drugs [1]–[3]. Polymer-based microparticles, especially based on lipids are of particular interest [4]–[9]. Different preparation techniques exist for lipid microparticles mainly based on emulsion or spray techniques [10] [11]. Molten lipids can be emulsified into an aqueous phase at higher temperatures forming solid, spherical microparticles upon cooling [11]. The solvent evaporation technique is based on an organic solution of lipid dispersed in an aqueous phase followed by solvent removal resulting in the formation of particles [12] [13]. The disadvantages of these techniques are problems arising from residual organic solvents and a high loss of water soluble drug due to partitioning into the aqueous phase [13]. Alternatively, a solution of lipid in organic solvent can be spray dried [14].

Spray congealing, in literature also referred to as spray cooling or spray chilling, is a process without the use of organic solvents characterized by the atomization of a lipid melt [15] [16]. Typically, the small droplets are formed by the use of internal mixing two-fluid nozzles using compressed air or nitrogen as spray gas [17] [18]. Depending on the lipid´s melting/solidification point, the droplets are sprayed into a chamber, which is kept at room temperature [19] or below [7]. In some cases a subsequent freeze-drying step is applied [7]. Alternatively, atomization of the lipid melt is performed with a Venturi effect-based wide pneumatic nozzle (WPN) [20]. Furthermore, by the use of an ultrasound-based system, molten lipid can be split up into small droplets upon contact with the sonotrode followed by solidification at room temperature [21]–[25]. In addition, centrifugal wheel [26] [27] and rotating disc atomizers [28] are described.

In the first steps of development of lipid-based microparticles for sustained release of G [6-D-Phe] by spray congealing, the influence of different process parameters in a two-fluid nozzle-based process was evaluated. Additionally, the use of different triglycerides was assessed. The results with placebo particles should form the base for the subsequent studies with model substances and the peptide drug.

2.3 Materials and Methods

2.3.1 Materials

Dynasan 116 (tripalmitin, D116), Dynasan 118 (tristearin, D118) and Dynasan 120 (triarachidin, D120) were kindly provided from Cremer Oleo (Witten, Germany). The thickening agent aluminum stearate was supplied from Fluka (Fluka, Sigma-Aldrich, Taufkirchen, Germany). Ultrapure deionized water with a conductivity of less than 0.055 µS/cm (Milli-Q Water systems, Millipore, MA, USA) was used throughout all experiments. Polysorbate 20 (PS 20) was purchased from Merck KGaA (Darmstadt, Germany).

2.3.2 Preparation of triglyceride microparticles by spray congealing

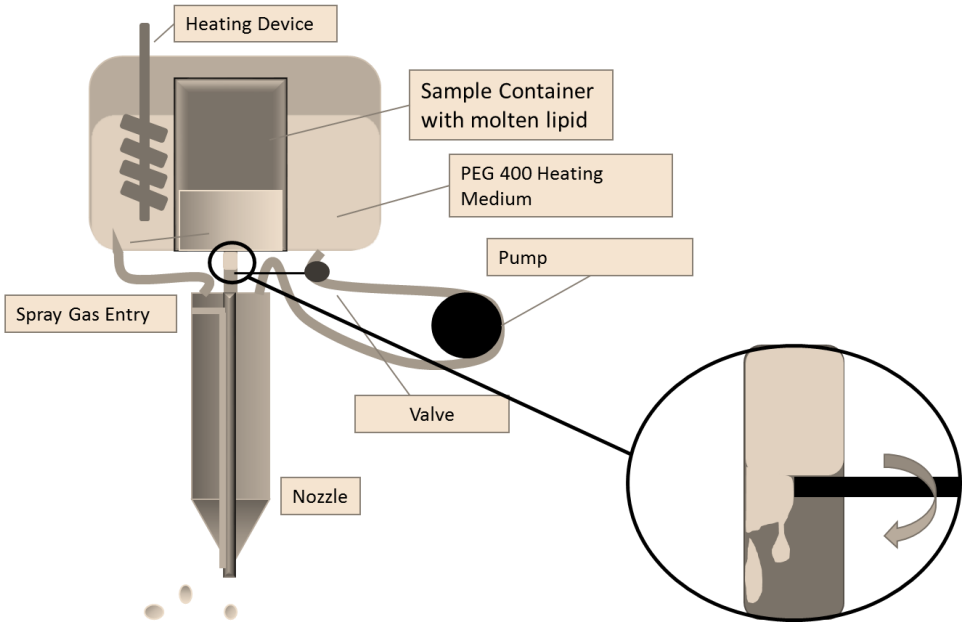

Figure 2-1: Schematic depiction of the spray congealing equipment additionally installed on top of a B-290 Mini spray dryer

12 g of triglyceride was melted on a hot plate and transferred into the sample container of a B-290 Mini spray dryer with an additional spray chilling setup (Büchi, Flawil, Switzerland) as visualized in *Figure 2-1* . The whole system was pre-conditioned for 1.5 hours prior to particle production (sample container 90 °C for D116 and 110 °C for D118 and D120, spray tower temperature 15 °C). The original sample container with a plane bottom by Büchi was used for production of microparticles. Additionally, a custom-made sample container with a coned bottom (tilt 13 °) to minimize material loss was tested for flow-out studies. The coned bottom container was made by the LMU fine mechanics department at the same dimensions as the original flat bottom container. The connector passage between sample container and nozzle was heated to 70 °C with a self-made resistance wire-based heating. The spray tower was fed with

pre-cooled air via an additional dehumidifier Deltatherm® LT (Deltatherm® Hirmer GmbH, Much, Germany). The lipid melt was sprayed using nitrogen at a pressure between 4 and 6 bar. A high-performance cyclone was used to separate the microparticles. Resulting microparticles were stored at 2-8 °C until further analysis was performed.

2.3.3 Determination of yield

Yield was determined by weighing the product container before and after microparticle production. Only microparticles collected in the product container were taken into account for yield calculation.

2.3.4 Evaluation of the product flow using different sample containers

The product flow into the nozzle depends on the temperature as well as viscosity of the used melt. D116 was used as lipid material. The valve was opened and the resulting product flow within 20 s collected and the flow calculated in ml/min. Both the flat bottom sample container as well as the coned bottom container were evaluated.

2.3.5 Determination of particle size

Approximately 20 mg of microparticles were suspended in 10 ml of an aqueous solution of 0.02 % PS 20. The sample was analyzed in the laser diffraction system Horiba Partica LA-950 (Horiba, Kyoto, Japan) while stirring.

2.3.6 Investigations on polymorphic behavior using differential scanning calorimetry (DSC)

Microparticles were analyzed using a Mettler DSC 821e (Mettler Toledo, Columbus, OH, USA). 5-15 mg were weighed into aluminum crucibles and analyzed at a heating and cooling rate of 10 K/min from 0 to 110 °C in two cycles against an empty crucible as reference. To determine polymorphic behavior upon tempering, particles were stored in glass vials at 2-8, 25 and 45 °C for 24, 48 and 72 h, respectively.

2.3.7 Characterization of particle morphology using scanning electron microscopy (SEM)

Surface characteristics of lipid microparticles were assessed by SEM using a Jeol JSM 6500-F SEM (Jeol, Tokyo, Japan) without additional coating at 150, 900 and 1500-fold magnification, after attaching on aluminum sample holders with double adhesive tape (Plano, Wetzlar, Germany).

2.4 Results and Discussion

2.4.1 Evaluation of the product flow into the nozzle using different sample containers

As the product flow into the nozzle depends on the used melt and temperature as well as valve opening, the product flow in ml/min was determined experimentally for D116 at 90 °C for both the flat bottom container, as well as the coned bottom container. A turnable metal screw controlled the flow out of the container into the nozzle. *Figure 2-2* shows the product flow depending on the valve opening out of the sample container.

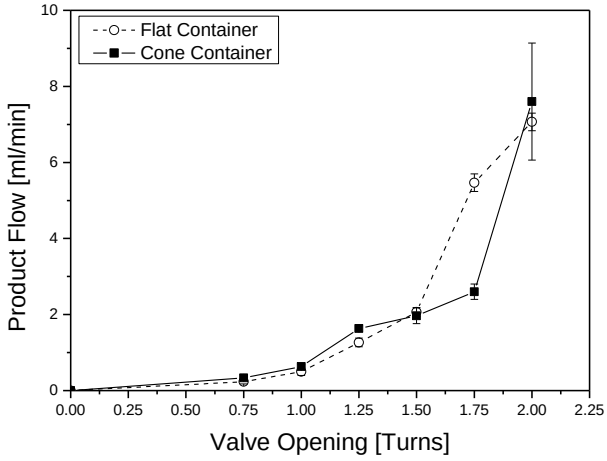

Figure 2-2: Product flow out of the sample container for D116 at 90 °C (mean and SD, n = 3)

Flowing of the melt started at 0.75 turns with 0.23 ml/min (flat bottom) and 0.33 ml/min (coned bottom) respectively. In general, both container configurations showed a similar flow rate. At a valve opening of 1.75 turns, the flat container showed a deviating product flow of 5.5 ml/min compared to the coned bottom container with 2.6 ml/min. Increasing the valve opening to 2.0 turns, maximum flow rates of 7.1 ml/min (flat bottom container) and 7.6 ml/min (coned bottom container) were reached. Thus, the flow from the sample container was only minimally affected of the container shape. The product flow increased almost exponentially with turning of the valve.

2.4.2 **Preparation of drug-free lipid microparticles by spray congealing**

Subsequently, microparticles were prepared by spray congealing using the triglycerides D116, D118 and D120. *Table 2-1* summarizes the tested settings, as well as yields and particle sizes. The attempt to generate larger particles by addition of 1 % aluminum stearate via increasing the viscosity led to valve blockages and was not further pursued.

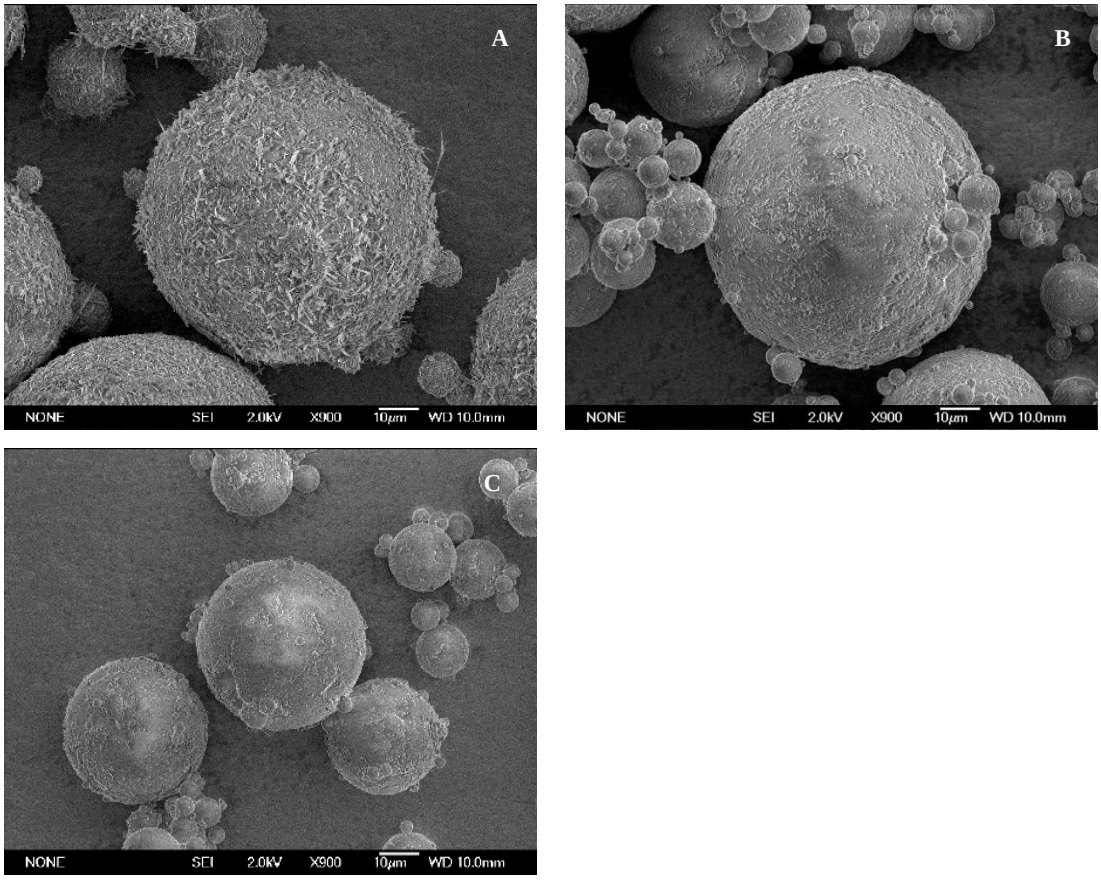

Figure 2-3: SEM images of triglyceride microparticles based on D116 (A), D118 (B) and D120 (C)

Particles showed a spherical shape and the use of D116 led to a flaky surface appearance whereas D118 and D120 particles obtained a very smooth surface (*Figure 2-3*).

Spray pressure is reported to be a factor of major influence on particle characteristics in spray congealing [29] [30]. Yield was determined as 39.0 % (4 bar), 43.7 % (5 bar) and 42.9 % (6 bar). Particle size did not change upon varying the spray pressure. Thus, spray pressure did not have major influence on product characteristics, as the actual spray gas volume (or spray flow) getting into the nozzle was regulated separately.

Another important parameter is the lipid feed rate. The feed rate was varied in a range between 0.75 and 2.0 valve turns, corresponding to an effective product flow rate between 0.23 and 7.1 ml/min. With application of a product flow above 3 ml/min corresponding to 1.75 turns, an

irregular spray cone and a high material loss at the spray tower wall could be monitored, which reasoned slightly lower yields.

Table 2-1: Overview of placebo microparticle batches produced with varied apparatus settings.

TG	Spray pressure [bar]	Temp. [°C]	Spray flow [Normlitres/h]	Valve opening [Turns]	Yield [%]	Mean particle size [µm]
D116	4	90	473	0.75	39.0	40.4 (± 12.0)
D116	6	90	473	0.75	42.9	66.7 (± 28.8)
D116	5	90	473	0.75	43.7	40.2 (± 15.5)
D116	5	90	473	0.75	43.7	40.2 (± 15.5)
D116	5	90	473	1	53.2	45.1 (± 14.8)
D116	5	90	473	1.125	51.3	42.9 (± 8.0)
D116	5	90	473	1.25	46.0	48.5 (± 10.9)
D116	5	90	473	1.5	43.7	40.0 (± 6.0)
D116	5	90	473	1.75	37.8	40.8 (± 5.5)
D116	5	90	473	2	38.3	47.4 (± 2.6)
D116	5	90	301	1	23.3	43.3 (± 3.4)
D116	5	90	357	1	21.1	45.6 (± 7.0)
D116	5	90	473	1	53.2	28.9 (± 16.3)
D116	4	90	473	1	42.2	49.3 (± 12.1)
D118	4	110	473	1	34.6	35.5 (± 14.1)

The spray flow, a factor with high influence on particle size, was assessed between 246 and 473 l/h [31]. At a spray flow below 301 l/h spray congealing was not possible because of inadequate droplet formation. At 301 and 357 l/h, yields of 22 % with particle sizes of approximately 45 µm could be achieved. The increase of spray flow to 473 l/h resulted in the highest observed yield of about 50 % and the size of the collected particles was reduced to 28.9 µm, indicating a more effective atomization and the formation of smaller droplets. Consequently, the spray congealing process was rather robust over a wide range of process settings. The strongest influence regarding yield and particle size was observed for the variation of spray flow.

2.4.3 **Evaluation of polymorphic behavior**

The lipid modification of the microparticles plays a crucial role [7] [13]. After the spray congealing process, D116 microparticles were present in the α-polymorph as indicated by the melting endotherm at 46 °C in the first and the second heating scan (*Figure 2-4 A*) [32]. This hexagonal modification with only minor and unspecific chain-chain-interactions is thermodynamically unstable upon storage [7] [33]–[35]. According to Lutton, it is only stable for several weeks at room temperature [36]. Upon further heating, the exothermic recrystallization to the higher melting β´-polymorph occurred [32]. This orthorhombic-arrayed polymorph is also metastable and not always detectable in DSC [33] [37]. Further energy led to the formation of the most stable β-form, which should be favored for reasons of long-term storage. In this triclinic modification, the carbon chains are parallel to each other with a high potential of interaction [33] [38]. The observed polymorphic behavior went in accordance with the behavior of spray congealed microparticles manufactured with different natural or synthetic lipids as well as lipid implants and solid lipid nanoparticles [6] [19] [39]–[42].

Tempering at 2-8 °C and room temperature did not lead to changes in the polymorph. Incubation at slightly increased temperatures induced a transition of the microparticles to the β-polymorph with only one single melting endotherm at 65 °C already after 24 h (*Figure 2-4 E*). Storage at 45 °C, which is slightly over the distinct melting point of the α-form, induces crystal lattice formation to the more densely packed β-form. This tempering of the lipid matrix is a suitable tool to induce formation in the thermodynamically stable β-modification for higher stability of the drug delivery systems [32] [39] [43].

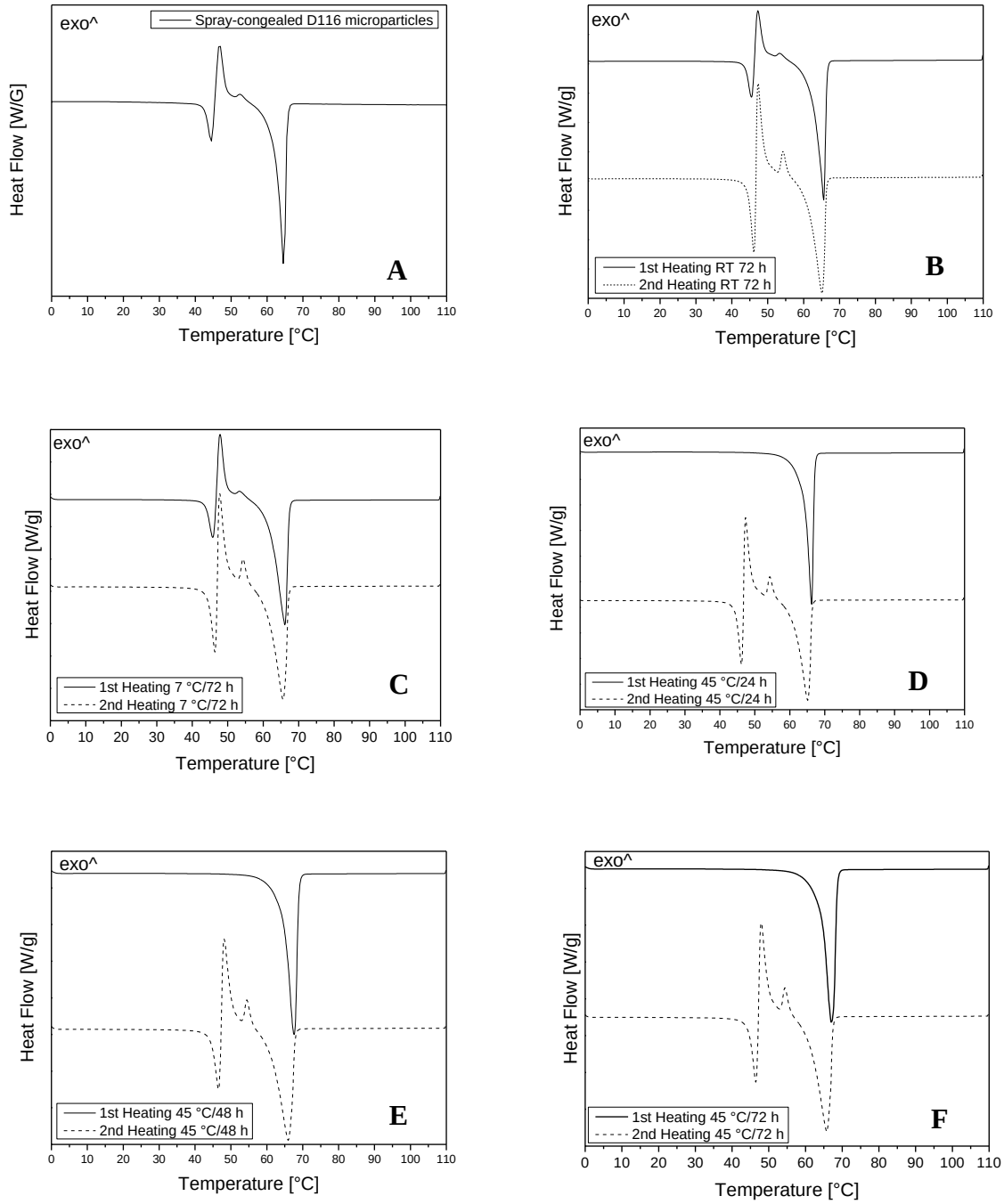

Figure 2-4: Polymorphic behavior of freshly prepared D116 microparticles (A) and after 72 h stored at room temperature (B), after 72 h at 7 °C (C), after 24 h at 45 °C (C), after 48 h at 45 °C (D) and 72 h at 45 °C (E). Representative heating scans are depicted of the first and second heating each. Curves are displaced along the ordinate for better visualization

2.5 Conclusion

It was possible to produce triglyceride microparticles by the means of spray congealing. The choice of sample container had no pronounced influence on the product outflow, but could possibly minimize material loss inside the container. The evaluation of process parameters showed that the process was rather robust against their variation. The apparatus settings spray pressure, product flow and spray flow are reported to have the highest influence, which could only be partly confirmed in our experiment. Low product and spray flow led to insufficient atomization which caused a high product loss in the spray dryer. The spray flow was evaluated to be the most influencing factor on particle size and yield. DSC measurements showed that microparticles were crystalline in the α-polymorph directly after production which did not change upon storage at 2-8 and 25 °C over 72 h. Storage at 45 °C induced the transformation to the thermodynamically stable and desirable β-form after 24 h.

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3 INCORPORATION OF HYDROPHILIC MODEL SUBSTANCES INTO SPRAY CONGEALED SOLID LIPID MICROPARTICLES

Parts of this chapter are intended for publication

Data of amino acid-loaded microparticles was collected and evaluated by **Katrin Mayer** during her master thesis entitled "***Development and in vitro evaluation of parenteral lipid-based depot formulations" (2014)***

3.1 Abstract

Two different hydrophilic model substances, the amino acid tryptophan and the dipeptide aspartame, were incorporated into spray congealed lipid microparticles as a surrogate for the decapeptide G [6-D-Phe]. Aspartame was lyophilized to mimic the properties of the freeze-dried drug. The spray congealing process as well as the release behavior from the obtained microparticles were evaluated using different triglycerides, also in combination with release modifying additives, such as emulsifiers and sugars. Additionally, the influence of lipases on drug liberation from the particles was investigated. The addition of emulsifying agents led to better spray performance. A slow and incomplete release resulted for pure triglyceride microparticles. Using tryptophan as model substance, a release for 20 d (D116 + 5 % GMS) with an initial burst of less than 20 % could be achieved. The formulation of D118 + 10 % GMS resulted in a sustained release for 24 d, whereas the initially released drug could be kept below 25 %. The addition of lipase to the release medium led to matrix changes visible in SEM images also leading to a faster release when GMS concentrations higher than 5 % were used. The released aspartame was different compared to tryptophan and could be controlled over approx. 10 d by the addition of Span 40 and Span 85.

3.2 Introduction

The incorporation of drugs into biodegradable matrices for parenteral application is an important field within pharmaceutical research [1]–[3]. Lipid drug delivery systems, like microparticles [4], solid lipid nanoparticles [5] and implants [6] [7] are suitable tools to prolong and control the delivery of drugs. Hydrophilic proteins and peptides, like somatostatin [8], insulin [4], thymocartin (TP-4) [9], ovalbumin [6], bovine serum albumin [10] and lysozyme [11] have successfully been loaded into lipid microspheres. The spray congealing process, technically evaluated in the previous chapter, has been reported to be a suitable technique for the production of lipid microparticles [4] [10] [12]. The absence of organic solvents, high yields and encapsulation efficiencies are benefits arising from this technique compared to other emulsion-based methods [4] [9] [13].

Widely used excipients for lipid matrices are for example solid triglycerides of different chain length [14]–[17] and waxes [18]. Additionally, the application of composed fats, consisting of mono-, di- and triglycerides in variable amounts, like Compritol® 888 ATO [19] [20] or Gelucire® -qualities, combining glycerides and hydrophilic polyethylene glycols [21] [22] are common.

Release kinetics achieved with lipid delivery systems range from several minutes up to months [23] [24]. With the use of spray congealed microparticles consisting of Gelucire® 50/13 loaded with diclofenac a complete dissolution below 10 minutes was achieved [24]. A sustained release was reported in case of implants consisting of high-melting triglycerides for over three months [14] [25]. The modification with partial glycerides improves surface wettability, as well as increased matrix swelling which enables drug dissolution [22] [26]. The addition of pore-formers, like polyethylene glycol [27] [28], sucrose [14] or trehalose [29], induces the formation of a connected pore network above a certain concentration. Test conditions, such as temperature [23] [30], addition of emulsifiers to the medium [31] [32] and the presence of lipases [33] [34] are important variables influencing release rates.

As a delivery system for release of G [6-D-Phe] over a 15 days' time frame should be developed, lipid microparticles produced by spray congealing were selected. The following study focused on the incorporation of model substances as a surrogate for the peptide drug. Surfactants, like monostearates, sorbitan esters and fat alcohols, as well as sugars like sucrose, were added to influence release rates and to adjust the formulation to the required release profile. Furthermore, suitable release conditions with or without the presence of lipase and a

suitable release method were evaluated based on previously reported dialysis membrane models for oily suspensions and other colloidal systems [35] [36].

3.3 Materials and Methods

3.3.1 **Materials**

The peptide drug aspartame (ASP) was obtained from Sandoz AG, Nuremberg, Germany. The amino acid tryptophan (TRP) was purchased from Sigma-Aldrich (Taufkirchen, Germany). Triglycerides Dynasan 112 (trilaurin, D112), Dynasan 114 (trimyristin, D114), Dynasan 116 (tripalmitin, D116) and Dynasan 118 (tristearin, D118) were kindly provided from Cremer Oleo (Witten, Germany). Glycerol monostearate (GMS) with a monoester content of 40-55 % was purchased from Caelo (Caesar & Loretz, Hilden Germany). Cetylstearyl alcohol (CSA, Lanette® O) and Poloxamer 188 (Lutrol® F 68) were purchased from BASF (Ludwigshafen, Germany). Span 40, 65 and 85 were kindly provided from Croda (Nettetal, Germany). PEG-24-glyceryl stearate (PEG-24 GS), tradename Cutina® E 24, was a kind gift from BASF (Ludwigshafen, Germany). Colloidal silicium dioxide (Aerosil®) was purchased from Evonik Industries (Essen, Germany). Sucrose was purchased from Südzucker (Mannheim, Germany). Ultrapure deionized water with a conductivity of less than 0.055 µS/cm (Milli-Q Water systems, Millipore, MA, USA) was used for preparation of buffers in this study. Visking dialysis tubings with a diameter of 1.6 mm and a cut-off of 12-14 kDa for performance of release studies were sourced from Serva Electrophoresis GmbH (Heidelberg, Germany). Centrifuge tubes were purchased from VWR International GmbH (Darmstadt, Germany). Lipase isolated from *rhizopus oryzae* was purchased from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals were of analytical grade.

3.3.2 Lyophilization of ASP

ASP was bulk lyophilized in a concentration of 0.5 % (w/v) on a tray without addition of further stabilizers using an Epsilon 2-6 D freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and the following cycle:

Step	Ramp [°C/min]	Temperature [°C]	Pressure [Torr]	Hold time [min]
Freezing	1	-50	750	60
Annealing	1	-20	750	240
Freezing	1	-50	750	60
Primary drying	0.5	-20	0.0675	2100
Secondary drying	0.1	30	0.0675	720

3.3.3 Cryogenic ball-milling of ASP

Prior to the milling step, ASP was blended with 0.5 % (w/w) Aerosil® to enable particle separation during the milling process. The cryogenic milling was conducted with the Cryomill (Retsch® Technologies GmbH, Haan, Germany) using a stainless steel beaker and two milling balls with a diameter of 10 mm. A precooling time of 10 min at 5 Hz was applied, followed by two milling cycles of 2 min each at a frequency of 20 Hz. The milled mixture was aliquoted and stored at 2-8 °C until spray congealing was performed.

3.3.4 Mortar grinding of TRP for suspension in the lipid melt

Approximately 7 g of lipid components consisting of triglycerides (D116, D118) and the additive (GMS or sucrose) were ground together with 130 mg (1 %) or 260 mg (2 %) TRP manually for 30 min using mortar and pestle without heating. The remainder of the lipid components (total batch volume 13 g) were added and the mixture heated up on a water bath to 90 °C. The mixture was homogenized for 1 min using an Ultraturrax T-10 basic (IKA Laboratory Technology, Staufen, Germany).

3.3.5 Preparation of model substance-loaded microparticles by spray congealing

For preparation of TRP-loaded microparticles, the lipid dispersion was filled in the sample container of the B-290 Mini spray dryer with additional spray chilling setup (Büchi, Flawil, Switzerland), which was pre-conditioned for 1.5 h prior to the production step (sample container 90-110 °C, spray tower temperature 15 °C). The additional equipment consisted of a sample container, surrounded by a bath filled with polyethylene glycol (PEG) 400, also heating the nozzle. The connection between nozzle and sample container was heated externally by a

resistance wire-based heating, generating a temperature of approximately 70 °C. The spray tower was fed with pre-cooled air produced by an additional dehumidifier Deltatherm® LT (Deltatherm® Hirmer GmbH, Much, Germany). The lipid melt was atomized using nitrogen as spray gas at a pressure of 6 bar and a modified nozzle with an inner diameter of 2.5 mm.

ASP-loaded microparticles were prepared by melting lipid components and adding the milled ASP under high shear mixing using an Ultraturrax for 2 min. The suspension was fed into the sample container of the spray dryer and atomized in analogy to amino acid-loaded microparticles. The theoretical drug load was 1.67 %.

3.3.6 Determination of particle size

Approximately 20 mg of microparticles were suspended in 10 ml of an aqueous solution of 0.02 % PS 20 prior to analysis. The dispersion was analyzed using the laser diffraction system Horiba Partica LA-950 (Horiba, Kyoto, Japan) while stirring.

3.3.7 Characterization of particle morphology using scanning electron microscopy (SEM)

Particle characteristics of lipid microparticles were assessed using a Jeol JSM-6500 F SEM (Jeol, Tokyo, Japan) without additional coating of the particles after attaching on aluminum sample holders with double adhesive tape (Plano, Wetzlar, Germany). Cross sections of particles were prepared by cutting the particles with the use of a surgical scalpel by hand.

3.3.8 Determination of encapsulation efficiency

30 mg of TRP-loaded microparticles were dissolved in 2.0 ml of methylene chloride and 2.0 ml of PBS pH 7.4 were added. Prior to analysis of the water phase, the two-phase system was shaken vigorously for 5 min and let stand afterwards for another 30 min. The aqueous phase was analyzed using the NanoDrop 2000 spectrophotometer at 280 nm [37] (Thermo Fisher Scientific, Waltham, MA, USA). Each batch was extracted in triplicate.

50 mg of ASP-loaded microparticles were dissolved in 0.5 ml of methylene chloride and the same amount of highly purified water was added. The mixture was incubated for 18 h on the GFL 3031 horizontal shaking incubator (Gesellschaft für Labortechnik, Burgwedel, Germany) at a temperature of 39 °C and 60 rpm. All microparticular batches were extracted in triplicate and measured using an Agilent UV-Vis spectrophotometer 8453 (Agilent Technologies, Santa Clara, CA, USA) at 258 nm [38].

3.3.9 Development of a suitable release model and evaluation of TRP and ASP release behavior

For release studies of TRP-loaded microparticles 100 mg with 2 % and 200 mg with 1 % drug load were weighed into dialysis tubings. After addition of 0.5 ml (100 mg microparticles) or 1.0 ml (200 mg microparticles) PBS pH 7.4 with 0.05 % NaN_3 and 0.02 % Poloxamer 188, the tubings were closed at both ends and placed in a 15-ml centrifuge tube, release medium was added to a total volume of 5.0 ml. Samples were incubated on a horizontal shaker at 39 °C and 60 rpm. Additionally, the same experiment was carried out with addition of lipase from *rhizopus oryzae* in a concentration of 1.0 U/mg microparticles (corresponding to a concentration of 20 or 40 U/ml, respectively) in the dialysis membrane. All release studies were performed in triplicate. Samples of 100 µl per time point were taken, replaced with fresh release medium and analyzed using a NanoDrop 2000 spectrophotometer at 280 nm. 100 mg of microparticles without amino acid were used as control. A solution of 1 mg/ml TRP in PBS was filled into the membrane as positive control to proof the permeability through the membrane.

100 mg of ASP-loaded microparticles were filled in dialysis tubings and 2 ml of PBS buffer pH 7.4 were added, tubings were closed with threads at both ends and placed in 15-ml centrifuge tubes. 3 ml of release medium was added and samples were treated according to TRP-loaded microparticles. Each sample was performed and analyzed in triplicate. Samples of 100 µl were taken for measurement of released drug using an Agilent spectrophotometer 8453 at a wavelength of 258 nm.

3.4 Results and Discussion

3.4.1 Preparation and characterization of model drug-loaded lipid microparticles by spray congealing

All TRP-loaded batches could be sprayed successfully resulting in different yield and particle size (*Table 3-1*). An increased spray flow of 473 l/h compared to 414 l/h did not influence the microparticle size. Both concentrations of TRP could be sprayed without affecting yield. Microparticles were obtained at yields in a range between 16.8 (D116) and 29.0 % (D118) with particle sizes between 30.2 and 53.9 µm. Addition of GMS resulted in slightly larger particles. With addition of GMS, yield was not affected for D118-based microparticles, but slightly increased for the formulations based on D116. The emulsifier GMS is reported to enable a more effective encapsulation, which may result in larger particles [39].

Table 3-1: Overview of TRP-loaded microparticle batches sprayed with different apparatus settings and with addition of GMS and sucrose

Model drug	Drug Load [%] (w/w)	Spray Flow [l/h]	TG	Excipient	[%]	Yield [%]	Mean Particle Size [µm]
TRP	2	414	D118	-	-	29.0	30.2
TRP	1	414	D118	GMS	5	22.9	49.7
TRP	1	414	D118	GMS	10	22.0	42.4
TRP	2	473	D116	-	-	16.8	41.8
TRP	2	473	D116	Sucrose	1	25.5	39.0
TRP	2	473	D116	GMS	5	28.8	50.6
TRP	2	473	D116	GMS	10	25.9	53.9

The addition of sucrose as pore former resulted in an irregular spray cone and valve blockages and was therefore not further investigated. *Figure 3-1* depicts SEM images of triglyceride-based microparticles loaded with TRP. Microparticles showed an overall smooth surface with small crystals. Cross-sections confirmed a dense, compact matrix without noticeable cavities. The incorporated TRP could not be visualized in contrast to other researchers [25] [40].

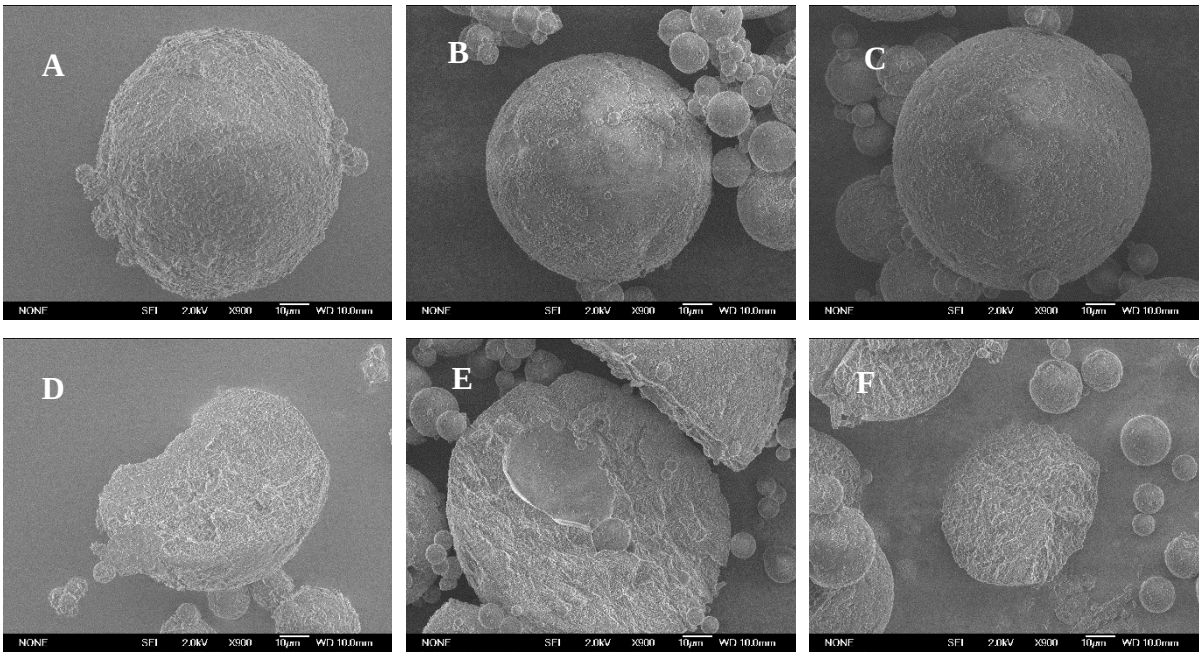

Figure 3-1: SEM images of TRP-loaded microparticles (D116: A, D, D118 + 1 % sucrose: B, E, D118 + 5 % GMS: C, F)

ASP lyophilisate could not be incorporated into spray congealed microparticles. Upon homogenization using Ultraturrax, the viscosity of the melt increased substantially and could not be sprayed. For this reason, all other batches were prepared using cryomilled ASP. These formulations could be spray congealed successfully (*Table 3-2*).

High yield of approx. 50 % were obtained for D116 with addition of 5 and 15 % CSA achieving values of 50 % usable product. Span 65 and 85 caused a decrease in yield. With addition of the low melting triglyceride D112 only 10 % product was obtained. This effect was less pronounced when combinations of D112 with GMS were used. The highest product yield was achieved with addition of 20 % PEG-24 GS. Particle size was exemplarily measured for triglyceride particles with addition of two intermediate and one very lipophilic emulsifiers. A slightly higher particle size was observed with the use of emulsifiers with lowest HLB values (Span 85, HLB 1.8 [41]) as well as the production with pure triglycerides. Using emulsifiers with higher HLB values (Span 40 and 65), particle size slightly decreased indicating a more ineffective drug encapsulation [42] [43].

Table 3-2: Overview of 1.67 % ASP-loaded microparticle batches (s = solid, l = liquid, ss = semi-solid); Three emulsifies with different HLB values were investigated concerning particle size exemplarily (data shown mean and SD, n = 3)

Triglyceride	Excipient	[%]	Yield [%]	Mean particle size [µm]
D116	GMS (s)	5	37.0	-
D116	GMS (s)	10	51.9	-
D116	GMS (s)	15 %	38.1	-
D116	CSA (s)	5	50.5	-
D116	CSA (s)	15	50.3	-
D116	Span 40 (s)	5	44.3	42.7 (± 1.6)
D116	Span 40 (s)	10	48.1	33.3 (± 1.1)
D116	Span 40 (s)	20	43.7	-
D116	Span 65 (s)	5	28.9	25.0 (± 1.6)
D116	Span 65 (s)	10	43.9	32.4 (± 2.3)
D116	Span 65 (s)	20	42.2	21.0 (± 1.9)
D116	Span 85 (l)	2	7.6	84.6 (± 5.0)
D116	Span 85 (l)	5	23.9	62.5 (± 12.9)
D116	Span 85 (l)	10	20.4	65.8 (± 27.7)
D116	PEG-24 GS (ss)	0.7	26.3	-
D116	PEG-24 GS (ss)	10	48.8	-
D116	PEG-24 GS (ss)	20 %	74.3	-
D116	D112 (s)	10	10.4	69.6 (± 3.2)
D116	GMS/D112 (s/s)	10/5	40.4	-
D116	GMS/D112 (s/s)	10/10	42.9	-
D114	GMS/D112 (s/s)	10/5	39.3	-
D114	GMS/D112 (s/s)	10/10	50.3	-

3.4.2 In vitro release behavior of TRP-loaded microparticles

Figure 3-2 shows the release profiles of TRP from pure triglyceride microspheres and with 5 or 10 % GMS. Pure triglyceride microparticles showed a very low initial burst release (≤ 10 %) within the first day, which was also stated by other researchers when investigating peptide and protein release from triglyceride microparticles and cylinders [9] [44]. A high amount of drug remained inside the particles and the total released drug after three weeks was about 15 %. With addition of 5 % GMS to D116 the burst release increased to approx. 15 %. A continuous release

phase followed until day 20 reaching 45 % total release. With 10 % GMS, 40 % of incorporated TRP was released within the first day of the study and a steady release which slowed down after approximately one week followed. The incorporated TRP was almost completely released. McCarron and co-workers also found enhanced release rates for lipid particles in combination with emulsifiers [45]. Penetration appears to be enhanced with the higher emulsifier content.

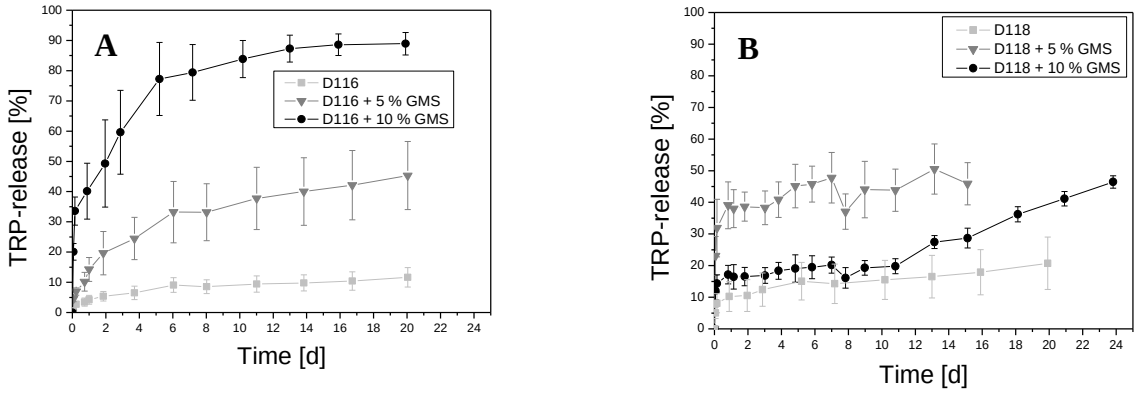

Figure 3-2: Release of TRP from D116 (A) and D118 (B) microparticles without and with 5 or 10 % GMS in PBS pH 7.4 (mean and SD, n = 3)

Particles based on pure D118 exhibited an initial TRP burst of less than 15 % and total drug release of approx. 20 %. Addition of 5 % GMS resulted in a higher burst of around 40 %, followed by further release of 5-10 % until day 6. In total, about 50 % of TRP was released. With 10 % GMS, the initial burst was low, followed by a lag time until day 10 and a continuous release phase until day 24. The total amount of drug released was 45 %. The incomplete drug release might be due to, on the one hand, an insufficient emulsifier concentration to facilitate the penetration of medium into the core of the particle or the adsorption to the lipid in solution [8] [46]. Incorporation of cholesterol and mono- or diglycerides has been described as suitable means to tailor release kinetics from triglyceride matrices [26] [47]. The concentration dependent emulsifier effect was also shown by other researchers who found enhanced release of hydrophilic drugs, like diclofenac [39]. Thus, the release can be adapted by the addition of a W/O surfactant.

3.4.3 **Influence of lipase addition to the release medium on TRP release**

Lipases are present in a variety of organs of the mammalian body and digest lipids typically by cleaving fatty acids from the triglyceride backbone [48]. The activity in the human body differs depending on the organ and is reported to be 0.01 U/ml in serum and subcutaneous fat [33]. In this study, the lipase concentration was set at 20 U/ml to see the effect during the 20 day-study period more clearly.

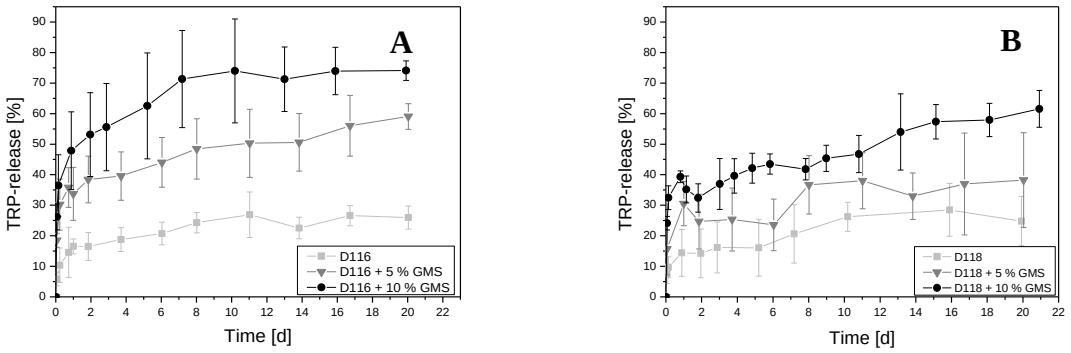

Figure 3-3: Release of TRP from D116 (A) and D118 (B) microparticles with 0, 5 and 10 % GMS in PBS pH 7.4 containing 40 U/ml lipase (mean and SD, n = 3)

Lipase addition resulted in slightly enhanced drug release from D116-based microparticles *(Figure 3-3*). Compared to the release in PBS buffer, the burst was increased by about 10 % and a continuous release could be monitored until day 11. In total, 25 % of incorporated drug were released, approximately 10 % more than in absence of lipase. For D116 particles with GMS, a general trend to a faster release could not be stated. Slightly enhanced release profiles could be observed regarding the pure triglyceride formulation and the combination with 5 % GMS. The most pronounced effect was noticed for microparticles formed with 10 % GMS. A burst of 40 % was followed by a continuous drug release until the end of the monitored time. The addition of higher amounts of monostearates enhanced the digestion. Surface structure was affected by the presence of lipase (*Figure 3-4*). Whereas microparticles in absence of lipase kept the initially observed smooth surface with slightly increased roughness potentially due to swelling [26], particle surface became uneven with larger flaky structures [47] and a more porous surface [33] [49] in presence of lipase. Keeping the lipase activity in the body in mind, the effect of lipase in vivo is most likely only minimal.

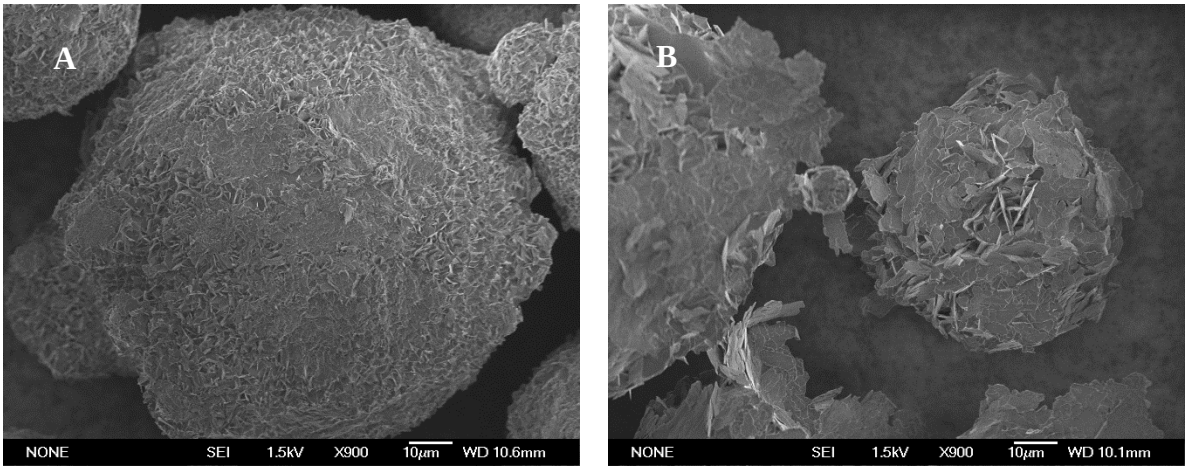

Figure 3-4: D118 + 5 % GMS-based microparticles after 3-weeks incubation in PBS pH 7.4 without lipase (A) and with 40 U/ml lipase (B)

3.4.4 Evaluation of lipid composition and additive concentration on in vitro release behavior of ASP from lipid-based microparticles

In addition to the release of TRP, particles containing the dipeptide ASP were tested. *Figure 3-5* shows selected ASP release profiles. Specifically, the influence of surfactants as release modifiers has been studied [50]. Vilivalam and Adeyeye showed, that the addition of stearic acid led to an enhanced release of diclofenac from waxy microspheres [39]. Furthermore, the hydrophilic and lipophilic properties of the drug influence the ability to be released from the matrix. More hydrophilic drugs are dissolved easier out of a lipid matrix than more lipophilic ones [20]. We studied the effect of both W/O or O/W-emulsifiers on the release behavior.

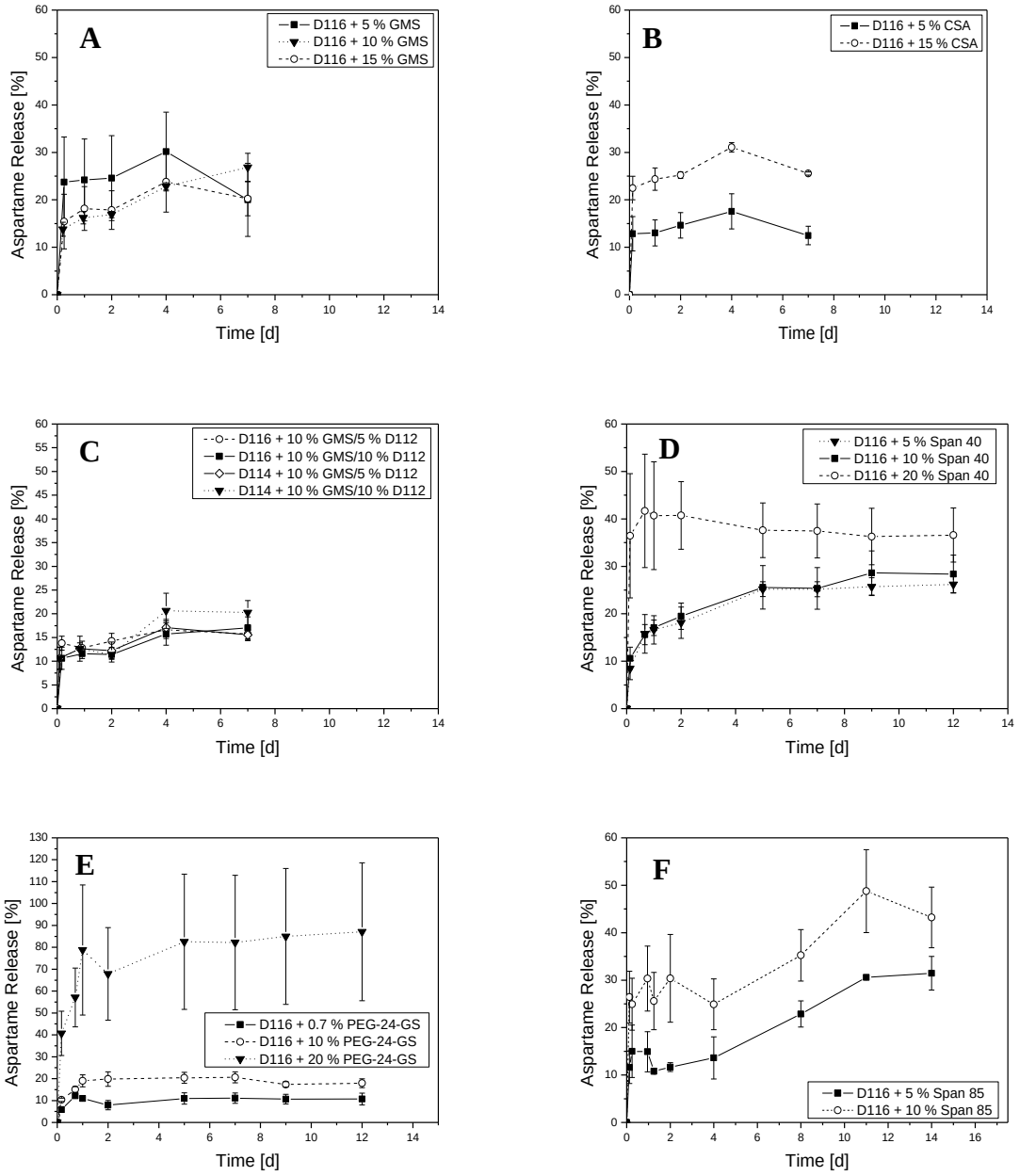

Figure 3-5: Release of ASP from D114 and D116 microparticles blended with emulsifying additives and/or low melting triglycerides in PBS pH 7.4 (mean and SD, n = 3)

The addition of GMS to D116 did not result in an improved release profile but only a burst of 15-25 % without further release. The use of CSA with a lower HLB value showed a comparable effect at both tested concentrations as a sustained release profile could not be obtained. To achieve a more complete ASP release, combinations of GMS and D112 were tested using the base lipids D116 and D114. Again, the desired profile could not be obtained. PEG-24 GS, an O/W emulsifier, led to a more complete drug release at higher concentrations, but did not render a sustained release profile. Khan and co-workers investigated the Gelucire® family, consisting of glycerides in combination with PEG-esters. Depending on the composition, a swelling and enhanced water penetration could be achieved leading to a faster drug release [51].

Most promising results could be obtained with the use of sorbitan fatty acid esters. Span 40, with an HLB value of 6.7 [41], resulted in promising profiles with a release up to 10 d when added in concentrations of 5 and 10 % to D116. 5 and 10 % Span 85, with an HLB value of 1.8 [41] rendered a sustained release profile for 12 d with an initial burst of 15 and 30 % respectively for the 5 and 10 %-addition. Thus, with ASP as model compound, no adequate release profile over 15 d could be achieved. Comparing the TRP and ASP release profiles led to the assumption, that the release behavior of G [6-D-Phe] is hard to predict. Drug properties appear to play an important role. Having been able to find the required release profile using TRP in a triglyceride formulation using GMS, this approach seemed most suitable for G [6-D-Phe]. An addition of Span 40 and 85 could form a promising base for further development.

3.5 Conclusion

The spray congealing process was determined as suitable production process for the preparation of TRP- and ASP-loaded microparticles. In general, microparticles with a spherical shape and smooth surface could be produced. Variation of process parameters did not result in pronounced changes regarding yield and particle size, confirming an adequate robustness of the process. Exclusive use of triglycerides D116 and D118 did not result in adequate release profiles, as a preferably low burst was not followed by a sustained release. With addition of 5 and 10 % GMS to D116 and D118, release profiles over 20 d could be obtained for TRP. The addition of lipases to the release medium led to a slightly accelerated and more complete release, but the enzymatic degradation is expected to have little influence in vivo. None of the tested formulations using ASP as model substance showed the aspired release profile. In most of the cases, a continuous release could not be observed. Formulations which could provide a springboard for the following study using G [6-D-Phe] could be based on D116 and D118 with 10 % GMS.

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4 OPTIMIZATION OF THE SPRAY CONGEALING PROCESS WITH FOCUS ON YIELD AND DRUG LOAD

Parts of this chapter are intended for publication

Data shown in this chapter was collected by **Kay Uwe Kraft** during his Bachelor thesis entitled ***"Optimization of the spray congealing process for lipid microparticles"*** **(2015)**

4.1 Abstract

The spray congealing process was evaluated concerning material loss, yield, particle size of different fractions deposited in the spray dryer and drug load using aspartame as model substance. The apparatus settings spray flow, aspirator power, melt temperature and installation of an additional stirrer were varied. Viscosities of lipid melts were determined using a rotational viscometer. Viscosity was mainly influenced by the temperature of the melt and only marginally by the lipid composition. At a spray flow of 414 l/h, a product yield of approximately 25 % resulted with substantial material loss in the spray tower and the melt container. Other components like high-performance cyclone, filter and connection between spray tower and cyclone were not identified as critical parts. Increasing the spray flow to 601 l/h resulted in higher product yield of approx. 45 % whereas a decrease in spray flow to 301 l/h and a reduction of the aspirator capacity reduced the yield. Melt temperature reduction (yield approx. 40 %) and installation of a stirrer within the sample container (yield approx. 50 %) had positive effects on product yield. Mean particle size changed with spray flow, from 70 (301 l/h) over 45 (414 l/h) to 20.7 µm (601 l/h). Highest encapsulation efficiencies in the product could be achieved with reduction of the spray flow to 301 l/h, as well as with the reduction of the melt temperature from 90 to 75 °C.

4.2 Introduction

The spray congealing process is a widely used technique suitable for encapsulation of drugs [1]–[3]. Having been able to develop a method suitable for the encapsulation of the amino acid tryptophan as well as the dipeptide aspartame, the process and influencing parameters should be further investigated. A lot of experimental data and knowledge is available on the influence of different nozzle types on the process and the product, but only little research has been done with the focus on yield distribution and material loss [4]–[7]. Particle size is typically influenced by spray flow and temperature difference between the melt and tower [8]. In general, for the spray congealing process rather high yields are reported [4], [9]–[11]. Additionally, the drug load depends on the apparatus settings. In general it is stated, that a faster solidification of microparticles leads to a desirably high entrapment efficiency [12] [13].

This chapter deals with the adjustment of the process with focus on the reduction of material loss, improvement of yield, a homogenous drug distribution within the melt, as well as particle sizes adequate for veterinary i.m. injection.

4.3 Materials and Methods

4.3.1 Materials

Triglycerides Dynasan 114 (trimyristin, D114) and Dynasan 116 (tripalmitin, D116) were kindly provided from Cremer Oleo (Witten, Germany). Aspartame (ASP) was purchased from Sandoz AG (Nuremberg, Germany). Glycerol monostearate (GMS) with a monoester content of 40-55 % was supplied from Caelo (Caesar & Loretz GmbH, Hilden, Germany). Colloidal silicium dioxide (Aerosil®) was purchased from Evonik (Hanau, Germany). Sorbitan monopalmitate (Span 40) was purchased from Croda (Nettetal, Germany). Polysorbate 20 (PS 20) was supplied from Merck KGaA (Darmstadt, Germany). All other chemicals were of analytical grade.

4.3.2 Cryogenic milling of ASP

ASP was blended with 0.5 % of colloidal silicium dioxide prior to being loaded into stainless steel milling beakers of the Cryomill (Retsch® Technologies GmbH, Haan, Germany). The powder was milled using two stainless steel milling balls with a diameter of 10 mm. A precooling time of 10 min at 5 Hz was followed by the actual milling step for 2 x 2 min at 20 Hz.

4.3.3 Preparation of triglyceride-based microparticles by spray congealing

Lipid components were melted on a hot plate, 100 mg (0.83 % drug load) or 200 mg (1.67 % drug load) ASP were added and homogenized using a T-10 basic Ultraturrax (IKA, Staufen, Germany) for 2 min (total batch volume 12 g). The melt was transferred into the sample container of the B-290 Mini spray dryer with additional spray chilling setup (Büchi, Flawil, Switzerland), which was pre-conditioned for 1.5 hours prior to the production step (sample container 75-90 °C, spray tower 15 °C), monitored with a PeakTech infrared thermometer (PeakTech Prüf- und Messtechnik GmbH, Ahrensburg, Germany). A modified nozzle with an inner diameter of 2.5 mm was used to avoid nozzle blockages. The connecting passage between sample container and nozzle was heated with a resistance wire-based heating to 70 °C. The spray tower was fed with pre-cooled air produced via an additional dehumidifier Deltatherm® LT (Deltatherm® Hirmer GmbH, Much, Germany). The lipid melt was sprayed using nitrogen at a pressure of 6 bar. A high-performance cyclone was used to separate the microparticles.

4.3.4 Determination of material distribution in the spray dryer

The formulation D116 + 5 % Span 40 with an ASP load of 0.83 % was sprayed as described in *4.3.3.* After the manufacturing process, the dryer was dismounted and the solidified material removed using a flexible soft rubber scraper. High-performance cyclone and filter were weighed before and after the experiment. Sampling points are depicted in *Figure 4-1*.

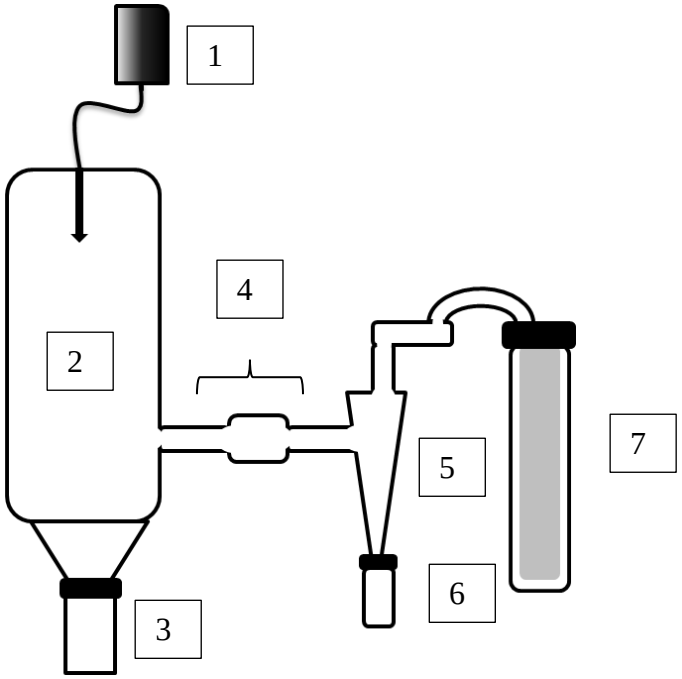

Figure 4-1: Schematic plan of the spray dryer. Particles were sampled at (1) sample container, (2) spray tower, (3) spray tower vessel, (4) connection, (5) high-performance cyclone, (6) product container and (7) filter

4.3.5 Determination of particle size

Approximately 20 mg of microparticles were suspended in 10 ml of an aqueous solution of 0.02 % PS 20 and analyzed using the laser diffraction system Horiba Partica LA-950 (Horiba, Kyoto, Japan).

4.3.6 Determination of encapsulation efficiency

Depending on theoretical drug load, 50 or 100 mg microparticles were dissolved in 1.0 ml of methylene chloride and the same amount of water was added. The tubes were incubated overnight on a horizontal shaker GFL 3031 (Gesellschaft für Labortechnik, Burgwedel, Germany) at a temperature of 39 °C at 60 rpm. Each sample was extracted in triplicate and measured using an Agilent Spectrophotometer 8453 (Agilent Technologies, Santa, Clara, CA, USA) at a wavelength of 258 nm [14].

4.3.7 Determination of lipid-melt viscosities

An MCR 100 rheometer (Physica, Anton Paar, Ostfildern, Germany) was used for determination of viscosity of the melt using the cone/plate method and the CP-50 1 measurement system at a logarithmic shear rate from 1-100 s^{-1}. The plate was preheated at the desired temperature of 75 or 90 °C.

4.4 Results and Discussion

4.4.1 Viscosity of the lipid melt

The viscosity of lipid melts can have an influence on the properties of the spray congealed solid lipid microparticles and the process [6] [9] [15]. In particular particle size of the generated spheres [4] [16], yield [9] and their time for solidification [17] are affected. Recently, Wong et al. investigated the temperature-dependent viscosity of lipids suitable for spray congealing, and revealed that viscosity of the melt decreases up to a certain temperature, above which the viscosity does not decrease any more with further heating [15]. Furthermore, the viscosity of triglyceride melts increases with longer side chains [18]. Consequently, the viscosities of differently formulated lipid melts at 75 and 90 °C were tested. D114 and D116-based formulations with addition of 5-10 % GMS and Span 40 showed viscosities between 10 and 25 mPas measured at 90 °C. With temperature reduction to 75 °C the viscosity slightly increased to 35 mPas (*Figure 4-2*), according to literature [15] [19]. When spraying was performed under a certain temperature, an increase in particle size upon spray congealing can be observed [15]. Formulation effects on the melt viscosity were not detectable during our experiments. Higher viscosities compared to pure lipid melts, as well as shear thinning are reported in literature for lipid suspensions [20].

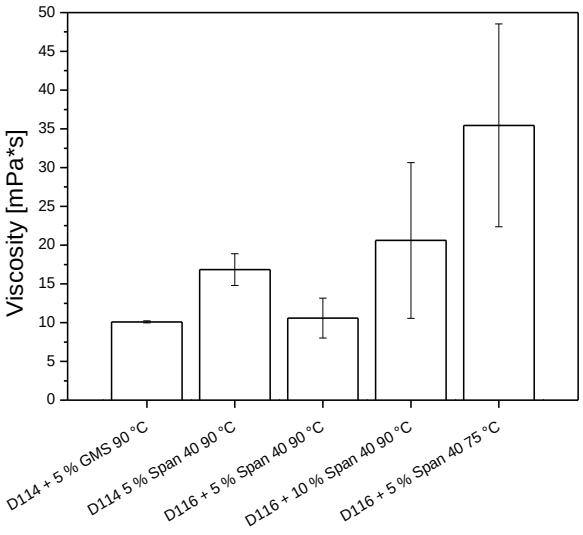

Figure 4-2: Viscosity of drug-free lipid melts (D116 and D114) with emulsifiers at 90 °C and 75 °C at a shear rate of 1 s^{-1} (Mean and SD, n = 2)

4.4.2 Yield distribution and material loss during the spray congealing process

Due to low yields of 20 to 50 % in the previously performed spray congealing experiments, it was necessary to evaluate where the high material loss occurred. Other researchers reported yields of over 90 % with the use of spray congealing [4] [9] [21]. With the standard conditions (inlet temperature 90 °C, spray pressure 6 bar, aspirator 100 %, spray flow 414 l/h) a yield of 26 % was exemplarily achieved during implementation of our spray congealing process (*Figure 4-3 A*). Highest material loss occurred in the spray tower itself (49 %), whereas only 1 % ended directly in the spray tower vessel. The material found in the tower was not completely solidified when getting in contact with the glassware. Another critical part for material loss is the sample container. Having a plane bottom and being construed for volumes of up to 300 ml [22], the container is prone for residual melt remaining inside when working with smaller batches. All other parts, like the connection, the high-performance cyclone, as well as the filter did not have a critical influence on material loss. A low material loss in the cyclone was also reported by Scott et al. [6].

The aspirator, generally responsible for drying of particles, is known to influence total batch yield in spray drying [23] [24]. The speed with which the droplets follow the airstream determines their contact time with the tempered air and thus the drying or solidification [25]. Reduction of aspirator power from 100 to 70 % led to a marginally reduced yield as observed with spray-dried insulin [26]. The material found in the spray tower was reduced to 36 %, whereas the particle amount found in the spray tower vessel increased. Obviously, the reduced aspirator force enabled a more effective solidification in the tower. Thus, the amount usually found in the spray tower was transferred to the spray tower vessel, also confirming the theory of a more effective solidification. Other parts of the spray dryer were only of minor importance (*Figure 4-3 B*).

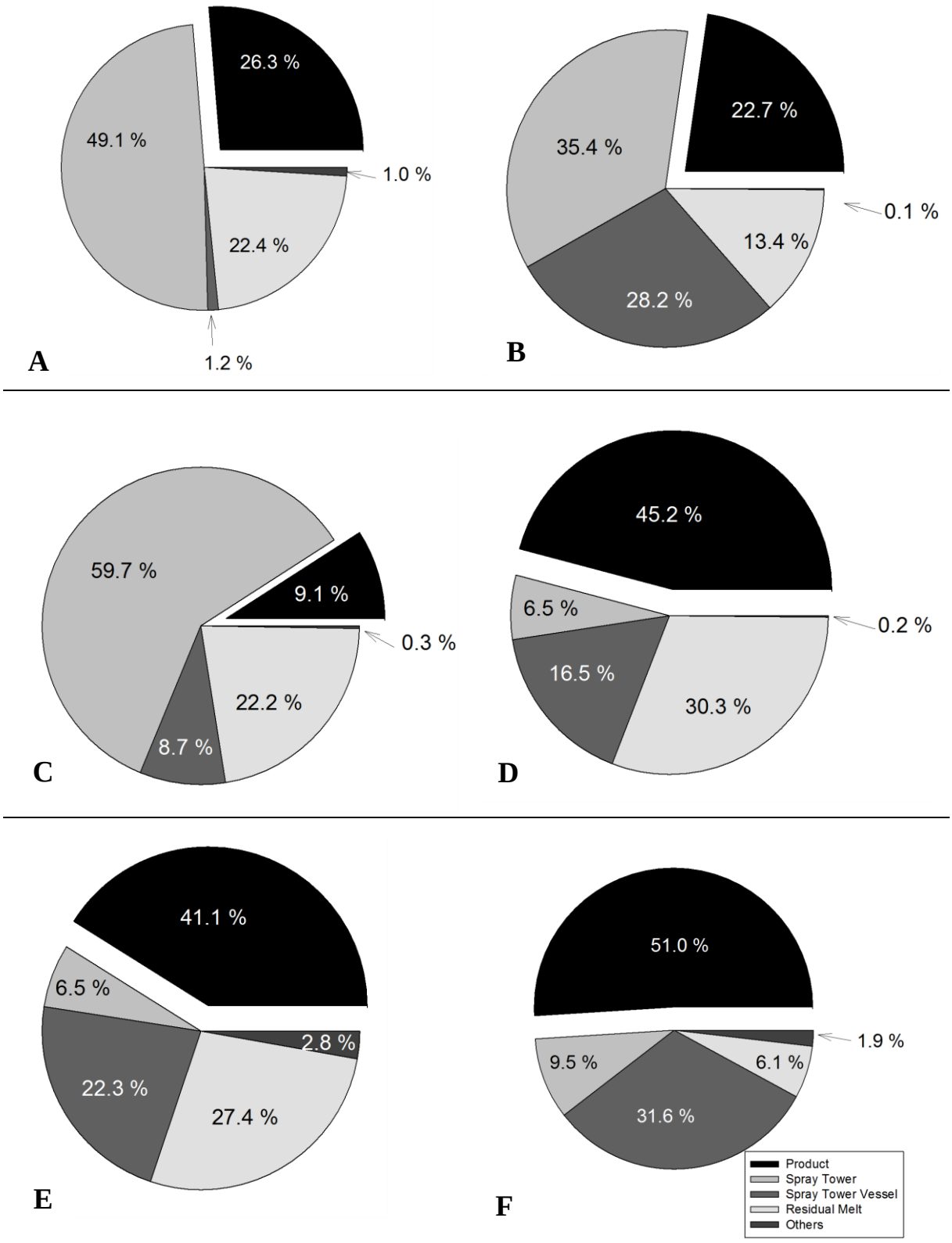

Figure 4-3: Material distribution after spray congealing experiments using standard conditions (spray flow 401 l/h, aspirator 100 %, 90 °C) (A), reduced aspirator power of 70 % (B), reduced spray flow of 301 l/h (C), increased spray flow of 601 l/h (D), melt temperature 75 °C (E) and installation of an additional stirrer (F). "Others" includes filter, high-performance cyclone and connection tube between tower and high-performance cyclone

An important factor in both spray drying and congealing is the spray flow, responsible for the effectiveness in atomization [27]. Reduction of spray flow to 301 l/h led to a remarkable reduction in product yield (*Figure 4-3*). Material loss in the spray tower was increased to about 60 %, whereas more than 8 % of the powder were found in the spray tower vessel, indicating the formation of larger droplets and incomplete solidification. Also, a high amount of residual lipid material was found in the sample container. Application of higher spray flow of 601 l/h resulted in an increased product yield of 45 %. Compared to standard conditions, the material lost in the spray tower was reduced to approximately 6 % and about 16 % of the material could be collected from the spray tower vessel. Surprisingly, again a high residual melt could be found in the sample container. A higher spray flow causes the formation of smaller droplets, which solidify more rapidly [9] [28]. Reduction of spray flow led to larger spheres, which could be responsible for lower yields, due to insufficient hardening and collision with the tower wall. Furthermore, larger spheres generated at lower spray flow values may not be able to follow the air stream [29].

Another approach to influence spray properties is the modification of the melt viscosity by changing the temperature (*Figure 4-3 E*) [4] [15]. A higher product yield of 41 % was reached with a reduction of the melt temperature from 90 to 75 °C. The amount collected in the spray tower could be reduced to approx. 6 %, due to a more effective solidification, as the temperature difference between liquid and solid state of the lipid was reduced. This also caused a higher amount of solidified product in the spray tower vessel. As a downside, a higher amount of residual melt of about 27.35 % resulted.

For an effective and homogenous spray congealing process an additional stirring device was installed. The stirrer led to an increase in product yield to about 51 %. Moreover, the material lost in the spray tower could be reduced to approx. 9 %, whereas the material collected in the spray tower vessel was increased to about 31 %. The residual melt could be minimized by the stirrer to approx. 6 %, as less material gathered at the sample container.

The tested parameters showed that material loss in spray congealing was sensitive to production parameters. Substantial loss of material could be noticed in the spray tower, as well as in the sample container. The most positive effect resulted from an increase of spray flow to 601 l/h and the installation of an additional stirrer.

4.4.3 **Investigations on particle size of drug-loaded particles**

Particle size is another critical parameter in the development of a spray congealing process. The size of the generated spheres affects drug release and encapsulation capacity [30] [31]. For this reason, particle fractions from the spray tower, spray tower vessel and product container were collected and analyzed for mean particle size (*Figure 4-4*).

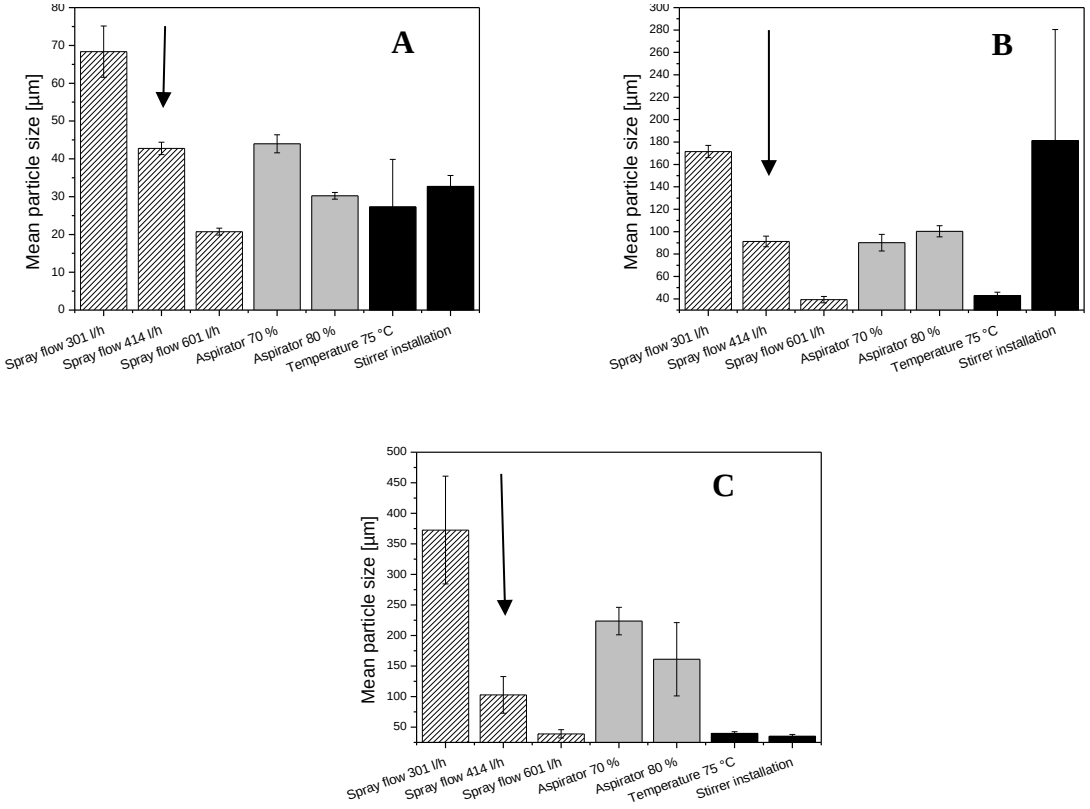

Figure 4-4: Mean particle size of samples collected in the product container (A), spray tower vessel (B) and spray tower (C). Standard settings were defined as spray flow 414 l/h, spray pressure 6 bar, aspirator 100 %, melt temperature 90 °C (black arrows)

Most important was the particle size of the product. The largest particles (> 65 µm) were formed at a reduced spray flow of 301 l/h. When applying standard spray flow of 414 l/h the achieved particles were about 45 µm in diameter. Increase of spray flow to 601 l/h led to reduction of the particle size to approx. 23 µm. This effect was reported for both spray drying and spray congealing processes [9] [27] [32] [33]. Besides the product, also the particles found in the spray tower and the spray tower vessel were affected by the spray flow, with larger particles found at a reduced spray flow. The reduction of aspirator power affected the size of the particles collected in the product and spray tower vessel only marginally, in contrast to Esposito et al. during spray drying. A remarkable increase in size of the particles from the tower was observed. These observations might be explained by a longer residence time in the airstream, which allow the solidification of larger droplets [25]. Thus, a possible reduced deposition of droplets on the

chamber wall can be achieved. Nevertheless, product size was barely affected. Generally, the product showed a smaller diameter compared to fractions collected in the spray tower and spray tower vessel. Due to their bigger size, particles were not able to follow the airstream and were deposited in the spray tower vessel.

A reduction of the melt temperature did not lead to an increase in particle size of the product, but the particle size of material collected from both the spray tower and the spray tower vessel was substantially reduced to under 100 µm.

The installation of an additional stirrer slightly reduced the product particle size to approx. 30 µm. Particles collected in the spray tower showed a reduced particle size, whereas the particles in the spray tower vessel were larger. These findings indicate that the additional vibration of the stirrer caused a slightly inhomogeneous atomization.

4.4.4 **Evaluation of drug distribution and encapsulation efficiency**

Ideally, the final product should show an encapsulation efficiency of 100 %. Commonly, high encapsulation efficiencies are reported for spray congealed delivery systems, especially in comparison to emulsion-based methods, where problems in drug encapsulation occur due to migration into the aqueous phase [9] [11] [34] [35].

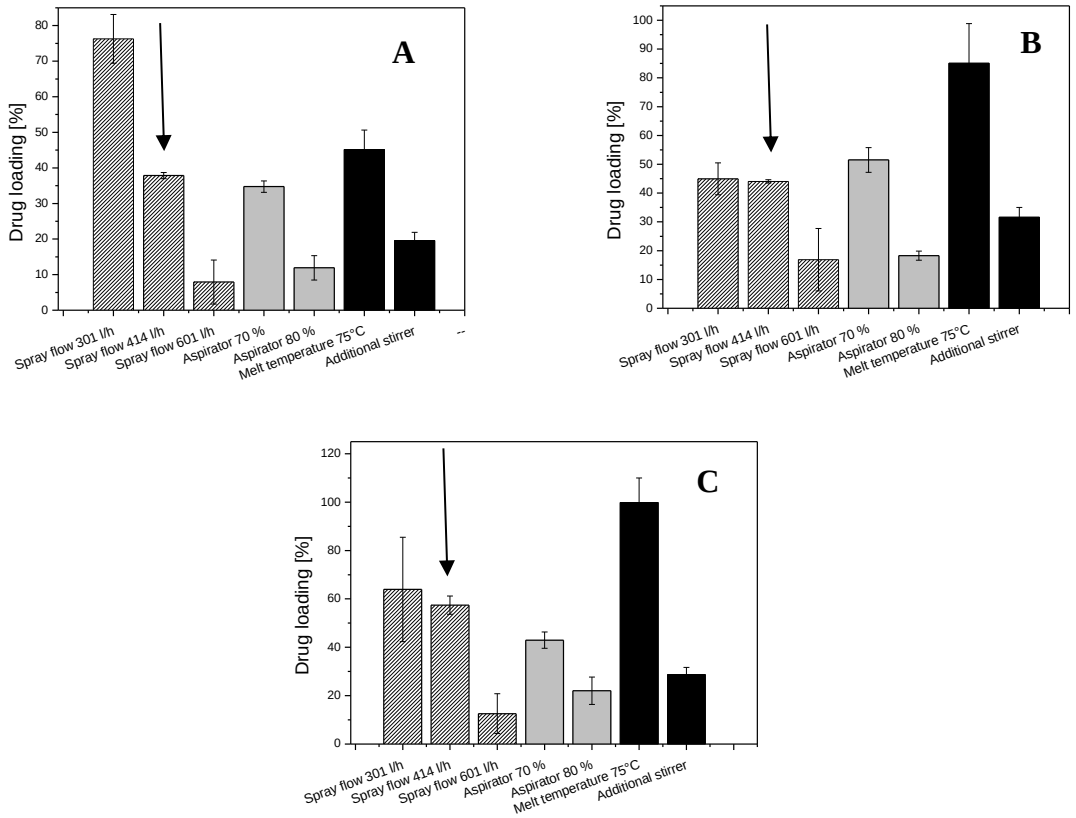

Figure 4-5: Drug loads of collected samples from the product container (A), spray tower (B) and spray tower vessel (C). Standard settings were defined as spray flow 414 l/h, spray pressure 6 bar, aspirator 100 %, melt temperature 90 °C (black arrows)

The application of standard conditions (*Figure 4-5 A)* led to an encapsulation efficiency of 38 % in the product and higher values of 45-60 % were obtained in the spray tower and spray tower vessel. Reducing the spray flow to 301 l/h resulted in a considerable increase in drug load to 80 %, which might go in accordance with the simultaneous increase in particle size, possibly enabling a better encapsulation [31] [36]. Spheres collected from the spray tower and vessel were not affected. Applying a spray flow of 601 l/h reduced the drug load in all sampled fractions. The reduction of aspirator capacity to 70 or 80 % respectively, affected drug encapsulation only marginally.

Melt temperature reduction to 75 instead of 90 °C increased the loading efficiency compared to standard conditions in the product as well as in the material collected in the spray tower vessel and the spray tower. The expectation of achieving better results with the installation of an additional stirrer were not fulfilled, all three fractions showed drug loads below 40 %.

In brief, with variation of apparatus settings it was possible to influence particle size and encapsulation efficiency to a certain extent. Generally, the spray congealing process can be regarded as complex process, with a variety of parameters having an influence. The reduction of spray flow was a suitable means to increase particle size, which also had positive effects on the encapsulation efficiency. On the other hand, an increase of spray flow affected the drug content negatively, due to the decreased particle size. Acceptable results concerning particle size and encapsulation efficiencies could be reached with the reduction of the melt temperature. Particle size remained constant with slightly enhanced encapsulation efficiencies in all sampled fractions. The installation of a vibrating stirrer led to opposite effects as expected, as an inhomogeneous atomization resulted and the product showed less encapsulated drug. The aspirator capacity did not affect the particle size and encapsulation efficiency substantially.

4.5 Conclusion

The spray congealing process was investigated concerning yield and material distribution, particle size and encapsulation efficiency. The parameters spray flow, aspirator power, melt temperature, as well as the influence of an additional stirring device were evaluated. Our investigations showed, that the highest material loss can be found in the spray tower, spray tower vessel as well as in the sample container. Filter, high performance cyclone and the connection between tower and cyclone were not evaluated as critical parts for material loss. The most pronounced effect on yield of the useable product could be determined by the increase of the spray flow to 601 l/h and by the melt temperature reduction to 75 °C. All other variations led to lower yields and a higher material loss. Evaluating particle size of the product, the variation of spray flow was determined as the most influencing factor. Drug distribution, which was an issue during previously performed experiments, could be improved by the reduction of melt temperature to 75 °C, as well as the reduction of spray flow. Viscosity measurements revealed, that the melt temperature was the mainly influencing factor, indicating that the decrease in melt temperature to 75 °C resulted in increased viscosity. Overall, the spray congealing process is influenced by a variety of parameters. Our results showed, that an increase in particle size is going hand in hand with a better encapsulation efficiency, which can be achieved by the reduction of spray flow. The increase in spray flow implemented the formation of smaller particles with considerably lower drug loadings. Increasing viscosity by the means of temperature reduction increased encapsulation efficiency due to faster solidification. Thus, a compromise needs to be found to assure an acceptable drug loading and process yields.

4.6 References

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5 INCORPORATION OF GONADORELIN [6-D-PHE] INTO SPRAY CONGEALED SOLID LIPID MICROPARTICLES AND EVALUATION OF RELEASE MECHANISMS

This chapter is intended for publication

5.1 Abstract

The decapeptide gonadorelin [6-D-Phe] was incorporated into spray congealed microparticles consisting of triglycerides of different chain lengths. As a continuous release in the desired time frame of 15 d could not be achieved with the exclusive use of triglycerides, different additives like GMS, CSA and Spans were applied to modify the release behavior. To investigate the underlying mechanisms influencing the release profile, polymorphic behavior, surface morphology and wettability, as well as water uptake were investigated. Crystallization of the α- or β-form and entrance of water were determined to be influenced by the emulsifier type and its concentration. Wettability of lipid surfaces was low regarding pure triglycerides and was increased when surfactants, especially monoglycerides, were added to the formulation. SEM images showed a particle breakdown upon incubation when liquid emulsifiers were used. It was possible to achieve a sustained release for 14 and 22 d with the addition of 10 % GMS to D116 and D118. Release duration of 20 d was obtained with the addition of 5 % Span 40 to D114. Thus, two formulations for subsequent in vivo studies in swine could be identified. The challenge of a high burst release could be minimized by suspending the particles in a thermosensitive poloxamer-based gel.

5.2 Introduction

The treatment with peptides or proteins often comes with frequent injection [1]–[4]. This can be a problem and limitation for application in human and as well veterinary medicine, due to compliance, efforts and cost issues compared to orally applied small molecule drugs. To circumvent these problems, sustained release systems for peptides and proteins are sought after [5]–[12].

One example for an immediate releasing biopharmaceutical formulation in veterinary use is Virbagen® Omega (Virbac), delivering interferon omega for the feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) in cats, as well as parvovirus in dogs [13]–[16]. Furthermore, GnRH-analogues are currently used for induction of ovulation in swine, cattle and mares [17]–[20]. Gonadorelin [6-D-Phe] (G [6-D-Phe]), active compound in Gonavet®Veyx (Veyx Pharma GmbH) with a molecular weight of 1000 Da, is a GnRH (LHRH)-I agonist with a higher affinity to GnRH receptors [21]–[24]. Other commonly used GnRH analogues for estrus control are buserelin (Receptal®, MSD Animal Health) [25] [26], lecirelin (Dalmarelin®, Fatro) [27] and peforelin (Maprelin®, Veyx Pharma GmbH) [28]. Besides the veterinary importance, GnRH analogues are used in several indications in humans, for example breast and prostate cancer, endometriosis and fertility disorders [29] [30].

In 1985, one approach for a sustained delivery was the application of nafarelin, a substance related to GnRH-I for estrus suppression in female dogs, via an osmotic pump system [12]. Current, sustained release delivery systems used in veterinary medicine are injectable suspensions, solid implants, in-situ forming implants and microparticles mostly based on lipid compounds [12] [31] [32] [33]. Lipid excipients inherit several advantages compared to other polymers, as biodegradability, high biocompatibility and low costs [34] [35].

The lipid-based implant Suprelorin® (Virbac), containing deslorelin acetate, is approved for the non-invasive castration in male dogs [36] [37]. The implant consists mainly of hydrogenated palm oil and is biodegradable, which omits surgical removal [37]. Besides the use in male dogs, a deslorelin-containing implant was also tested in female dogs [36] and was able to achieve a suppression of estrus for 27 months [38].

Estrogen and progesterone-loaded, polymer-based microparticles have been investigated in terms of estrus synchronization in mares [39]. Additionally, ProHeart® 6 (Zoetis) is a microparticular delivery system based on tristearin for the antibiotic substance moxidectin,

which has the FDA approval in the USA and provides a 6-month protection against the canine heartworm disease [32] [40] [41].

Consequently, we developed an injectable sustained release system based on triglyceride microparticles for G [6-D-Phe] via spray congealing. The incorporation of the drug G [6-D-Phe] and the adaption of the process was the focus of this work. Additionally, the in vitro release of G [6-D-Phe] was to be investigated to achieve a sustained release over two weeks suitable for estrus synchronization in swine [42]. In order to understand the underlying release mechanism, lipid polymorphism, surface wettability and water uptake were evaluated.

5.3 Materials and Methods

5.3.1 Materials

Lyophilized G [6-D-Phe] was donated by Veyx Pharma GmbH (Schwarzenborn, Germany). Triglycerides Dynasan 114 (trimyristin, D114), Dynasan 116 (tripalmitin, D116) and Dynasan 118 (tristearin, D118) were kindly provided from Cremer Oleo (Witten, Germany). Glycerol monostearate (GMS) with a monoester content of 40-55 % was purchased from Caelo (Caesar & Loretz, Hilden, Germany). Cetylstearyl alcohol (CSA, Lanette® O) was purchased from BASF (Ludwigshafen, Germany). Span 40, 60, 80, 85 were kindly provided from Croda (Nettetal, Germany). Poloxamer 407 and Hydranal® for Karl-Fischer titration were purchased from Sigma-Aldrich (Taufkirchen, Germany). Ultrapure deionized water with a conductivity of less than 0.055 µS/cm (Milli-Q Water systems, Millipore, MA, USA) was used throughout all experiments. Centrifuge tubes were purchased from VWR International GmbH (Darmstadt, Germany). Visking dialysis tubings with a diameter of 1.6 mm and a cut-off of 12-14 kDa for performance of release studies were sourced from Serva Electrophoresis GmbH (Heidelberg, Germany). All other chemicals were of analytical grade.

5.3.2 Cryogenic milling of G [6-D-Phe]

G [6-D-Phe] was milled using a Retsch® Cryomill (Retsch Technology, Haan, Germany) equipped with two stainless steel milling balls (diameter 10 mm) with a precooling time of 10 min at 5 Hz followed by the actual milling step of 4 min at 20 Hz. The obtained powder was aliquoted under nitrogen atmosphere.

5.3.3 Preparation of G [6-D-Phe]-loaded microparticles by spray congealing

Lipid compounds were melted on a hot plate at 90 °C, the peptide was added and homogenized using a T-10 basic Ultraturrax (IKA Laboratory Technology, Staufen, Germany) for 2 min. A Büchi B-290 Mini spray dryer (Büchi, Flawil, Switzerland) with additional spray chilling setup, was pre-conditioned for 1.5 hours prior to the production step (sample container 90 °C/110 °C, spray tower 13-15 °C). Additional equipment consisted of a sample container surrounded by a polyethylene glycol (PEG) 400 bath. The connection between sample container and nozzle was heated externally by a resistance wire-based heating to approximately 70 °C. The spray tower was fed with pre-cooled air produced by an additional dehumidifier Deltatherm® LT (Deltatherm® Hirmer GmbH, Much, Germany). The lipid melt was filled into the sample container and sprayed using nitrogen and a modified nozzle with a channel diameter of 2.5 mm using the following apparatus settings:

Spray parameter	Value
Spray pressure [bar]	6
Aspirator power [%]	100
Valve opening [turns]	1.25-1.5
Temperature oil bath [°C]	90 (D114, D116), 110 (D118)
Inlet temperature [°C]	13-17
Pressure filter [mbar]	92
Spray flow [l/h]	414

All produced microparticle batches had a theoretical drug loading of 1.8 %.

5.3.4 Determination of particle size

20 mg of microparticles were suspended in 10 ml of an aqueous solution of 0.02 % PS 20 and measured with the laser diffraction system Horiba Partica LA-950 (Horiba, Kyoto, Japan).

5.3.5 Investigations on polymorphic behavior using differential scanning calorimetry (DSC)

Microparticles, triglycerides and lipid mixtures were analyzed using a Mettler DSC 821e (Mettler Toledo, Columbus, OH, USA). 5-15 mg sample were weighed into aluminum crucibles and analyzed at a heating and cooling rate of 10 K/min between 0 °C to 110 °C in two cycles. Incubated particles were dried on a lint-free cloth for 1 h at room temperature.

5.3.6 X-Ray powder diffraction (XRPD) of lipid microparticles

XRPD measurements were conducted with 2 different systems:

- Small sample quantities were analyzed with a PANalytical Empyrean (PANalytical, Almelo, the Netherlands) equipped with a copper anode (45 kV, 40 mA, Kα1 emission λ = 0.154 nm) and a PIXcel3D detector between 5 to 50 ° 2-Theta with a step of 0.039 °.
- To analyze multiple samples and to benefit from an autosampler, samples were scanned between 10 and 40 ° 2-Theta at a step size of 0.05 ° with a XRD 3000 TT (Seifert, Ahrensburg, Germany) equipped with a copper anode (λ= 0.15418 nm, 40 kV, 30 mA).

5.3.7 Karl-Fischer titration

10 mg microparticles were weighed into 2 R glass vials, closed with rubber stoppers and analyzed with an Aqua 40.00 titrator (Analytik Jena, Jena, Germany) with a headspace module at 100 °C. The analysis of incubated particles in PBS was performed after drying on a lint-free cloth for 1 h at room temperature.

5.3.8 Characterization of particle morphology using scanning electron microscopy (SEM)

A Jeol JSM 6500-F SEM (Jeol, Tokyo, Japan) was used for SEM-images at a voltage of 2 kV. Microparticles were fixed on aluminum sample holders with black, double adhesive tape (Plano, Wetzlar, Germany) and analyzed without an additional graphite coating. After release studies, particles were washed five times and dried for 48 h in a vacuum dryer (Memmert GmbH, Schwabach, Germany) at 25 mbar and 25 °C.

5.3.9 Contact-angle measurements for prediction of lipid surface wettability

Contact angle measurements were performed using the Krüss Drop Shape Analyzer DSA 25 (Krüss, Hamburg, Germany). 0.5 g of lipid components were melted at 80 °C, poured onto glass microscopy slides and cooled for solidification. A drop volume of 2 µl water was placed on the lipid surface at a speed of 1 ml/s. Measurements were performed in triplicate after a wait time of 20 s (mean values of left and right contact angle). The ellipse-tangent-1 fit was used for calculation of contact angles.

5.3.10 Determination of drug-content using RP-HPLC after extraction

20 mg microparticles were weighed into 30 ml-centrifuge tubes, dissolved in 2.0 ml of methylene chloride and the same amount of water was added. The tubes were placed on a horizontal shaking incubator GFL 3031 (Gesellschaft für Labortechnik, Burgwedel, Germany) at 39 °C for 12 h. Samples of 1 ml were taken from the water phase and analyzed using RP-HPLC. Extractions were performed in triplicate. RP-HPLC analysis was performed using an Agilent RP-HPLC system (Agilent, Santa Clara, CA, USA), supplied with a 250 x 4.6 mm Luna (5 µm) C-8 column (Phenomenex, Aschaffenburg, Germany) and a SecurityGuard KJO-4282 C-8 pre-column (5 µm) (Phenomenex, Aschaffenburg, Germany). Columns were maintained at 40 °C using the following gradient:

Time [min]	A [%]	B [%]
0	65	35
1	65	35
21	65	35
22	10	90
26	10	90
35	65	35

Mobile Phase A: 1000 ml highly purified water + 1 ml trifluoracetic acid

Mobile Phase B: 800 g Acetonitrile + 200 g water + 1.2 ml trifluoracetic acid

5.3.11 In vitro release behavior of G [6-D-Phe] from lipid microparticles

Approximately 50 mg of microparticles were transferred into dialysis tubings, 2.0 ml of phosphate-buffered saline (PBS) pH 7.4 preserved with 0.05 % NaN_3 were added, followed by the addition of glass balls which served as additional weight. The closed tubings were placed in 50 ml centrifuge tubes, 15 ml PBS were added and incubated on a horizontal shaking incubator at 39 °C and 60 rpm. When poloxamer gels were used as suspension medium, the centrifuge tubes were pre-filled with PBS and incubated overnight at 39 °C. Dialysis tubings with an aliquot of microparticles and 2 ml of poloxamer gels were closed and put into pre-heated surrounding buffer. At predetermined time points, samples of 1 ml volume were taken and removed solution was replaced by fresh PBS. Samples were analyzed with RP-HPLC as described in *5.3.10*.

5.3.12 Preparation of poloxamer gels

Poloxamer 407 (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in water in a refrigerator at 2-8 °C overnight.

5.3.13 Temperature-dependent viscosity measurements using a rotation viscometer

For analysis of temperature-dependent gelling behavior, an MCR 100 rheometer (Physica Anton Paar, Ostfildern, Germany) with a plate/plate system PP-25 and a temperature ramp from 4 to 34 °C was used at a shear stress of 1 Pa. Each measurement was performed in triplicate.

5.4 Results and Discussion

5.4.1 Preparation of drug-loaded lipid microparticles by spray congealing

Based on previous spray congealing tests with placebo and model substance-loaded microparticles, a first set of spray congealing conditions and formulations for G [6-D-Phe] was selected *(Table 5-1*). Initial criteria for the performed pre-tests were yield, particle size and encapsulation efficiency (EE).

Table 5-1: First generation G [6-D-Phe]-loaded formulations produced by spray congealing

TG	Additive	Concentration [%]	Yield [%]	EE [%]
D 118	-	-	13.8	61.3
D 116	-	-	20.6	56.7
D 118	GMS	10	17.8	61.4
D 118	GMS	20	16.6	40.4
D 118	GMS	40	3.1	15.9
D 118	GMS	60	4.9	23.5
D116	GMS	10	26.1	38.9
D116	GMS	20	30.6	37.0
D116	GMS	40	21.0	41.0
GMS	-	-	≤ 1	23.5
D116	CSA	10	32.3	47.1
D116	CSA	20	19.1	56.8
D116	CSA	60	26.6	54.8

G [6-D-Phe]-loaded microparticles could be sprayed in analogy to placebo and model substance-loaded particles. Compared to the incorporation of model substances, the peptide led to a more persistent and continuous spray cone and less valve blockages. The use of pure GMS resulted in needle-like, inadequate particles, which showed pronounced electrical charging. All other batches showed higher yields. Pure triglyceride formulations showed yields below 21 % and GMS addition led to slightly increased product yields. More than 30 % product could be obtained with 10-20 % GMS. Generally, obtained yields were lower as reported in literature [43] [44] [45], since we only considered the powder in the product vessel.

EE was about 60 % for pure triglycerides. The addition of 10 % GMS to D118 led to comparable results whereas EE was slightly decreased when added to D116. With higher amounts of monoglycerides added to D118 the drug load decreased. The addition of higher amounts of GMS and CSA to D116-based microparticles did not affect the drug load, which

went in accordance with data reported by Eustáquio Matos-Jr. et al. comparing hydrogenated palm oil and glycerol monostearate particles [45].

Table 5-2: Second generation G [6-D-Phe]-loaded formulations produced by spray congealing

TG	Additive	Concentration [%]	Yield [%]	EE [%]
D114	GMS	10 %	33.9	78.4
D114	GMS	5 %	22.7	78.7
D114	Span40	5 %	28.8	51.2
D114	Span 40	10 %	12.1	21.1
D114	Span 40	30 %	1.6	n.d.
D114	Span 85	10 %	17.8	60.9
D114	Span 85	20 %	16.2	46.5
D114	Span 80	10 %	2.1	57.4
D114	Span 80	20 %	16.4	21.9
D114	Span 85	30 %	3.4	21.1
D116	Span 85	10 %	15.9	71.9
D116	Span 85	20 %	17.8	72.4
D116	Span 85	30 %	7.6	71.4
D116	D112	10 %	7.9	81.8
D116	Span 40	5 %	15.6	29.9
D116	Span 40/D112	10 %	4.1	14.4
D116	GMS/ D112	5 %/10 %	22.88	64.34
D116	GMS/ D112	10 %/10 %	18.22	65.30
D116	Span 80	10 %	7.27	40.15
D116	Span 80	30 %	10.59	43.56

Second generation of lipid microparticles was produced using short-chain triglycerides and a broader spectrum of surfactants to achieve a faster release [46]. D114 rendered adequate yields between 23 and 34 % upon spray congealing in combination with 5 and 10 % GMS, respectively. The encapsulation efficiency was raised to 79 %. The addition of Span 40 with a HLB value of 6.7 [47] [48] caused an irregular spray cone due to the formation of foam, also reasoning lower yields and decreased EE. The use of surfactants with lower HLB-values, Span 80 and 85 [47], also reduced process yield. EE was acceptable (71 %) when Span 85 was used in combination with D116 and reduced when combined with D114 (21-60 %). Span 80 led to lower EE. D112 was previously used as low melting component in the manufacturing of solid lipid implants, where a faster degradation rate was obtained [49] [50]. A lower yield but

acceptable EE of 81.75 % was achieved. Triple combination of D112 with GMS and Span 40 resulted in appropriate results regarding GMS, but both reduced yield and drug content when Span 40 was added.

Sprayability, yield and EE are critical parameters in the production of spray congealed lipid microparticles. The parameters yield and EE seemed to be influenced by the concentration and state of emulsifier, also affecting solubility of the drug in the lipid [51]. In general, for spray congealed lipid microparticles high yields and encapsulation efficiencies above 90 % are reported in literature, which could not be achieved during our experiments [44] [52]. No pronounced effects regarding EE were found by Mccarron et al., comparing different emulsifier-triglyceride compositions [53]. Possible approaches to enhance EE were achieved by addition of Aerosil® and a followed increased viscosity [54], bigger particle sizes [55] and higher drug-lipid ratio [56].

5.4.2 Characterization of polymorphic behavior using DSC and XPRD

The polymorphic behavior of the microparticles was analyzed by DSC and XRPD. The heating curve of D116 showed a melting endotherm with an onset of 42 °C corresponding to the α-polymorph [57] followed by an exothermic recrystallization and subsequent melting of the thermodynamically stable β-polymorph (*Figure 5-1*) [58]. Particles with 5 % Span 40 showed similar trace. A single sharp peak corresponding to the β-form could be observed when Span 85, GMS or the lower melting D112 were added. The small endotherm at 45 °C for microparticles of 80 % D116, 10 % D112 and 10 % GMS was either due to melting of D112 or the α-polymorph of D116 [57] [59]. For pure GMS, polymorphic transformation is also known with the α-polymorph melting at 67.9 °C and β-polymorph at 71.9 °C [60]. An event in this temperature region could not be detected in the GMS-containing microparticles.

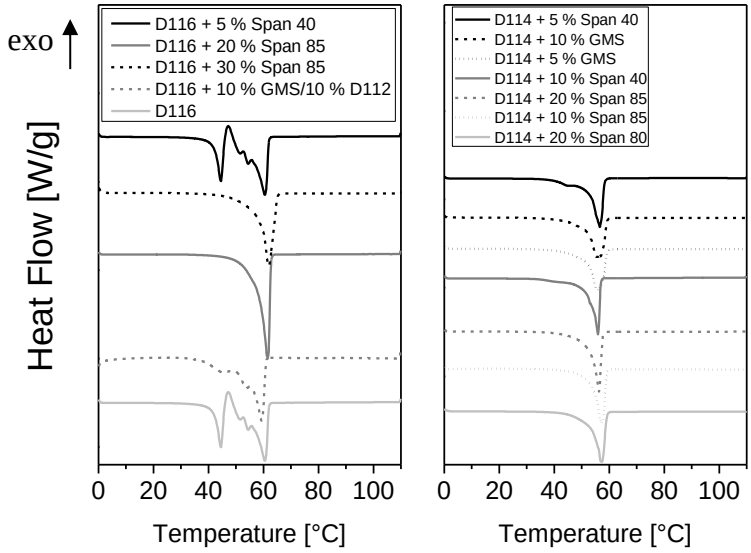


Figure 5-1: DSC thermograms of G [6-D-Phe]-loaded microparticles (n = 2). Thermograms represent the first heating scan of one sample exemplarily and were shifted on the ordinate for better visualization

The addition of 5 and 10 % Span 40, 80 and 85 to D114 resulted in crystallization of the thermodynamically stable β-polymorph with a melting onset of 53 °C and a melting peak of 55-58 °C [61] [62]. The instable α-form with a melting point of 32 °C [62] was not detected in any sample. Whittam and Rosano observed that the transformation to the β-modification was already completed after 31 min at room temperature [62].

To further investigate the crystallization behavior of lipids, DSC measurements were conducted after incubation in PBS. D114-based samples showed the β-polymorph with a melting temperature of 57 °C [58] [63] after incubation, irrespective of the additive. D116-based microspheres with addition of GMS/D112 showed a small endotherm at 46 °C [64] prior to incubation. Upon incubation, re-crystallization to the β-polymorph melting at 60 °C occurred, which is a slightly lower melting point compared to literature [62]. Furthermore, a wider peak was observed. When Span 85 was added, no changes were observed.

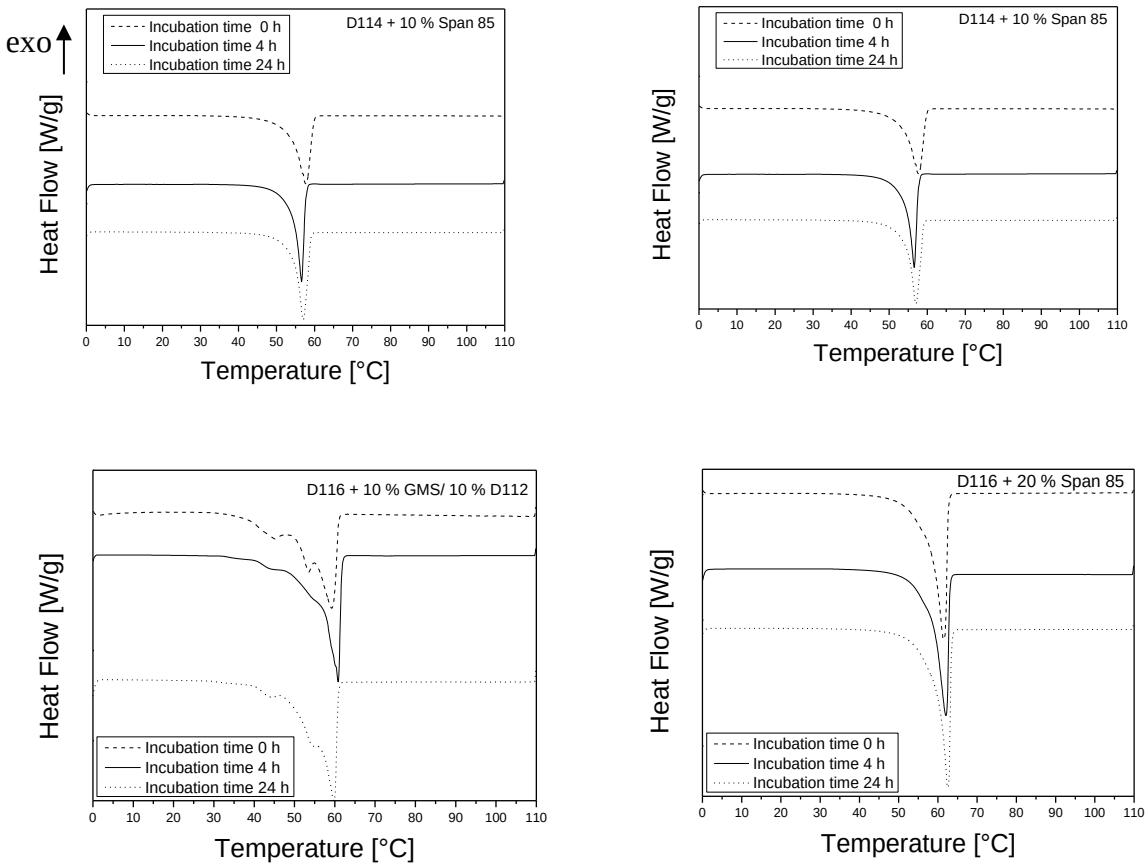


Figure 5-2: Representative DSC thermograms of G [6-D-Phe]-loaded triglyceride microparticles before and after incubation (4 h, 24 h, n = 3) in PBS buffer pH 7.4 at 39 °C. Scans were shifted on the ordinate for better visualization

XRPD measurements were conducted to further investigate the polymorphs (*Figure 5-3)*. D114 raw material showed the characteristic triglyceride pattern of the β-polymorph with three peaks at 18, 22 and 23 ° 2-Theta [65]. This pattern was also visible upon analysis of spray congealed particles of D114 with 10 % Span 40 and D114 + 5 % GMS. GMS raw material revealed two peaks at 18 and 24 ° 2-Theta also indicating the β-polymorph [66]. D116 raw material showed the peaks of the stable β-modification [65]. Microparticles with 5 % Span 40 addition as well as pure Span 40 showed one single peak at 21 ° 2-Theta, confirming a different crystal structure [57]. In contrast, D116 microparticles with 10 % Span 85 clearly exhibited the β-polymorph. As already indicated in DSC measurements, crystallization of the stable modification was not completed in the microparticle formulation D116 + 10 % GMS/10 % D112.

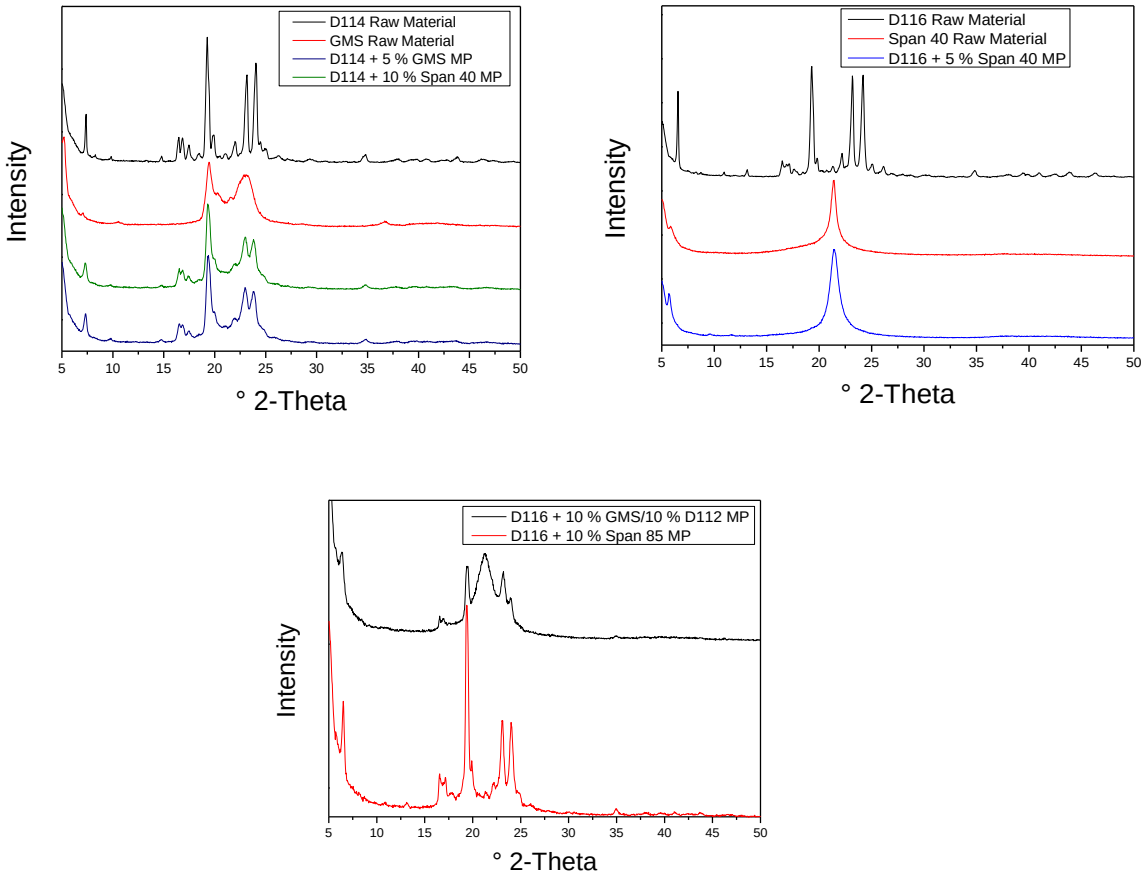


Figure 5-3. Representative XRPD measurements of lipid raw materials and spray congealed lipid microparticles (n = 3). Diffraction patterns were shifted on the ordinate for better visualization

Crystallization behavior of lipid-based delivery systems is influenced by a variety of exogenic factors, especially when manufactured at higher temperatures. Underlying principles are not yet fully understood. In our study, the crystallizing polymorph depended on the used triglyceride itself, as well as the emulsifier. The delay or promotion of crystallization in the thermodynamically most stable β-form depends on the ability of the emulsifier to interact with the crystal lattice of the lipid [67]. D114 with its shorter fatty acid side chains seemed to have an enhanced property to crystallize in the β-polymorph upon spray congealing, irrespective of the emulsifier. D116 showed a partial complete formation of the α-polymorph in combination with 5 % Span 40, as well as in combination with GMS and D112 which indicates a stabilization of α-crystal lattice with these emulsifiers.

5.4.3 Dependency of water uptake of G [6-D-Phe]-loaded microparticles from the lipid composition

To evaluate moisture of freshly prepared particles and their water uptake, the produced formulations were investigated by the means of Karl-Fischer titration. Water uptake between 3 and 30 % was reported for triglyceride cylinders depending on cholesterol content over six months [68]. For triglyceride implants, a water uptake of maximum 10 % depending on the chain length could be demonstrated [69]. The higher water uptake of microspheres compared to cylinders can be explained by a higher surface to volume ratio [68]. Investigations on Gelucire® 50/13 tablets revealed that a continuous water uptake over 8 h to more than 80 % was possible [70]. In our case, particles showed an initial water content between 0.05 % (D114 + 10 % GMS) and 0.19 % (D114 + 20 % Span 80, *Figure* 5-4) after production.

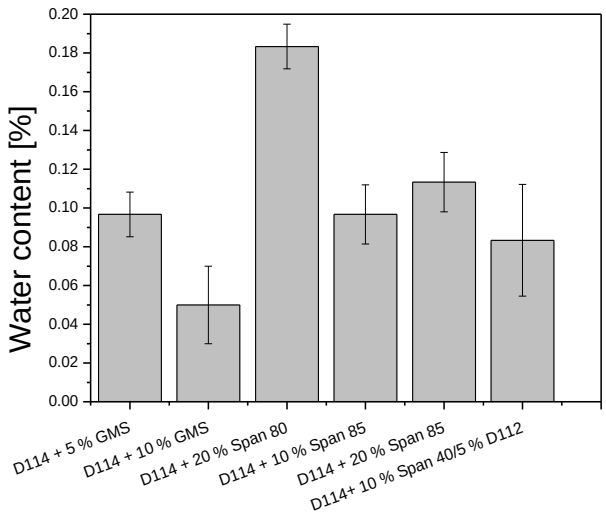


Figure 5-4: Water content of spray congealed microparticles consisting of D114 with different emulsifying additives (mean and SD, n = 3)

After 24 h of incubation, water content of D116-based microparticles with Span 40 and 85 stayed below 3 % (*Figure 5-5*). A higher water uptake between 10 and 30 % was found when D114 was combined with Span 80 and 85. A water content below 10 % was obtained with 5 % Span 40, whereas the combination of 10 % Span 40 with D112 increased the incorporated water to more than 40 %. Vogelhuber et al. identified the chain length of triglycerides as possible tool for swelling and release modification [68]. Addition of GMS to D114 led to an increased water uptake, whereas 5 % GMS addition did not enhance water penetration. In general, D114-based microparticles showed higher water uptake especially in combination with liquid and low melting emulsifiers compared to D116 microparticles.

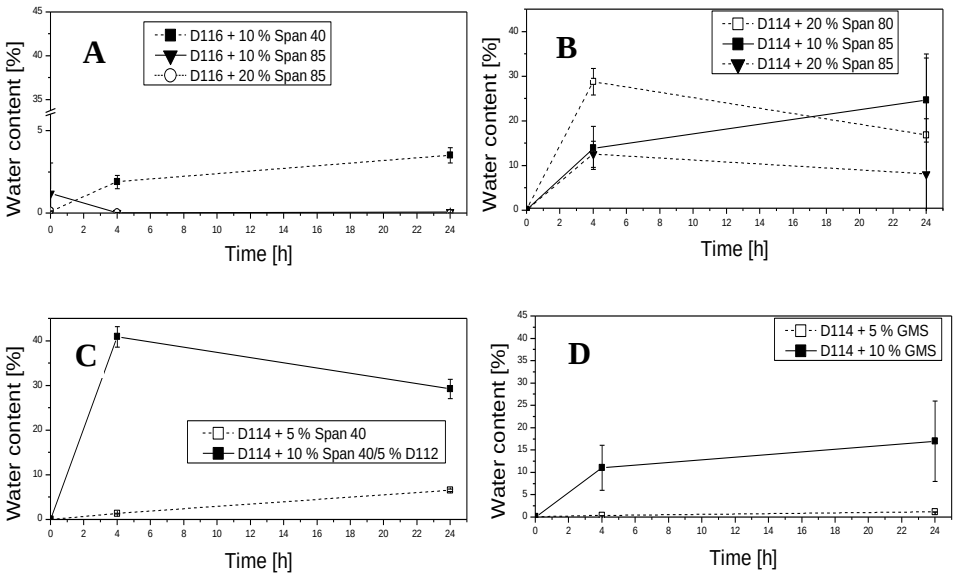


Figure 5-5: Water uptake of spray congealed lipid microparticles after incubation in PBS at 39 °C determined by Karl-Fischer titration (mean and SD, n = 3)

5.4.4 **Impact of lipid-composition on contact angle and surface wettability**

To understand release behavior from lipid matrices, surface wettability can be a helpful tool [71] [72]. In our case, the preparation of flat lipid surfaces was possible with all lipid mixtures. *Figure 5-6* shows the contact angle of highly purified water on formulations based only on mono- or triglycerides. Resulting contact angles are complex results of different influencing factors, like surface roughness and surface tension [72]–[74]. The highest contact angle of 114 ° was observed for the triglyceride with the shortest fatty acid chain, D114. Tripalmitin (D116), tristearin (D118) and triarachidin (D120) showed lower contact angles and following better wettability. Glycerol monostearate showed the best wettability with a contact angle below 90 °. Koennings et al. found out, that wettability of lipid matrices decreases with increasing chain lengths [72]. This trend could not be observed in our experiments. One reason might be, that the lipid was not casted out of an organic solution on the microscopy slide to achieve similar conditions and contraction behavior compared to the spray congealing process. This procedure seemed suitable for our purpose, as the surface structure and the crystallization behavior affect wettability [75]. This was also suggested by Del Curto et al., who reported improved wettability of co-melted particles compared to those prepared by solvent-stripping [4].

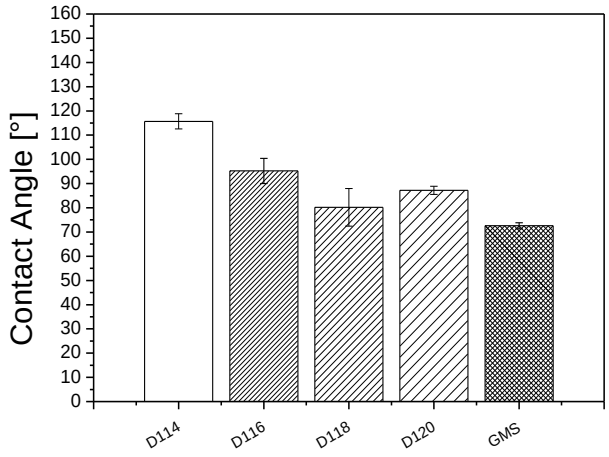


Figure 5-6: Contact angle of highly purified water on triglycerides (D114, D116, D118, D120) and GMS (mean and SD, n = 3)

Figure 5-7 shows the contact angles of D114-emulsifier mixtures. GMS addition led to improved wettability at 10 %, whereas the contact angle was increased at only 5 %. Concentrations above 20 % Span 40 and 10-20 % Span 80 reduced the contact angle dramatically. No pronounced effect was visible for a Span 85 addition.

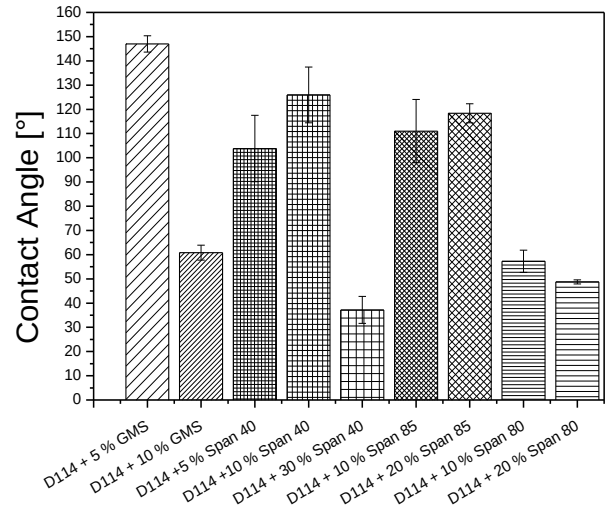


Figure 5-7: Contact angle of highly purified water on D114-emulsifier mixtures (mean and SD, n = 3)

Replacing D114 by D116, no pronounced effect on contact angles was observed in combination with 5 % Span 40 and 10 % D112 (*Figure 5-8*). A stepwise reduction was found when using Span 85. Formulation with the low melting triglyceride D112, the contact angle was raised to 123.61 °. A remarkable reduction in contact angle was observed using GMS and Span 60 as well as Span 85 in higher concentrations.

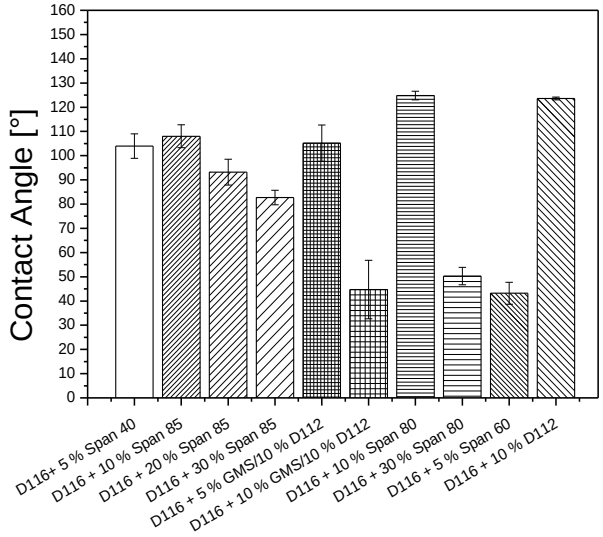


Figure 5-8: Contact angle of highly purified water on D116-emulsifier mixtures (mean and SD, n = 3)

Admixing emulsifiers to triglycerides led to enhanced wettability depending on type of triglyceride and emulsifier type and its concentration. Surface roughness and the so called "blooming" of triglycerides needs to be taken into account, when investigating the wettability of lipid surfaces. The smooth surfaces reported for the α-modification [76] are known to obtain increased wettability compared to those of the platelet- or flake-like surface of the β-polymorph [77] [78]. Consequently, polymorphic transformations need to be taken into consideration, as we showed that crystallization behavior also depended on emulsifier-triglyceride composition.

5.4.5 In vitro release behavior of G [6-D-Phe] from lipid microparticles

Microparticles based on pure D116 and D118 were investigated concerning their G [6-D-Phe] release behavior. In both cases a low burst between 10 and 25 % resulted, followed by only minimal release within the subsequent two weeks (*Figure 5-9)*, according to literature [2]. This goes in line with the fact that the pure triglyceride microparticles do not take up substantial amounts of water and G [6-D-Phe] only dissolves at the surface. Comparable release profiles from lipid microparticles were observed for desmopressin [79] [80]. Additionally, the used theoretical drug load of 1.8 % was insufficient to form a connected pore network. In literature, drug loads above 10 % are reported for implants to be sufficient for a formation of connected pores and to achieve sustained protein release [81].

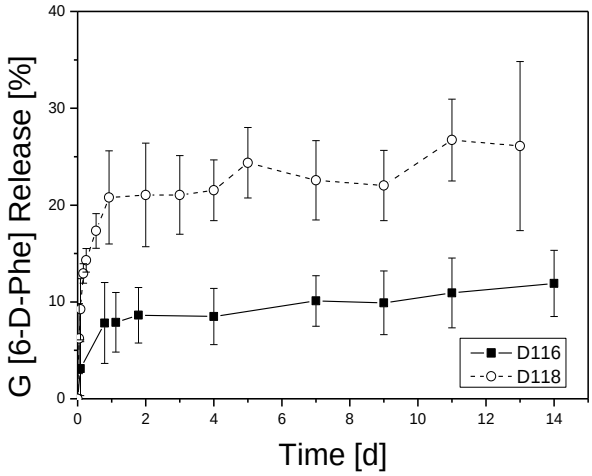


Figure 5-9: G [6-D-Phe] release from triglyceride microparticles (D116 solid line, D118 dashed line; mean and SD, n = 3)

To enhance peptide release, combinations of triglycerides with non-ionic emulsifiers were tested. Glycerol mono- and distearate have been reported to enhance peptide release from lipid microspheres [46] [79]. *Figure 5-10* gives an overview on the influence of a CSA- and GMS-addition to triglycerides on G [6-D-Phe] release. The addition of CSA to D116 led to an increased burst compared to pure D116 already at 10 %. Increasing the CSA content above 20 %, about 85 % of the drug were already released as burst. Microparticles prepared from 10 % GMS and 90 % triglyceride exhibited a high burst release of 55 %, followed by a continuing release until day 14. In total, 70 % of the incorporated drug was released. With 20 % GMS, the burst release was comparable and the sustained release could be monitored until day 12. An immediate release was obtained when 40 % GMS were added.

SEM micrographs of freshly prepared D118 microparticles with 10 % GMS showed particular shape before and after incubation, with increased roughness after incubation, indicating possible matrix changes like “blooming” (*Figure 5-11*) [78].

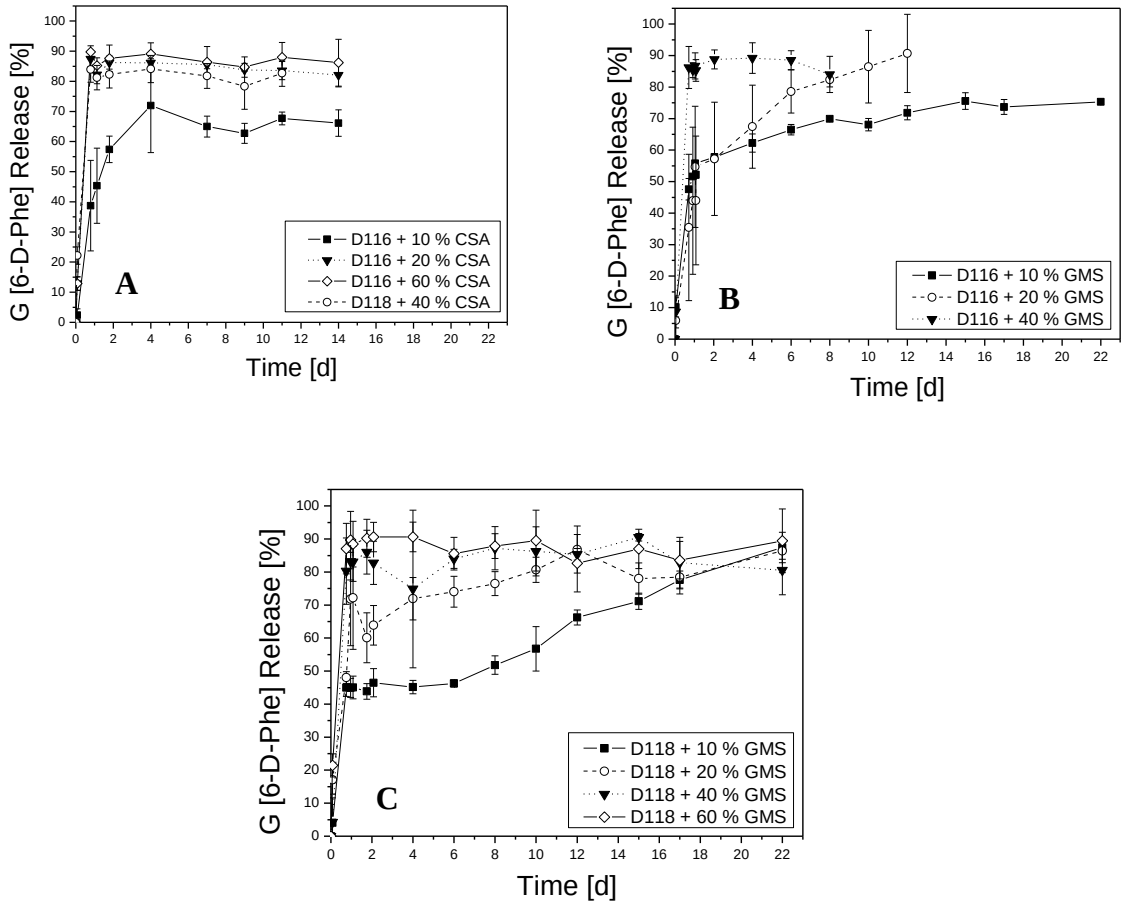


Figure 5-10: G [6-D-Phe] release from triglyceride microparticles with varied amounts of CSA (10, 20, 40 %) (A). G [6-D-Phe] release from D116 (B) and D118 (C) microparticles with varied concentrations of GMS (10 %, 20 %, 40 %, 60 %), mean and SD, n = 3

The use of D118 with 10 and 20 % GMS was most promising, resulting in a burst release between 45 and 60 % and a continuous release over 14 to 22 d releasing more than 80 % of the incorporated drug. Further increase of GMS did not lead to a sustained release profile, possibly due to a monoglyceride-induced matrix instability (*Figure 5-10 C*) [79]. Incomplete drug release (only 80-90 %) is frequently observed for lipid-based drug delivery systems [82], possibly due to an incomplete water accessibility of the core [83] [84] or an adsorption to matrix material [80] [85].

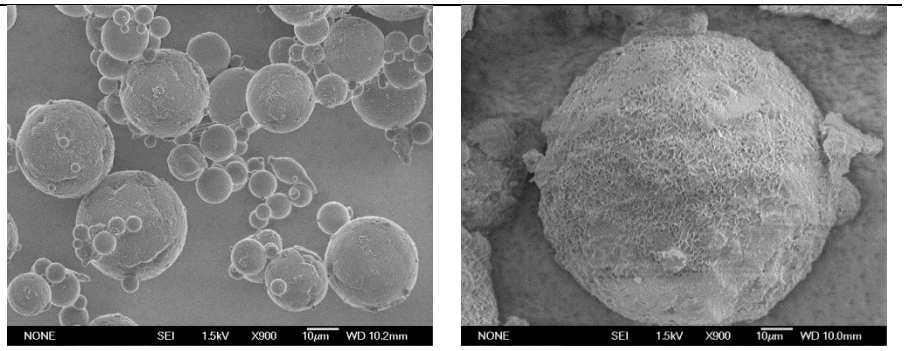
D118 + 10 % GMS	

Figure 5-11: SEM images of freshly prepared microparticles of D118 + 10 % GMS (left) and after a 4-week incubation in PBS at 39 °C (right)

To generate particles with a shorter release than 22 d additional mixtures of triglycerides with emulsifiers were tested. Therefore, a variety of emulsifying substances with different HLB-values were introduced. Furthermore, the low melting lipid D112 was added to enhance release rates as reported for solid lipid implants [49]. *Figure 5-12* gives an overview on release behavior of screened batches.

Having achieved adequate results with the combination of D116 with GMS, this additive was tested in combination with D114. Release from D114 microparticles with as well 5 and 10 % GMS was completed after 3 d releasing 70 % to 90 %. SEM images revealed that the GMS-containing microparticles still remained their shape (*Figure 5-13* exemplarily shows data of D114 + 10 % GMS). An increased roughness upon incubation could be observed, indicating swelling of the matrix followed by an enhanced porosity and water uptake [68] [86].

The addition of the liquid emulsifiers Span 80 and 85 with low HLB values did not show concentration dependent effects. The faster and more complete release could be explained by the liquid state of Span 85 leading to a faster drug liberation and complete degradation of the microparticles, independent of the added concentration as visualized by SEM images (*Figure 5-13* exemplarily shows D114 + 20 % Span 85). Overall, an almost complete drug release could be observed in all three tested formulations.

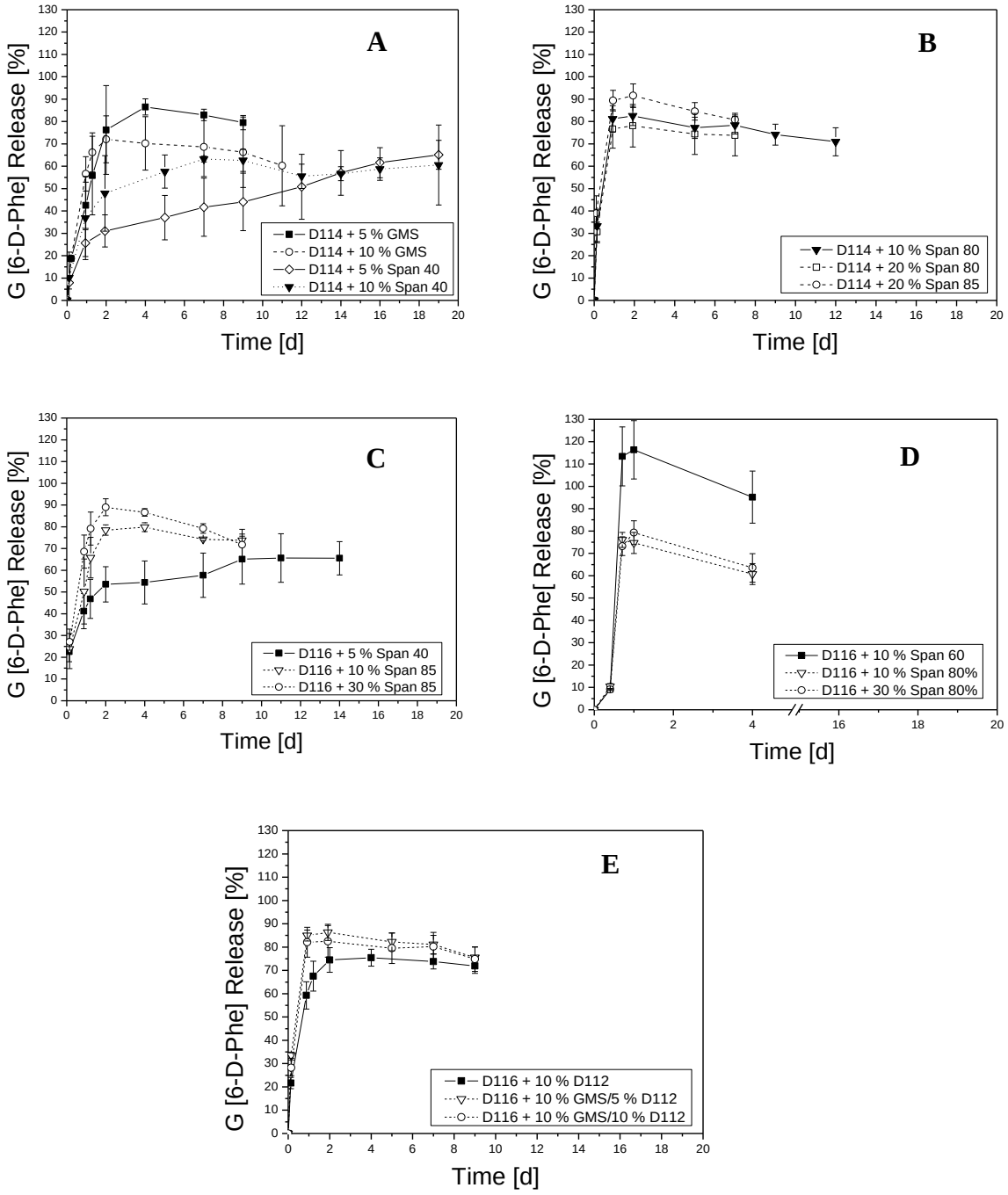
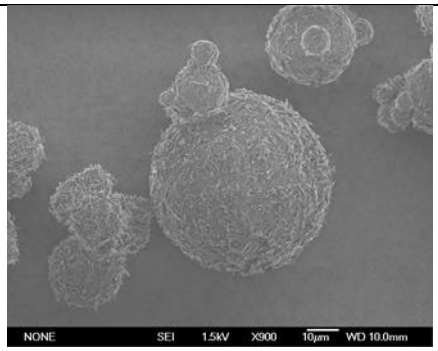
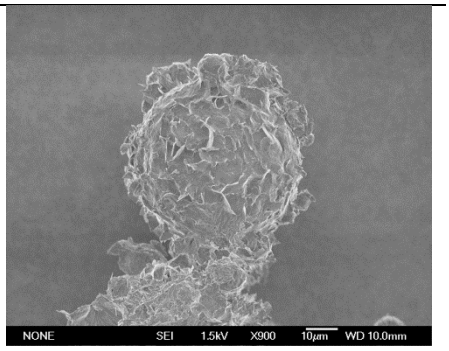
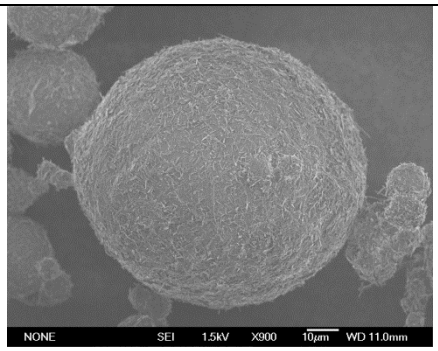
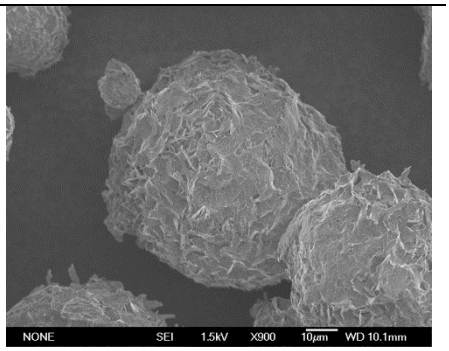
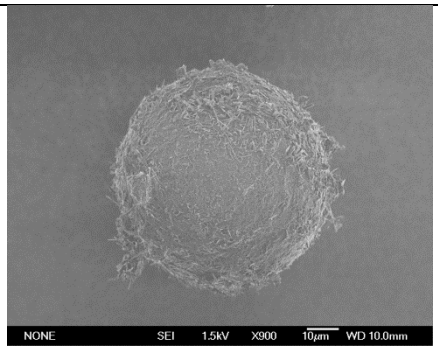
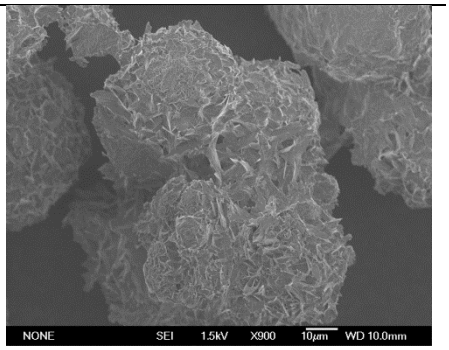
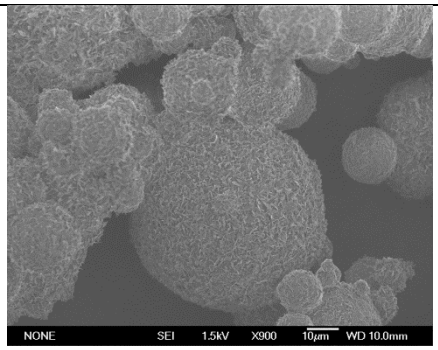
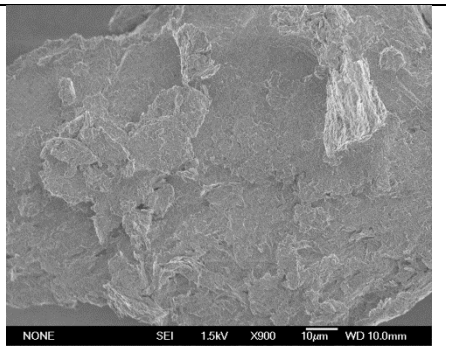
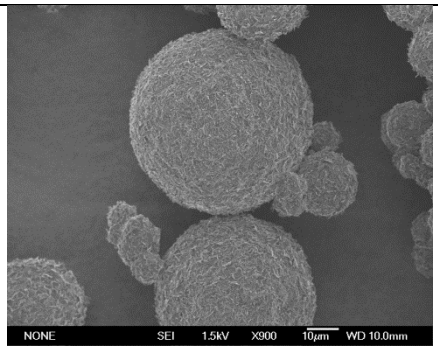
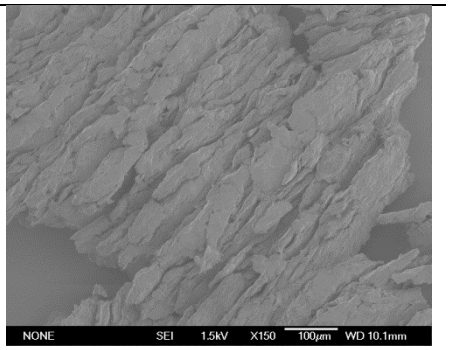
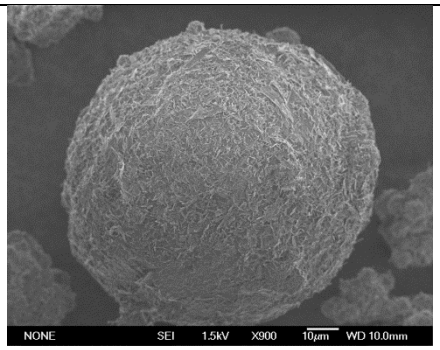
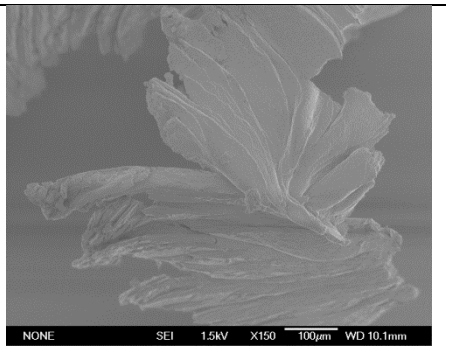
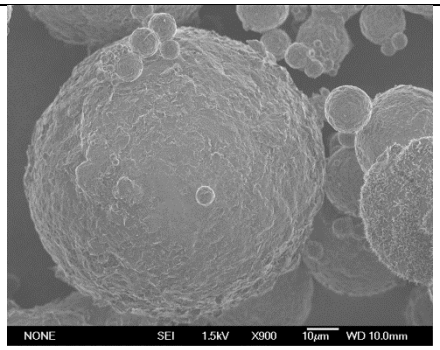
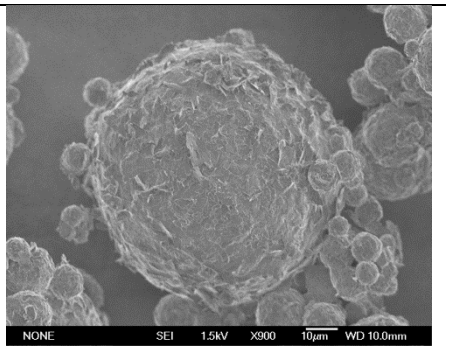
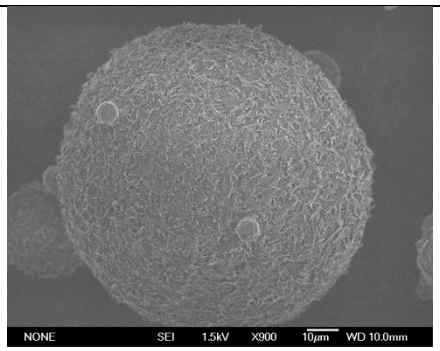
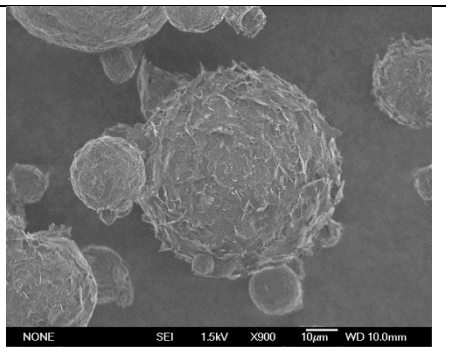
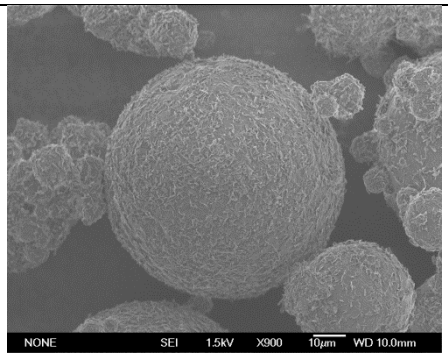
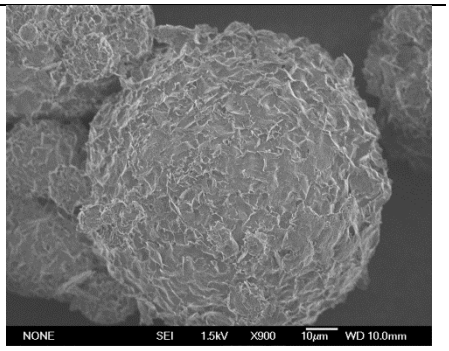
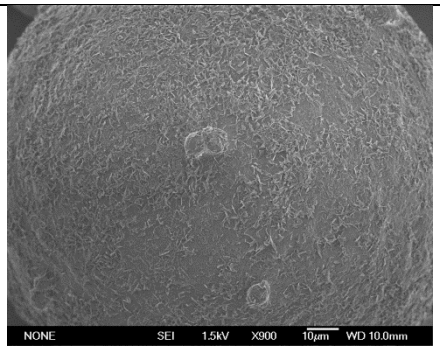
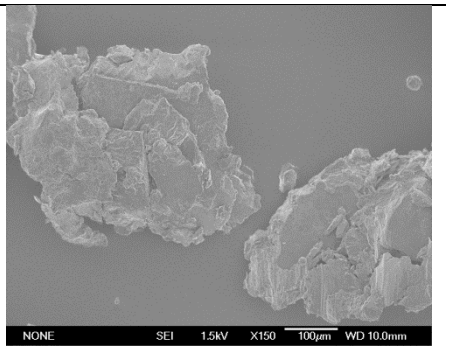


Figure 5-12: G [6-D-Phe] release from D114 microparticles with addition of Span 40 and GMS (A) and Span 80 and 85 (B), D116 microparticles with addition of Span 85 (C) and Span 80 (D) and with addition of D112/GMS and D112 (E), mean and SD, n = 3

The addition of Span 85 to D116 instead of D114 resulted in almost complete drug release within one day, whereas the particles were stable over two weeks exemplarily shown for D116 + 30 % Span 85 (*Figure 5-13*). Addition of Span 60 and 80 (*Figure 5-12 D*), as well as the combination of GMS and D112 (*E*) also resulted in a high burst release of 80 to 100 % from D116 microparticles. Only in case of the Span 60 containing microparticles the high burst release could be explained by complete disintegration of the matrix. The other combinations resulted in microparticles which kept their shape.

Adequate release until day 20 could be observed for D114 microparticles with 5 % Span 40. Using D116 as base lipid, release was limited to 9 days. (*Figure 5-12 C*). Both particle qualities remained intact (*Figure 5-13*). A G [6-D-Phe] fraction remained inside the particles due to insufficient interconnectivity of pores. The development of a rougher surface upon incubation reflects the enhanced water uptake into the core of the microparticles [83].

D114 + 10 % GMS		
D114 + 5 % Span 40		
D114 + 10 % Span 40		
D114 + 20 % Span 85		
D114 + 20 % Span 80		

D 114 + 10 % Span 40/5 % D112		
D116 + 30 % Span 85		
D116 + 10 % D112		
D116 + 5 % Span 40		
D116 + 5 % Span 60		

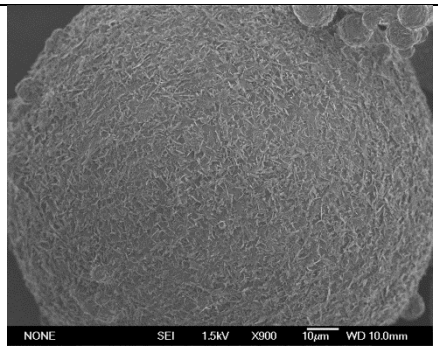
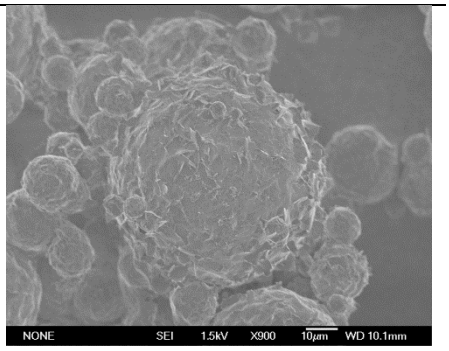
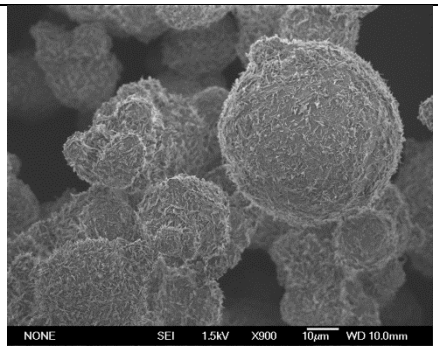
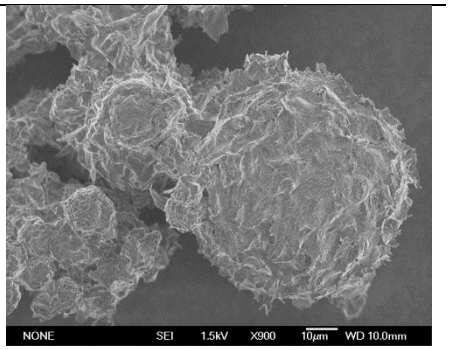
D116 + 30 % Span 80		
D116 + 10 % GMS/ 10 % D112		

Figure 5-13: SEM images of freshly prepared (left) and incubated D114 and D116-based particles with addition of Span 40, 60, 80, 85, GMS and D112 for 2 weeks in PBS buffer at 39 °C (right)

Consequently, pure triglyceride matrices did not lead to appropriate release profiles as they do not allow substantial water uptake. Furthermore, the drug load is low and thus an interconnected pore network cannot form upon drug dissolution. Thus, the addition of surfactants plays a major role [84] [87]. Mixing emulsifying agents with triglyceride base materials led in general to an enhanced drug release. Some emulsifiers like Span 40, GMS and CSA showed concentration dependent effects. GMS is reported to either retard [83] [84] or enhance drug release [82] [88] from lipid-based microparticles. We noticed the latter in our study.

Furthermore, the parameters wettability, polymorphic state as well as surface structure seem to influence the release behavior. In some test formulations, we noticed particle breakdown as a consequence of good wettability (D114 + 10 % Span 60). In other formulations, the particle collapsed despite insufficient wettability (D114 + 10 % Span 85), indicating that incubation time and temperature caused the mechanical breakdown.

Crystallization processes occurring in lipid matrices upon accelerated temperatures are reported to influence release behavior [67] [75] [78]. It is not fully understood, whether the polymorph itself or the rearrangements of the crystal lattice are responsible for changes in drug liberation [44] [78]. The influence of the polymorphic state on release could be explained by different mechanisms. On the one hand, the less densely packed hexagonal α-modification may lead to an enhanced drug release compared to the more dense, triclinic β-form [44] [89]. On the other

hand, structural changes upon re-crystallization like blooming, are supposed to influence the drug release [78]. An appropriate release profile could be found throughout the study in microparticles crystallizing in either α- or β-modification. As shown for the D116, GMS and D112-composed particles upon incubation at 39 °C, a transition to the stable polymorph is enabled. Appropriate release profiles over two weeks could be achieved in vitro with microparticles containing either 10 % GMS or 5 % Span 40. These particles kept their shape and had the ability to take up adequate amounts of water.

The ideal candidate for a potential sustained release delivery system for G [6-D-Phe] should guarantee a sustained peptide release of 15 days to ensure cycle blockage in gilts. Based on this requirement, in the first pre-clinical study we focused on the D116 and D118 formulations with 10 % GMS which provided an in vitro release over 22 days with a high burst (*Figure 5-14 A*). Taking the presence of lipases in the mammalian body [90] [91] and a possibly higher degradation rate of the lipid microspheres, body movement and temperature into account, a longer in vitro release than 15 d may result in a promising in vivo effect [50] [92].

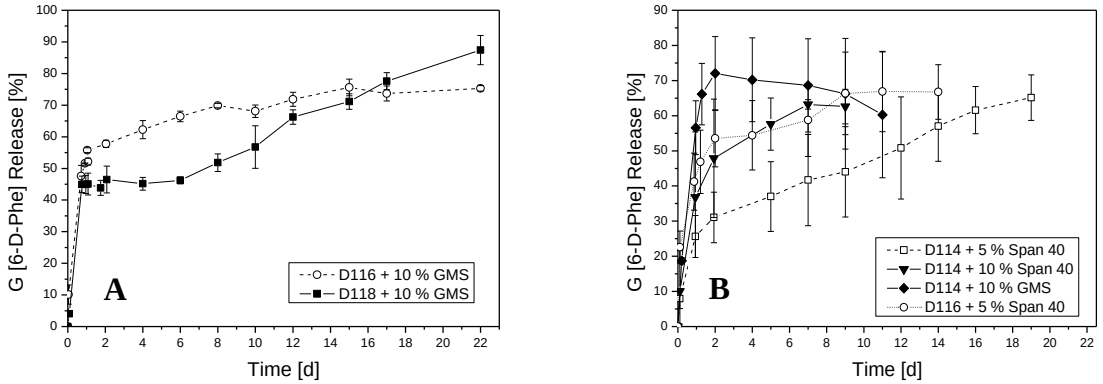


Figure 5-14: Identified candidates for clinical studies. (A) Suitable microparticle batches based on D116 and D118 with 10 % GMS applied in pre-clinical study I (September 2014). (B) Formulations prepared of D114 and D116 with different amounts of Span 40 and GMS used in pre-clinical study II (August 2015)

In the second pre-clinical study, microparticles with a faster release based on the combination of D114 with 5 and 10 % Span 40, 10 % GMS as well as D116 + 5 % Span 40 were evaluated (*Figure 5-14 B*).

5.4.6 G [6-D-Phe] release from lipid-based microparticles suspended in thermosensitive poloxamer gels

In order to cope with the problem of a high initial burst, suspensions of microparticles in a thermosensitive poloxamer-based gel were evaluated. Wenzel et al. reported the delivery of deslorelin and GnRH in a thermosensitive poloxamer gel assuring delayed plasma peak levels for the gel compared to the solution after injection in cattle [93].

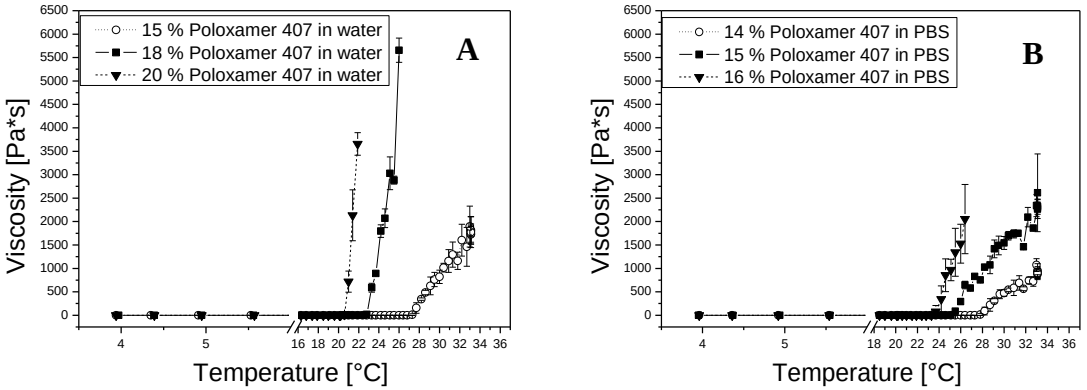


Figure 5-15: Viscosity measurements of PG in water (A) and PBS pH 7.4 (B) using a temperature ramp from 4 to 34 °C (mean and SD, n = 3)

Viscosity measurements revealed a temperature-dependent gelling behavior controlled by poloxamer concentration, whereas gelling showed an earlier onset when PBS was used (15 % poloxamer 407, *Figure 5-15*) confirming findings from literature [94]. The formulation should show a low viscosity at ambient temperature for application and a rapid gelling when brought into the tissue. Thus, formulations with 18 and 20 % poloxamer 407 were thought not feasible for our purpose, as they already showed an increased viscosity at 24 °C and 20 °C. Kempwade et al. found gelling temperatures of 32-34 °C for 18 % and 26 °C for 20 % poloxamer gels [95]. Compared to the aqueous solution of G [6-D-Phe], the release through the gel was delayed. According to other studies, the in vitro release from poloxamer 407 gels is rather short in a range of a few days [96]. 80 % of rIL-2 was released within 8 h from a poloxamer 407 gel in vitro [97]. The prolonged release of G [6-D-Phe] from the gel observed in our study may be caused by protection of the gel from dissolution by the dialysis tubing and the incubation temperature of 39 °C [98] [99]. As free fatty acids are reported to enhance mechanical strength of poloxamer gels, possible interactions with lipid microparticles and surfactants cannot be excluded [94].

The initial burst of microparticles could be reduced compared to suspension in PBS. D114 + 5 % Span 40 did not reveal a reduced burst, but an overall decelerated release (*Figure 5-16*).

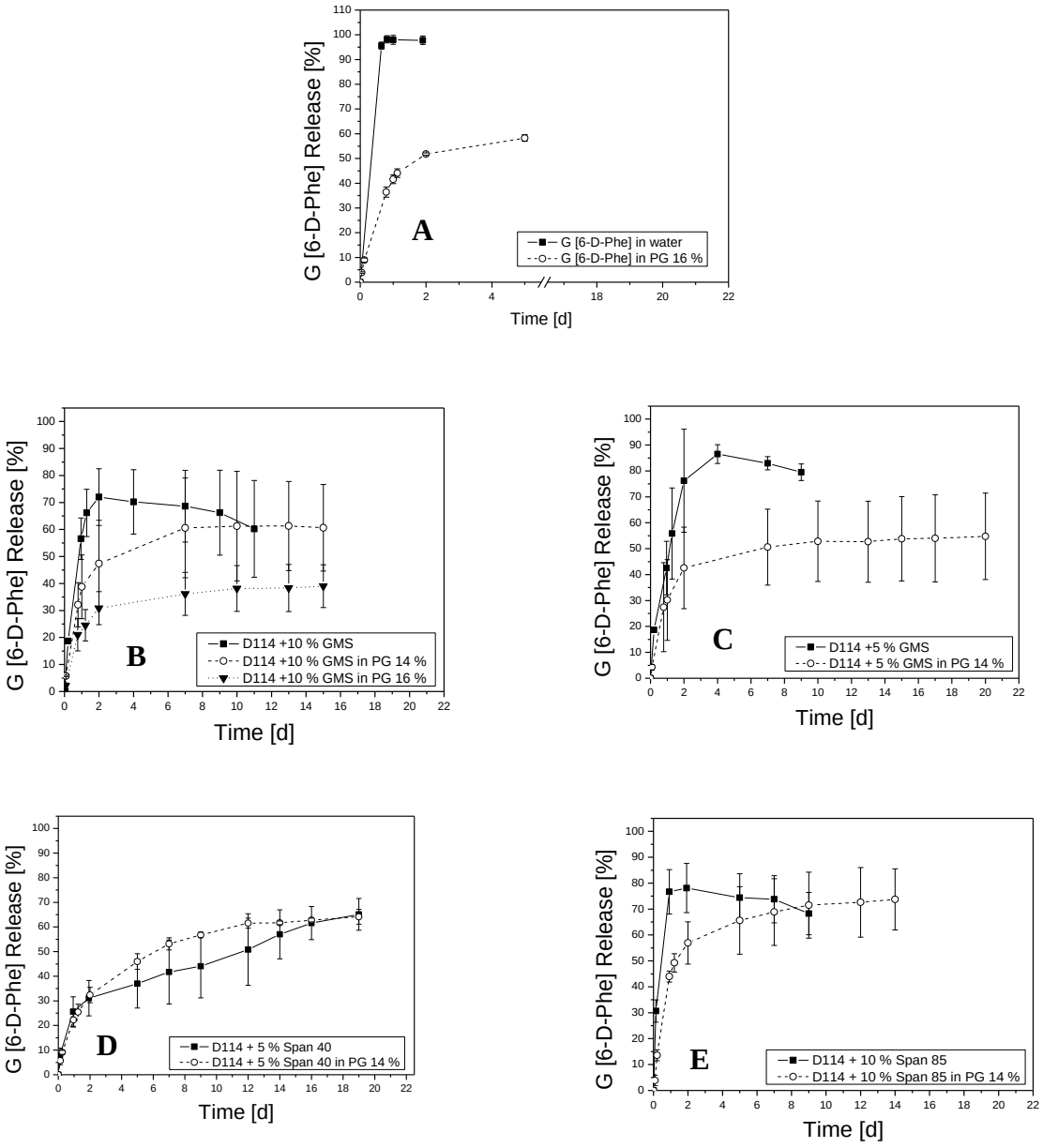


Figure 5-16: Release profiles of G [6-D-Phe] dissolved in water and PG 16 % (A) and drug-loaded microparticles after suspension in thermosensitive poloxamer gels (PG) with concentrations of 14 and 16 % (B-E) mean and SD, n = 3

5.5 Conclusion

The aim of this chapter was to investigate whether it is possible to incorporate G [6-D-Phe] into lipid-based microparticles to achieve a continuous release over 15 days, as required for estrus synchronization. Based on previous results with hydrophilic model substances, different triglycerides and surfactants were tested concerning in vitro release behavior, wettability of the lipid matrix as well as particle morphology before and after incubation. The use of pure triglycerides did not result in appropriate release behavior. The addition of release modifying substances was mandatory. With addition of emulsifiers it was possible to tailor the release kinetics concerning burst and completeness of drug release. With the use of GMS and Span 40 it was possible to achieve a release duration of 22 (D118) and 20 d (D114). The burst release could be reduced by the use of suspensions of triglyceride microparticles in thermosensitive poloxamer gels.

To understand the underlying release mechanisms from lipid-based microspheres, DSC and XRPD measurements gave an insight into the crystallized polymorph. In addition, Karl-Fischer titration showed that water uptake and swelling behavior of triglyceride matrices could not be generalized for surfactant type or HLB-value and was more pronounced for short-chain triglycerides in combination with liquid emulsifiers. Surface wettability of triglyceride matrices was insufficient and could mostly be improved by addition of surfactants. The combination of different triglycerides resulted in higher contact angles compared to the individual triglyceride formulation.

5.6 References

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6 EX VIVO PRE-EVALUATION OF PARTICLE- AND MODEL-DRUG DISTRIBUTION IN CARCASSES

Parts of this chapter are intended for publication

Investigations on swine carcasses were performed with the help of ***Dr. Haukur Lindberg Sigmarsson*** at the University of Leipzig (**December 2013**)

6.1 Abstract

In order to understand the distribution behavior of spray congealed lipid microparticles, dye-loaded microparticles were injected after suspension in a hydrophilic solvent into the neck or caudal thigh muscles of swine carcasses. The injection was performed using 2-ml syringes with 16 or 18 G needles and with an injection revolver for the application of multiple units. The aqueous resuspension medium spread rapidly, confirmed by ultrasound as well as opening the injection site by incision. The microparticles could be found at the injection site upon incision. With the help of ultrasound, it was not possible to detect the microparticles in the muscle.

6.2 Introduction

Microparticulate drug delivery systems are typically applied subcutaneously or intramuscularly [1] [2]. Aiming mostly for a systemic effect with a controlled or delayed release [3]–[5], it is important to investigate the distribution of microparticles after injection. This includes whether the microparticles are enclosed by a fibrous capsule, and if a local inflammatory reaction or transport takes place in vivo [6] [7]. A general problem arising from a depot formation may be a high local concentration of the residual drug [8]–[11]. The injection of depot formulations may also impair meat quality at the injection site on food producing animals [12]. The use of biodegradable microspheres or liposomes may minimize tissue lesions in food producing animals [11] [10].

Our purpose was the development of a delivery system, which is able to control the release of the peptide Gonadorelin [6-D-Phe] in the porcine muscle tissue and which is easy to inject. The reconstituted microparticles are intended for application via a syringe with a 16 or 18 G needle, but for a more effective injection for multiple animals, the use of an injection-revolver should be preferred. This device is commonly used for veterinary products with a higher importance of multiple-dose delivery systems, also mirrored in a variety of patents [13]–[15]. Consequently, the distribution of microparticles was to be studied in carcasses in order to understand the spreading behavior and to gain a preliminary insight into suspension performance in the injection device for the upcoming pre-clinical studies. Additionally, using the dye-loaded microparticles, the dye distribution in the surrounding tissue was to be assessed.

6.3 Materials and Methods

6.3.1 Materials

Methylene blue and crystal violet, as well as polysorbate 20 (PS 20) were purchased from Merck KGaA (Darmstadt, Germany). Dynasan 116 (tripalmitin, D116) was kindly provided from Cremer Oleo (Witten, Germany). The gelling agent Tylopur® C 300 (sodium carboxymethylcellulose, Na CMC) was kindly donated from Clariant (Muttenz, Switzerland). The suspended microparticles were applied via a 2-ml syringe equipped with a 16 or 18 G-needle (B. Braun Melsungen AG, Melsungen, Germany) or an injection revolver HSW ECO-MATIC® (Henke Sass Wolf GmbH, Tuttlingen, Germany) with an injection volume of 2.0 ml. The needle used in this study had a length of 40 mm and a diameter of 2 mm.

Two swine carcasses used in this study were fully mature German Landrace x Piétrain and were kindly donated by the Faculty of veterinary medicine of the University of Leipzig.

6.3.2 Preparation of dye loaded lipid microparticles by spray congealing

For preparation of 1 % dye-loaded microparticles, the lipid dispersion was filled in the sample container of the B-290 Mini spray dryer with additional spray chilling setup (Büchi, Flawil, Switzerland), which was pre-conditioned for 1.5 h prior to the production step. The additional equipment consisted of a sample container, surrounded by a bath filled with polyethylene glycol (PEG) 400, also heating the nozzle. The connection between nozzle and sample container was heated externally by a resistance wire-based heating, generating a temperature of approximately 70 °C. The spray tower was fed with pre-cooled air produced by an additional dehumidifier Deltatherm® LT (Deltatherm® Hirmer GmbH, Much, Germany). Lipid melt was sprayed using nitrogen as spray gas and the following apparatus settings:

Spray-parameter	Value
Spray pressure [bar]	5
Spray flow [l/h]	357
Aspirator power [%]	100
Valve opening	1.25 turns
Temperature oil bath [°C]	90
Inlet temperature [°C]	15
Pressure filter [mbar]	96

The produced lipid microparticles were separated using the high-performance cyclone from Büchi and stored at 2-8 °C.

6.3.3 Scanning Electron Microscopy

A Jeol JSM 6500-F SEM (Jeol, Tokyo, Japan) was used at 2 kV. Particles were fixed on aluminum sample holders with double adhesive tape (Plano, Wetzlar, Germany) and were analyzed without an additional graphite coating.

6.3.4 Preparation of the reconstitution medium

Na CMC was added to an aqueous 0.02 % PS 20 solution under stirring in concentrations of 0.5, 1.0 and 2.0 % (m/V) until a clear solution was obtained.

6.3.5 Application of microparticles

Approximately 50 mg of microparticles were mixed with 7 ml of reconstitution medium and shaken until a homogenous suspension was obtained. For ultrasound investigations, it was tried to visualize the microparticles in a higher concentration by suspension of 50 mg in 4 ml of the reconstitution medium. 2 ml of the suspension were injected into the lateral neck (*m. rhomboideus cervices, m. rhomboideus capitis, m. semispinalis capitis, m. complexus, m. serratus ventralis cervices*) or caudal thigh muscles (*m. biceps femoris, m. semitendinosus, m. semimembranosus*) via a 2-ml syringe using a 16/18 G needle or the injection revolver. The muscle tissues were examined concerning visibility of a bolus, distribution of hydrophilic color and localization of the microparticles. Ultrasonographic examination was performed using an esaote myLab ultrasonic device (Esaote Biomedica Deutschland GmbH, Köln, Germany).

Additionally, the injection sites were examined surgically. Therefore, the skin was cut crosswise with a scalpel. The cross-section was carefully held open to examine and to document the color of the spreading dye and the localization of the microparticles.

6.4 Results and Discussion

None of the tested suspensions with 0.5, 1 or 2 % Na CMC showed sedimentation effects during the handling procedure within the study and could be loaded into 2-ml syringes and into the injection revolver. Suspensions of methylene blue-loaded microparticles showed a light blue color approximately 10 min after preparation (*Figure 6-1 B)*, indicating a surface burst release of the dye into the surrounding medium. Suspensions of crystal violet showed a minor coloration and were therefore used for the determination of the particle behavior within the tissue (*C*).

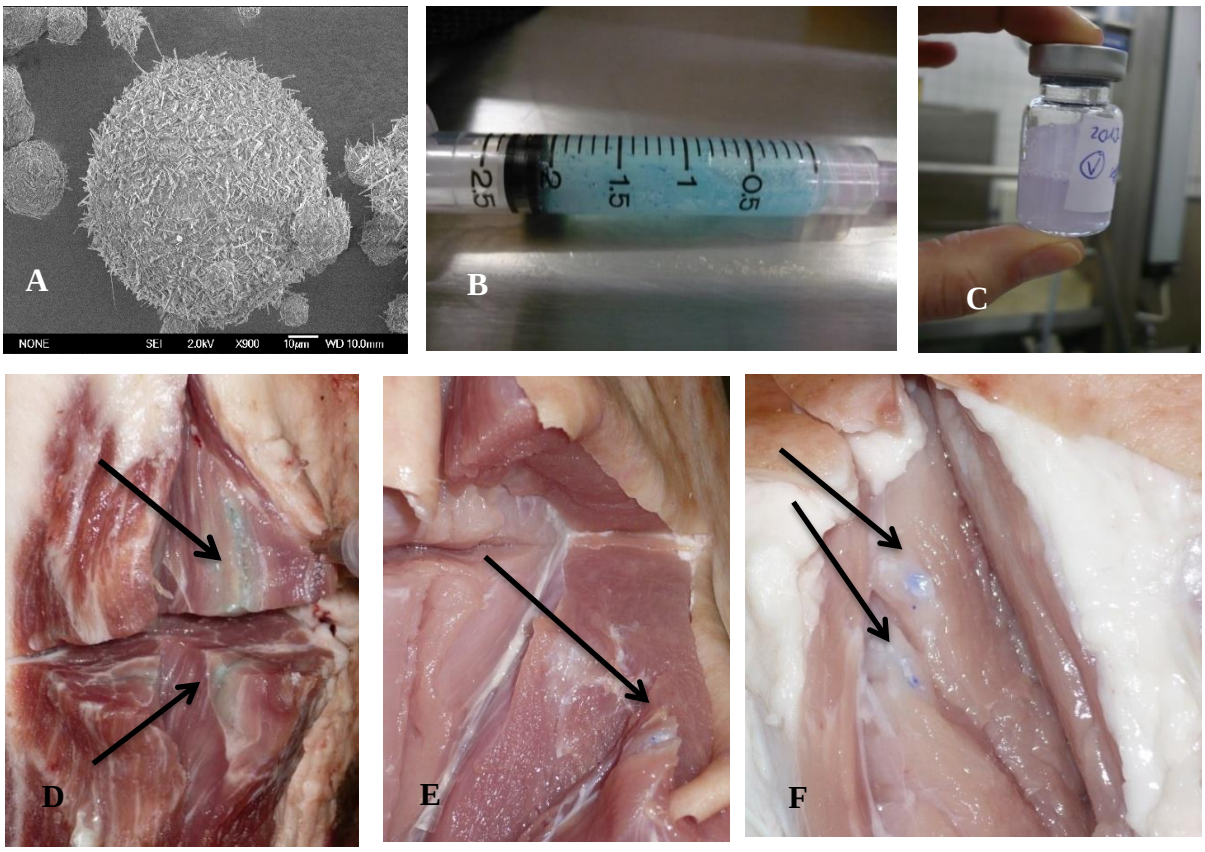

Figure 6-1: SEM image of methylene blue-loaded microparticles (A). Suspension of methylene blue-loaded lipid microparticles prior to application via a syringe (B); crystal violet-loaded lipid particles prior to application (C) distribution of methylene blue along the muscle fibers in the lateral neck muscles after injection and longitudinal and horizontal incision (D); crystal violet-loaded-lipid microparticles after injection in the lateral neck muscles (E) and after injection in the caudal thigh muscles (F)

Both suspensions of methylene blue- and crystal violet-loaded microparticles were easily syringeable via 16 and 18 G needles, as well as via the injection revolver into the lateral neck muscle. *Figure 6-1 D* shows the distribution of the dye along the muscle fibers, indicating a rapid diffusion of hydrophilic dye into the surrounding tissues although using a carcass. The diffusion might even be enhanced when additional movement and body temperature are given. Particle residues were not carried away from the injection site and remained in place (*Figure 6-1 E, F*). Generally, lesions at injection sites are reported in literature for different kinds of food producing animals, which can affect the meat quality concerning tenderness and texture [16]. Moreover, it is a fact that a more severe damage in food producing animals is caused by the injection of suspensions compared to the injection of solutions [10] [12].

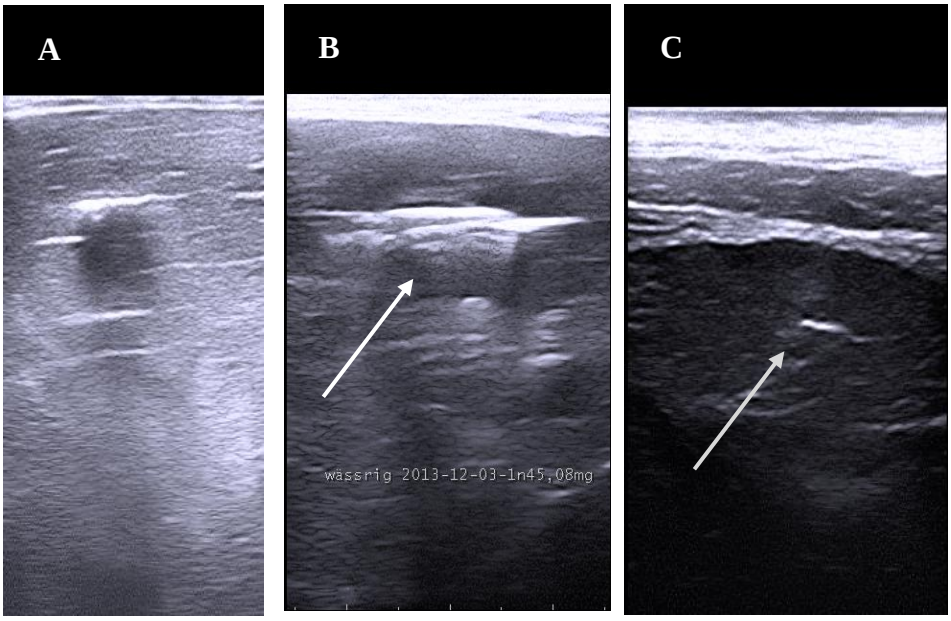

Figure 6-2: Ultrasonographic examination of the lateral neck muscles (A), the caudal thigh muscles directly after injection (B) and 1 h after injection (C)

Additionally, both injection sites, lateral neck and caudal thigh muscles, were characterized via ultrasonography (*Figure 6-2 A-C*). The pig skin and underlying fat layers demonstrate several peculiarities in contrast to other mammalian skin. The fat layer consists of five sublayers with different thicknesses and fatty acid compositions [17] [18]. Since it was not possible to visualize the placement of microparticles after injection into the lateral neck muscles, as they are covered by a thicker fat layer, the caudal thigh muscles seemed to be a more suitable site for injection and monitoring by ultrasonography. Ultrasound images showed faint shadows along the muscle fibers indicating the reconstitution medium, underlining the results obtained by incision [19]. Immediately after injection, the liquid reconstitution medium was clearly noticeable (*Figure 6-2 B*), but almost faded away after one hour (*Figure 6-2 C*). Nevertheless, the tested microparticles remained in the muscle tissue. The formation of a temporary depot in the tissue

due to enhanced viscosity of the reconstitution medium, as visible after injection of oily suspensions or in-situ forming implants [20], was not observable in both injection sites. For oily suspensions, the depot-effect is dependent on the solubility of the drug in the medium and the surrounding tissue [21].

6.5 Conclusion

The ex vivo pre-testing studies gave us first insights into the behavior of lipid microparticles after injection into the two major injection sites in swine. Furthermore, it was possible to evaluate the injection via a single-dose injection with a syringe, or a multiple-dose device, the commonly used injection revolver. In both cases, the injection could be easily performed.

The tested hydrophilic dye was partly dissolved out of the lipid particles upon reconstitution and could be found along the muscle fibers, whereas the lipid particles remained on the injection site. These findings could be confirmed by investigations using ultrasounds, whereby a visualization of the microparticles was not possible. The reconstitution medium consisting of Na CMC in concentrations of 0.5, 1.0, 2.0 % (m/V) and PS 20 0.02 % (m/V) seemed to be a good candidate for further development and optimization, with respect to pH and isotonicity for a following use in the in vivo study. In addition, it would be beneficious to keep the amount of injected microparticles in a low range to avoid tissue lesions leading to minor meat quality.

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7 IN VIVO EVALUATION OF G [6-D-PHE]-LOADED SOLID LIPID MICROPARTICLES WITH SPECIAL REGARD ON ESTRUS CONTROL

Parts of this chapter are intended for publication

Data and results in this chapter were gained in cooperation with *Prof. Dr. Johannes Kauffold* and the **Faculty of Veterinary Medicine of the University of Leipzig**. Investigations on swine were performed by *Catherine Poser* and *Rosa Stark*, PhD students of *Prof. Dr. J. Kauffold* with the help of *Dr. Haukur Lindberg Sigmarsson* and *Dr. Matthias Hoops*. Analysis of the obtained data with corresponding statistics was performed by me at the LMU in Munich.

7.1 Abstract

For evaluation of an ecologically compatible alternative intended for cycle blockage and estrus control, G [6-D-Phe]-loaded lipid microparticles were tested in vivo. Two different reconstitution media were applied to investigate applicability and syringeablilty of the suspension, revealing suspension properties for a PVP containing medium. In general, lipid-based microparticles were well tolerated throughout the studies with only mild reactions on injection sites. To evaluate the appropriate dose, 750 µg per animal and 3750 µg per animal were administered via different formulations. The low peptide concentration was sufficient for a reliable cycle blockage in swine. It was possible to achieve a cycle blockage between 6.4 ± 0.2 d with D114 + 5 % Span 40 and 14.2 ± 4.9 d with D118 + 10 % GMS. Onset of follicular growth could be controlled between 7 and 17 d depending on the formulation, with significantly delayed onset of follicular growth in the group treated with D114 + 5 % Span 40 microparticles. Ovulation was significantly reduced using the high dose formulations compared to placebo. The incidence of temporary and permanent ovarian cysts was higher in the treatment groups compared to the controls, without statistical evidence. Thus, it could be demonstrated that G [6-D-Phe] loaded lipid microparticles are a promising environmentally sustainable alternative for cycle blockage and estrus control in swine.

7.2 Introduction

For economic reasons in livestock breeding, animals are estrus synchronized to allow a fixed-time insemination at a predictable time point [1]–[4]. A variety of gestagens is currently used to achieve a cycle blockage [5]–[7]. Allyl trenbolone, also referred to as altrenogest, was one of the first synthetic progestogens daily administered to influence the estrus in gilts. A highly reliable estrus control can be achieved when fed in doses between 10 and 20 mg/d per animal over 14 to 19 days [8]–[11]. Further investigations indicate, that a treatment with altrenogest positively influences fertility and litter size of treated animals [12].

Besides the use in livestock breeding, gestagens like levonorgestrel and drospirenone are also active ingredients of hormonal contraceptives for women [13] [14]. Concerns about the intensive use of gestagens arise from their entry into the environment harming fish reproduction which results in infertility and masculinization [15]–[17]. Carlsson and co-workers tested a variety of drugs towards their effect on the water environment and identified the gestagen norethisterone as a substance with high toxicity for water organisms with possible long-term effects [18]. The problem is more pronounced due to the absent or if only minimal metabolization [16] [19] [20]. Therefore, there is a need for alternatives in the food-producing animal industry, where large amounts of gestagens are fed daily and which can enter the surrounding runoff, ground and water [19] [21]–[23]

To achieve estrus control with minimal effects on the environment, the application of GnRH analogues, like G [6-D-Phe] is of high interest. The original GnRH decapeptide pGlu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10}-NH_2 and G [6-D-Phe] are completely metabolized to non-toxic components [24]–[26]. The introduction of bulky and hydrophobic amino acids in position 6 of the peptide backbone is an important structural change to reduce degradation and increase bioactivity [25] [27] [28]. G [6-D-Phe] with D-Phe in position 6 instead of Gly is a so called superagonist for GnRH I, which has a 2.5-fold longer half-life compared to GnRH and shows higher resistance to degradation, due to modification in the cleavage point for endogenous peptidases [26] [27] [29].

A drawback of G [6-D-Phe] against gestagens in estrus synchronization is the need for parenteral application. Since daily injection is not desirable in food animal health, a sustained release system appears mandatory. Different delivery systems for GnRH analogues have been reported in literature for a diversity of mammalian species [30] [31]. Wenzel et al. incorporated GnRH in a sustained release poloxamer gel to achieve a sustained release after depot injection

in cattle and to protect the drug against degradation [32]. Further approaches with a successful achievement of synchronized estrus was reported in beagle bitches with the incorporation of leuprolide acetate into PLGA microparticles followed by treatment with fertirelin [33]. Furthermore, a long-term suppression of sexual functions in male and female dogs can be achieved with Suprelorin® (Virbac), a deslorelin containing implant based on hydrogenated palm oil and Phosphatidylcholine. Similarly, the sexual functions in the macaque could be controlled for 3 to 5 months with the delivery of buserelin via an implant [30] [31] [34]–[36]. These approaches and promising results from previously performed in vitro studies on G [6-D-Phe] loaded microparticles pathed the way for in vivo testing of these systems in swine. Effects on cycle blockage, ovulatory behavior, side effects and biocompatibility were to be evaluated [37]. Based on a previous study on continuous application of G [6-D-Phe] via pumps, consequently 750 µg or 3750 µg per animal were tested [38].

7.3 Materials and Methods

7.3.1 Materials

Gonadorelin [6-D-Phe] (G [6-D-Phe]) was donated by Veyx Pharma GmbH (Schwarzenborn, Germany). Triglycerides Dynasan 114 (trimyristin, D114), Dynasan 116 (tripalmitin, D116) and Dynasan 118 (tristearin, D118) were kindly provided from Cremer Oleo (Witten, Germany). Glycerol monostearate (GMS) with a monoester content of 40-55 % was purchased from Caelo (Caesar & Loretz, Hilden, Germany). Sorbitan monopalmitate (Span 40) was purchased from Croda (Nettetal, Germany). Mannitol and centrifuge tubes were purchased from VWR International GmbH (Darmstadt, Germany). Distilled water produced with a distillation apparatus (Wagner und Munz, München, Germany) was used for preparation of reconstitution media. Highly purified water was produced using a Milli-Q Water system, (Millipore, MA, USA). The gelling agent Tylopur® C 300 (sodium carboxymethylcellulose, Na CMC) was kindly donated from Clariant (Muttenz, Switzerland) and Kollidon® 90F (Polyvinyl pyrrolidone, PVP) was acquired from BASF (Ludwigshafen, Germany). Polysorbate 20 (PS 20) and polysorbate 80 (PS 80) were purchased from Merck KGaA (Darmstadt, Germany).

7.3.2 Animals

German Landrace x Piétrain gilts with a mean weight of 143.8 kg and an average age of 211 d at the beginning of the study were used. The animals were weaned from the mother after 28 d and kept at a flat deck for further 49 d. Afterwards, the animals were housed in a separate stable in pens à 2 animals and fed dependent on the age according to the guidelines of the Deutsche Landwirtschaftliche Gesellschaft (DLG) [39].

7.3.3 Preparation of G [6-D-Phe] loaded microparticles by spray congealing

Lyophilized G [6-D-Phe] was filled in high grade stainless steel beakers and milled using a Retsch® Cryomill (Retsch Technology, Haan, Germany) with two stainless steel balls (10 mm diameter) applying a precooling time of 10 minutes at 5 Hz followed by the actual milling step of 4 minutes at 20 Hz. The obtained powder was aliquoted in 6 R vials, closed with a rubber stopper and crimped. Lipid compounds were melted on a hot plate at approximately 90 °C (D114, D116) and 110 °C (D118), the aliquot of peptide was added and homogenized using a T-10 basic Ultraturrax (IKA Laboratory Technology, Staufen, Germany) for 2 min. The lipid dispersion was poured into the sample container of the B-290 Mini spray dryer (Büchi, Flawil, Switzerland) with additionally installed spray congealing equipment, which was pre-conditioned for 1.5 h prior to the production step. The lipid melt was atomized using nitrogen at a pressure of 6 bar. A spray flow of 414 l/h was used resulting in a filter pressure of 92 mbar.

The aspirator power was set to 100 % throughout all experiments. A dehumidifier Deltatherm® LT (Deltatherm® Hirmer GmbH, Much, Germany) was installed keeping the inlet temperature at 13-15 °C.

7.3.4 Determination of drug content

20 mg microparticles were weighed into centrifuge tubes, 2.0 ml of methylene chloride and 2.0 ml of highly purified water were added and the tubes were placed on a horizontal shaking incubator GFL 3031 (Gesellschaft für Labortechnik, Burgwedel, Germany) at 39 °C and 60 rpm for 12 h. Samples of 1 ml were taken from the water phase and analyzed using RP-HPLC. All extractions were performed in triplicate. RP-HPLC analysis was performed using an Agilent RP-HPLC system (Agilent, Santa Clara, CA, USA), supplied with a 250 x 4.6 mm Luna C-8 column(5µm) (Phenomenex, Aschaffenburg, Germany) and a Security Guard™ KJO-482 pre-column (5 µm) (Phenomenex, Aschaffenburg, Germany). Columns were maintained at 40 °C using the following gradient:

Time [min]	A [%]	B [%]
0	65	35
1	65	35
21	65	35
22	10	90
26	10	90
35	65	35

Mobile Phase A: 1000 ml highly purified water + 1 ml trifluoroacetic acid

Mobile Phase B: 800 g Acetonitrile + 200 g water + 1.2 ml trifluoracetic acid

7.3.5 Aliquoting of the particles under laminar air flow

Based on the drug content (*see 7.3.4*) microparticles delivering 750 µg or 3750 µg per animal for the treatment of six animals were weighed under laminar air flow (LAF) into sterile 10 R vials, closed with rubber stoppers and crimped.

7.3.6 Preparation of the reconstitution medium

For the pre-clinical studies, two different reconstitution media were used. Firstly, the reconstitution medium consisted of 1 % Na CMC and 0.02 % PS 20. The solution was filtrated through a 5 µm-syringe filter into sterilized 10 R glass vials under LAF closed with sterile rubber stoppers and sterilized in a table autoclave GTA 50 (Medizin und Labortechnik Fritz Gössner, Hamburg, Germany). Optimized reconstitution medium was prepared under LAF

conditions using the above-mentioned apparatus by adding 0.5 % PS 80, mannitol for isotonization and 3 % Kollidon® 90 F to distilled water while stirring followed by a sterile filtration through a 0.22 µm syringe filter. Each vial contained 20 ml of reconstitution medium and was closed with sterilized rubber stoppers and crimped. The vials were stored in the refrigerator until application.

7.3.7 Viscosity measurements

Both reconstitution media were analyzed on a rotation viscometer MCR 100 rheometer (Physica, Anton Paar, Ostfildern, Germany) applying the cone-plate-method at 25 °C. Shear rates between 1 s^{-1} and 100 s^{-1} were applied. Both pure reconstitution medium as well as particle suspension was tested. 100 mg of particles were suspended in 4.0 ml of reconstitution medium. Each measurement was performed in triplicate.

7.3.8 General study set-up and pre-treatment of the animals

Both in vivo studies were conducted at the Versuchsgut Oberholz of the University of Leipzig between September 2014 and August 2015. Gilts specifically bred for this purpose at age of 180 days were brought in contact with a boar to detect possible estruses. The study started with the ultrasound examination to assure onset of puberty in all participating animals. If this was not the case, puberty was induced using Intergonan® 240 IE/ml (equine chorionic gonadotropin, eCG, Intervet, Unterschleißheim, Germany). This treatment was only necessary for six animals out of 25 in the first study. These animals were again examined for detection of puberty status 8 d later [39].

Prior to application, all gilts were estrus-synchronized to assure that every animal reached day 12 of the sexual cycle with the 46 day of the study. For cycle synchronization 20 mg/animal Regumate® (altrenogest, Intervet, Unterschleißheim, Germany) were applied starting on day 11 for 28 d. 40 h after the last altrenogest application, 1.000 IE Intergonan® were injected i.m. to induce follicular growth. Ovulation was induced by intramuscular injection of 50 µg/ml Gonavet® Veyx (G [6-D-Phe], Veyx Pharma GmbH, Schwarzenborn, Germany). With day 46 of the study, gilts were randomized into five groups, consisting of four treatment groups and one control group. The injection of the different formulations started on the same day. *Table 7-1* gives an overview on formulations tested in the first and second pre-clinical study.

Table 7-1: Overview of applied treatments and G [6-D-Phe] concentrations per group. Groups 1-5 were investigated during the first pre-clinical study in September 2014, groups 6-10 were investigated in August 2015 in the second study

Pre-clinical study 1	**Triglyceride**	**Additive/concentration**	**G [6-D-Phe] concentration [µg/animal]**
1	D118	-	0 (Placebo)
2	D116	GMS/10 %	750
3	D118	GMS/10 %	750
4	D116	GMS/10 %	3750
5	D118	GMS/10 %	3750
Pre-clinical study 2	**Triglyceride**	**Additive/concentration**	**G [6-D-Phe] concentration [µg/animal]**
6	D114	Span 40/5 %	0 (Placebo)
7	D116	Span 40/5 %	750
8	D114	GMS/5 %	750
9	D114	Span 40/5 %	750
10	D114	Span 40/10 %	750

From day 34 on, the gilts were examined clinically and sonographically with focus on cycle state. Moreover, an additional detection of possible estruses was performed with the presence of a boar. Injection of microparticle formulations was performed on day 46 followed by daily visible inspection of injection sites. All animals were investigated at least 25 days and maximum 31 days after injection of the formulations to detect the end of cycle blockage and following follicular growth and ovulation [39].

7.3.9 Reconstitution and application of the suspension

Microparticles and reconstitution medium were delivered in two separate vials and mixed prior to application. In general, 50 to 100 mg microparticles (depending on the actual drug load) were mixed with 2.0 ml of corresponding reconstitution medium. Each vial contained single doses for potentially six animals. The vials were gently shaken for homogenization avoiding foam formation. Previous experiments suggested a manual shaking for 2 min, whereas the practical application needed reconstitution times of 15 min until homogeneity was reached with the high dose formulations. The multi-dose containers were assembled with the injection revolver and the respective amount of suspension was injected into the lateral neck muscles. High dose

formulations (3750 µg/animal) were suspended in 4 ml of reconstitution medium and 2 x 2 ml were injected [39].

7.3.10 General examination and evaluation of injection sites

Gilts were investigated concerning interior body temperature with the means of rectal temperature measurement, food intake, general condition and clinical signs of disease between days 34 to 72/78. Injection sites were evaluated with special focus on redness, pain, swelling and temperature. Results were documented photographically [39].

7.3.11 Determination of estrus control

Besides contact with a boar, estrus was detected via redness and swelling of the genitals, as well as beginning and end of "standing" according to a pre-defined schedule. "Standing" was detected via established tests, e.g. back-pressure test by instructed personnel. This data was gained as additional information to assure a gilt´s estrus [39].

7.3.12 Ultrasound examination of ovaries

Ultrasound examinations were performed using a Fazone CB device (Fujifilm, Tokyo, Japan) equipped with a convex transducer type C 9-3, achieving a frequency of 3-9 MHz. For our investigations, a frequency of 6 MHz was used, which assured a penetration depth of 10 cm. Overall gain was set at 84 dB. For examination, the animals were brought into a custom-made box and immobilized by feeding. Ultrasound was performed transcutaneously according to Kauffold et al., by positioning the transducer from the right ventro-lateral abdominal wall in dorsal direction to the last pair of teats and cranial to the hind leg [40]. Structures like follicles, *corpora haemorrhagica* and *corpora lutea* were monitored and documented. The term ovarial cysts was used for structures consisting of growing follicles with a diameter larger than 10 mm showing no ovulation. These structures were persistent besides ovulating follicles (solitary cysts) or exclusively, then called polycystic ovary degeneration (POD). If they were persistent solely over a defined period without influencing the cycle blockage, they were called temporary cysts. If monitored over the whole-time period, they were called permanent cysts. Ovulation was set as complete, if pre-ovulatory follicles vanished and just *corpora haemorrhagica* were present. Each measurement consisted of at least three representative follicles and if present, three *corpora lutea* to depict the onset of new follicle growth [41]. This value consisted of the mean value out of three single measurements for each day, respectively [39].

7.3.13 Statistical Analysis

Statistical Analysis was carried out using SigmaPlot (Systat Software Inc., San Jose, CA, USA).

7.4 Results and Discussion

7.4.1 Characterization of reconstitution behavior

Viscosity measurements were conducted to evaluate applicability of the particle suspensions (*Table 7-2).*

Table 7-2: Viscosities of reconstitution media and final suspensions

Formulation	**Viscosity [Pas]**	**Shear rate [s^{-1}]**
Na CMC 1 % medium	0.041 (± 0.002)	1
Na CMC 1 % suspension	20.9 (± 13.1)	1
Na CMC 1 % medium	0.036 (± 0.002)	100
Na CMC 1 % suspension	0.050 (± 0.006)	100
PVP 3 % medium	0.014 (± 0.007)	1
PVP 3 % suspension	8.0 (± 4.2)	1
PVP 3 % medium	0.007 (± 0.004)	100
PVP 3 % suspension	0.012 (± 0.002)	100

In literature, shear thinning and dilatancy are reported for suspensions, depending on medium and concentration of the dispersed phase [42] [43]. Both reconstitution media and suspensions showed shear thinning behavior. Shear thinning was more pronounced when Na CMC was used as thickening agent compared to PVP, possibly due to linear orientation of the colloids like cellulose upon mechanical stress [44]. Na CMC based reconstitution medium showed a higher viscosity without particles at low shear rates (0.041 ± 0.002 Pas), as well as after suspension (20.9 ± 13.1 Pas) compared to the PVP based medium (0.014 ± 0.005 Pas without particles, 8.0 ± 4.2 Pas with particles). With suspension of particles, both media showed a remarkably reduced viscosity after exposure to shear stress in comparison to the unsheared sample indicating an orientation of the particles upon shearing. Foam formation was only noticeable upon shaking the Na CMC based formulation with the potential of particle loss at the surface and inhomogeneity (*Figure 7-1*). Thus, the reconstitution medium based on PVP appeared to be more suitable for reconstitution of the particles, especially with a suspension start viscosity of 8 Pas, and a marked shear thinning to 0.012 Pas, which should enable easy syringeability and injectability.

7.4.2 **Applicability of the final suspensions in vivo**

For both studies two different compositions of reconstitution media were evaluated.

Figure 7-1: Particles suspended in reconstitution medium (Na CMC 1 %, PS 20 0.02 %) with formation of foam due to manual shaking and absorption of particles to the surface (A) kindly provided from the Faculty of Veterinary Medicine, University of Leipzig. Placebo formulation (D114 + 5 % Span 40) during homogenization in reconstitution medium (PVP 3 %, PS 80 0.5 %, mannitol 5 %) (B). Homogenous suspension of particles in PVP-based reconstitution medium (C)

Na CMC-based medium did not show satisfying reconstitution properties specifically for the high dose formulations (3750 µg/animal). Particles showed a marked tendency towards agglomeration (*Figure 7-1 A*). To achieve a homogenous suspension, the vials needed to be shaken manually for 15 to 30 min, which is not practicable for a market product. Furthermore, the formation of foam made it difficult to withdraw the desired volume, which necessitated improvement for the second pre-clinical study. Formation of foam could be reduced by exchanging Na CMC with PVP, as well as PS 20 by PS 80 and despite increasing the concentration of emulsifier from 0.02 to 0.5 %. All verum formulations of the second pre-clinical study could be suspended and injected. The placebo formulation still required longer reconstitution times (*Figure 7-2*).

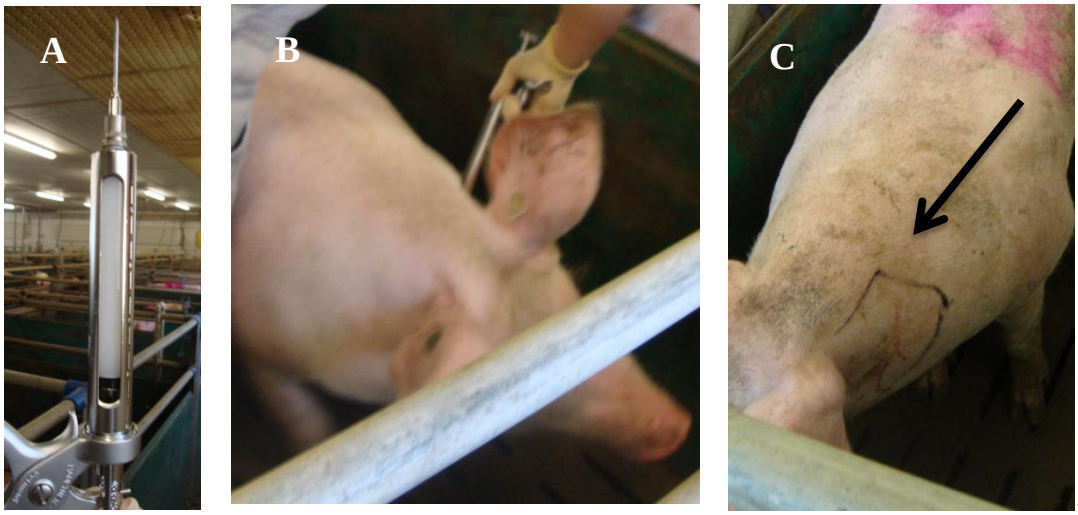

Figure 7-2: Particle suspension in optimized reconstitution medium (PVP 3 %, PS 80 0.5 %, mannitol 5 %) prior to the application in the injection revolver (A). Injection of the suspension into the pig´s lateral neck muscle (B). Marking of the injection site for further examination and documentation of adverse reactions (C)

7.4.3 **Biocompatibility and documentation of adverse drug reactions**

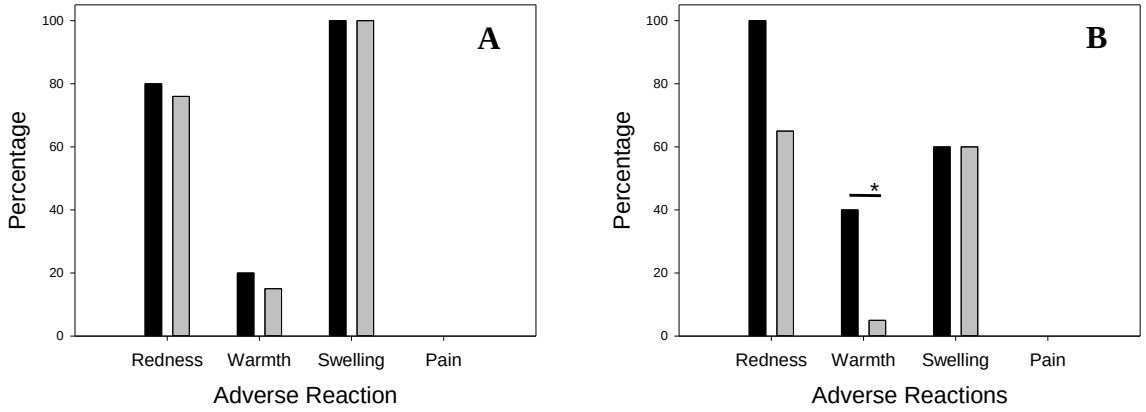

Figure 7-3: Adverse reactions documentated in first (A) and second pre-clinical study (B). All values are calculated as percentage of the treated animals (control: 5, black bars; treatment 20 animals, grey bars). Statistics were performed using a Mann-Whitney-Rank-Sum-test

The lipid microparticles were well tolerated, which went in accordance with observations from other researchers [45]–[47]. None of the gilts showed pain at the injection site in both control and treated groups. Throughout the first study, both control and treatment groups did not show signs of illness or fever. In the second study one gilt showed fever symptoms directly after injection and another after 24 d. Redness of the injection site could be detected in both studies, without significant difference between control and treatment groups, possibly reasoned by rapid injection without fixation of the animals. In both pre-clinical trials, more animals of the control groups showed redness at the injection sites. A significant difference (P-value: 0.04) was observed concerning warmth of injection sites in the second study, where a higher percentage of the control group showed increased temperature, potentially due to reconstitution problems.

Mild swelling was observed in all animals in both control and treatment groups during the first study, which could be minimized to 60 % due to better reconstitution properties. Two animals showed an intermediate swelling persisting for 4 d (treatment) and for 13 d (control). Isotonization with 5 % mannitol and replacement of Na CMC with PVP might be reasons for the better compatibility. In general, the treatment with G [6-D-Phe] did not show a significant increase in occurrence of adverse reactions compared to control.

7.4.4 Effects on cycle blockage in vivo using different formulations and G [6-D-Phe] concentrations

The sustained release G [6-D-Phe]-microparticles were evaluated with respect to minimal effective concentration as well as effect on cycle blockage and estrus control. Both placebo formulations of the first (D118) and second pre-clinical study (D114 + 5 % Span 40) did not influence the cycle (*Figure 7-4*).

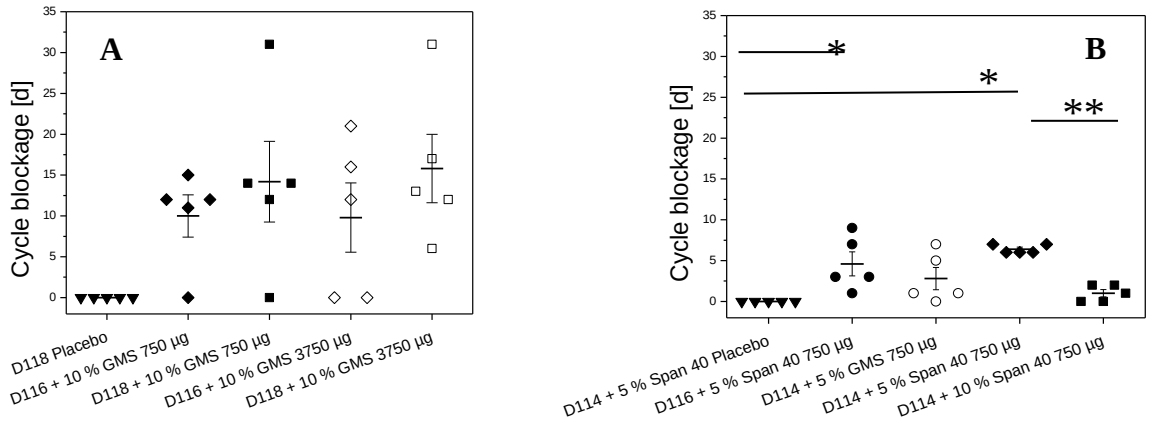

Figure 7-4: Duration of cycle blockage after injection of lipid based microparticle formulations. Results of the first pre-clinical study using G [6-D-Phe] 750 µg/animal and 3750 µg/animal in D116/D118 formulations with 10 % GMS (A) as well as D114/ D116 and G [6-D-Phe] 750 µg/animal with addition of GMS and Span 40 tested in the second study (B). Data shown are individual results with mean and SEM. Statistical analysis was performed by one-way ANOVA followed by pairwise multiple comparisons (Holm-Sidak-method)

Using the formulation D116 + 10 % GMS 750 µg/animal, a cycle blockage of 10.0 ± 2.6 d could be achieved with one animal not responding to the therapy. A longer cycle blockage of 14.2 ± 4.9 d was observed in animals receiving the longer chain triglyceride D118 + 10 % GMS 750 µg/animal, with one animal not showing a cycle blockage in the observed time. Increasing the therapeutic dose to 3750 µg/animal resulted in a cycle blockage of 9.8 ± 4.3 d with two gilts not responding to the therapy when treated with D116 + 10 % GMS. The delivery of 3750 µg/ animal G [6-D-Phe] formulated in D118 + 10 % GMS led to a cycle blockage of 15.8 ± 4.2 d in all animals. Due to high variation within the groups receiving the drug containing microparticles, no significant difference between placebo and treatment was observed. The

treatment with the higher dose of G [6-D-Phe] did not lead to a longer cycle blockage and did only in case of D118 + 10 % GMS result in a higher response within the group (5/5). In this group, one gilt showed a cycle blockage until the end of the observed time (31 d). In the treatment group with 3750 µg/animal (D116 + 10 % GMS) two animals did not show a response to drug application. Formulations tested in the first study evidenced, that the achievement of cycle blockage with the means of lipid based microparticles is generally possible using the low dose concentration, confirming the study with osmotic pumps [38]. Increasing the G [6-D-Phe] content did not lead to a significant improvement in therapy success or higher synchronicity. The highest synchronicity could be observed in the group of D116 + 10 % GMS 750 µg/animal. Furthermore, the delivery of a higher dose seemed to have the consequence of a permanent cycle blockage, as it was only observed in one of the high-dose groups.

For the second pre-clinical study, four different formulations were tested delivering 750 µg/animal of G [6-D-Phe]. The use of D116 + 5 % Span 40 resulted in a cycle blockage of 4.6 ± 1.5 d, with all animals responding to the therapy but a high variability within responses. The effect of cycle blockage was significantly different compared to placebo (P-value: 0.017). The group treated with D114 + 5 % GMS showed a cycle blockage of 2.8 ± 1.4 d with one animal not showing a cycle blockage. Formulation consisting of D114 + 10 % Span 40 led to a cycle blockage of 1 day ± 0.5 days with two animals not responding to the therapy. Best results throughout all studies could be achieved with the formulation D114 + 5 % Span 40, where all treated gilts showed a cycle blockage and the highest synchronicity could be observed (5/5). Hence, a cycle blockage of 6.4 ± 0.2 d was achieved with a significant difference to the control group (P-value < 0.001) and to the group treated with the formulation D114 + 10 % Span 40 (P-value: 0.005). Consequently, duration of cycle blockage was closest to the desired cycle blockage of 15 days using D116 + 10 % GMS 750 µg/animal, whereas highest synchronicity was observed when D114 + 5 % Span 40 was used. These findings confirmed that it was possible to achieve a cycle blockage with the use of a sustained release G [6-D-Phe] formulation.

7.4.5 **Influence on follicular growth**

Onset of follicular growth is another parameter indicating success of the therapy. The follicular growth started after 7 to 7.5 d in both control groups, which represented the regular duration time between diminishment of altrenogest and onset of follicular growth. After treatment with D116 + 10 % GMS 750 µg/animal, follicles started to grow after 14.4 ± 2.8 d. Exchanging the triglyceride to D118 + 10 % GMS 750 µg/animal led to an onset of follicular growth after

14.8 ± 3.1 d, leading to a permanent cycle blockage in one treated animal. An increase in G [6-D-Phe] dose to 3750 µg/animal resulted in onset of follicular growth after 14.2 ± 4.1 d (D116 + 10 % GMS) and 17 ± 2.0 d (D118 + 10 % GMS), where one animal did not show follicles after the monitored time. Due to considerably high variability within the groups, no significant difference between treatment and control groups could be detected, although follicular growth was delayed.

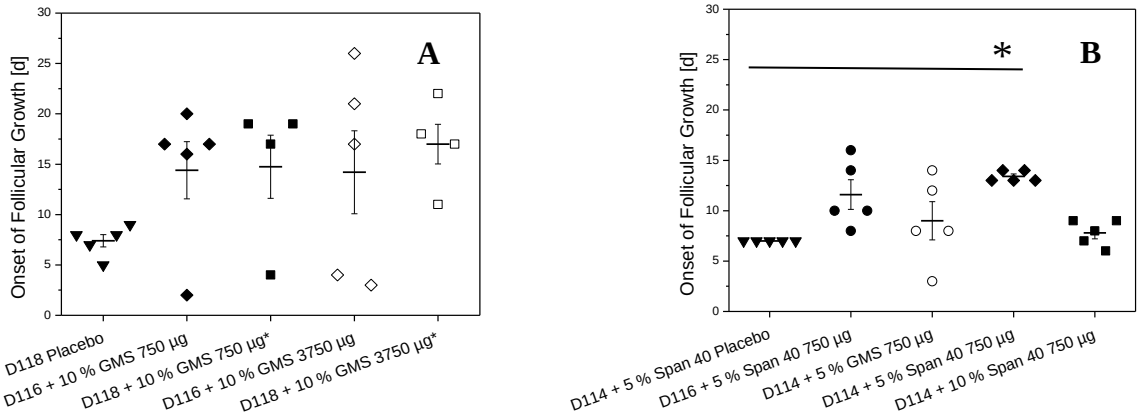

Figure 7-5: Onset of follicular growth after treatment with lipid microparticle formulations. (A) Results of the first pre-clinical study using of G [6-D-Phe] 750 µg/animal and 3750 µg/animal) in D116/D118 formulations with 10 % GMS. (B) Results of follow-up study using D114/D116 with addition of GMS and Span 40 and 750 µg/animal G [6-D-Phe]. Data shown are individual results with mean and SEM. Statistical analysis was performed by Kruskal-Wallis-one-way ANOVA on ranks (A) and by one-way-ANOVA followed by pairwise multiple comparisons (Tukey-test) (B). * one animal with persistent cycle blockage observed

In the second study, treatment with D116 + 5 % Span 40 resulted in an onset of follicular growth after 11.6 ± 1.5 d. After the treatment with D114 + 5 % GMS follicles occurred after 9 ± 1.9 d. Most promising results were achieved with the use of D114 + 5 % Span 40 particles, where an onset after 13.4 ± 0.2 d was monitored. This difference compared to the control group was significant (P-value < 0.05). Increasing the concentration of Span 40 to 10 % shortened the interval between discontinuation and onset to 7.8 ± 0.6 d. Growing follicles were visible in all treated animals in this study. Thus, the formulation D114 + 5 % Span 40 showed in cycle blockage, synchronicity and onset of follicular growth the most promising results.

7.4.6 **Influence of treatment on ovulatory behavior**

Another important parameter for a successful reproduction is the occurrence of ovulation of growing follicles. Treatment with altrenogest is known to improve ovulation rates and following litter sizes compared to controls [10]. GnRH and its analogues are indicated in ovulation induction in human and veterinary medicine [48]–[50]. Therapy-associated ovulation within all groups was compared in order to investigate, if the treatment affected ovulation

behavior in gilts. Both control groups, D118 and D114 + 5 % Span 40 resulted in a 100 %-ovulation observation within the ten tested animals. In the group treated with D116 + 10 % GMS 750 µg/animal only 60 % of treated gilts showed an ovulation. This value was reduced to 40 %, when the formulation D118 + 10 % GMS 750 µg/animal was used instead. A complete anovulatory behavior was observed in the treatment group consisting of D116 + 10 % GMS 3750 µg/animal where none of the treated gilts showed ovulatory behavior. Here, a significant difference compared to the placebo group was monitored (P-value < 0.008). Since the other high dose group did also show a decreased ovulation behavior with statistical significance (20 % of the gilts in this group ovulated, P-value 0.043), the application of the high G [6-D-Phe] dose could be regarded as responsible for this effect.

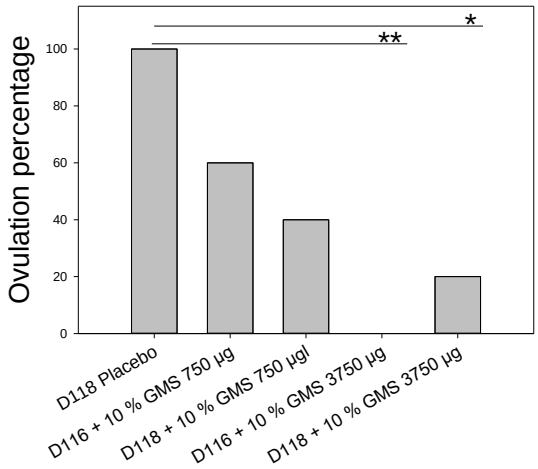

Figure 7-6: Observed ovulation percentage after treatment with lipid-based microparticles in the first study. Each bar represents the percentage out of 5 animals per group. Statistical analysis was performed using the one-way ANOVA test followed by a Holm-Sidak pairwise comparison.

In the second study, treatment with D116 + 5 % Span 40 and D114 + 5 % GMS resulted in reduced ovulation percentage to 20 %. The formulation with best performance concerning cycle blockage and follicular growth, D114 + 5 % Span 40, led to an ovulation in 40 % of treated animals. The highest percentage regarding ovulation was observed with the use of D114 + 10 % Span 40 (80 %). In the second study, no statistical evidence of reduced ovulation compared to control was given. Nevertheless, a higher incidence of anovulatory behavior was observed upon treatment with sustained release G [6-D-Phe], whereas only the high dose treatment led to significantly reduced occurrence of ovulation. The possible connection between sustained release treatment with a GnRH-analogue and absence of ovulation and development of ovarian cysts needs to be clarified due to a possible disruption in down-regulation mechanism [51] [52].

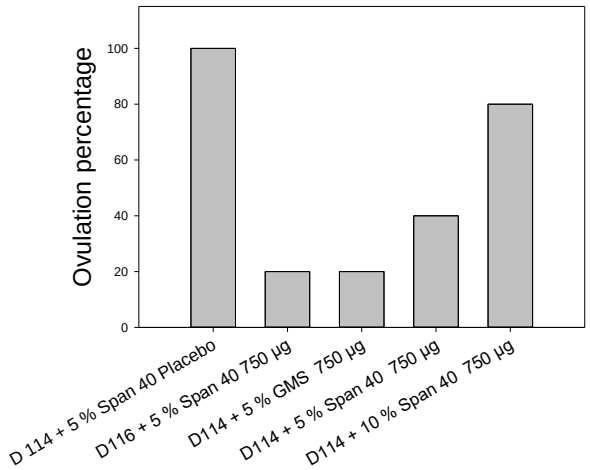

Figure 7-7: Observed ovulation percentage after treatment with lipid-based microparticles in the second pre-clinical study. Each bar represents the percentage out of 5 animals per group. Statistical analysis was performed using the Kruskal-Wallis one-way ANOVA on ranks followed by a pairwise multiple comparison (Tukey-test)

7.4.7 Evaluation of the relation between treatment and the occurrence of temporary and permanent cysts

The occurrence of ovarian cysts is a critical point in estrus synchronization, as it has negative influence on performance of ovaries and success of insemination. Although GnRH analogues are used for the treatment of ovarian cysts [53] [54], therapy-induced formation of abnormal follicular structures is reported in literature in both human and veterinary medicine [51] [55] [56]. Polycystic ovarian degeneration (POD) is characterized by the occurrence of numerous liquid-filled cysts accompanied by an absence of *c. lutea* [40]. A higher incidence of POD was observed in estrus synchronization when lower doses (16 mg) of altrenogest were compared to higher doses (20 mg) [4]. Consequently, the parameter cyst formation was included in evaluation of treatment with a sustained release G [6-D-Phe] delivery system. In both control groups, none of the gilts showed permanent or temporary cysts (*Figure 7-8*). In contrast, treatment with D116/D118 + 10 % GMS 750 µg/animal led to the development of permanent cysts in 40 % of treated gilts (*Figure 7-8 A black bars*). Increasing the dose to 3750 µg/animal resulted in the development of permanent and temporary cysts in 20 % of treated gilts when treated with the formulation D118 + 10 % GMS 3750 µg/animal.

The formulation D118 + 10 % GMS 3750 µg/animal did not lead to the occurrence of cysts at all. During the second pre-clinical study, three treatment groups (D116 + 5 % Span 40, D114 + 5 % GMS, D114 + 5 % Span 40) showed an occurrence of permanent cysts in 40 % of the animals per group. A lower value of 20 % resulted with the formulation D114 + 10 % Span

40. Only the treatment with D116 + 5 % Span 40 microparticles resulted in the development of temporary cysts in 20 % of the animals.

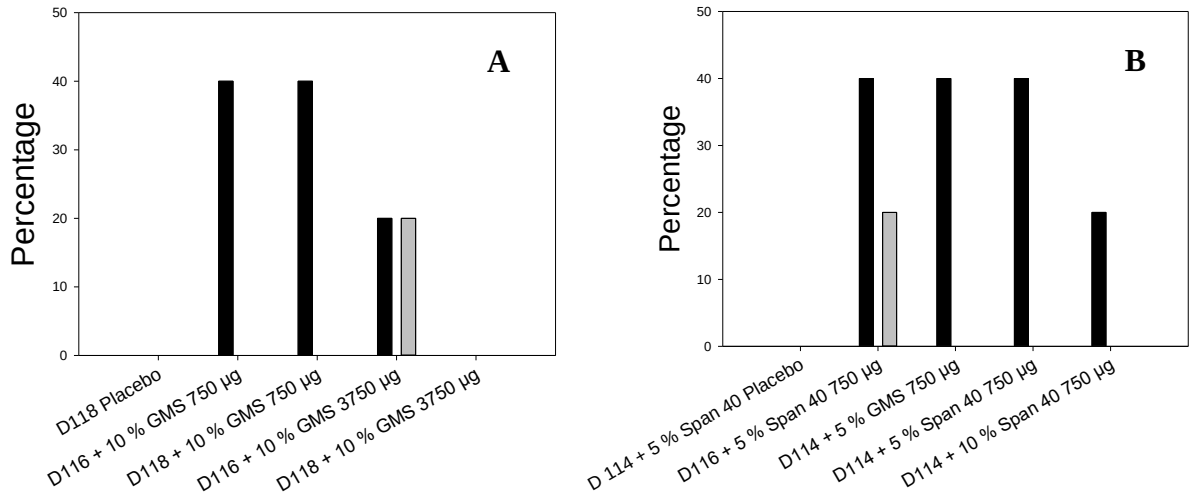

Figure 7-8: Percentage of observed cysts (permanent: black bars, temporary: grey bars) during the treatment with G [6-D-Phe]-loaded lipid based microparticles monitored in the first pre-clinical study (A) and the follow-up study (B). Each bar represents the percentage out of the population of 5 animals per group. Statistical analysis was performed using the Kruskal-Wallis one-way ANOVA test on ranks

Kauffold et al. reported a very low incidence of the severe POD in gilts compared to sows upon estrus synchronization with gestagens and gonadotropins [40]. Besides the occurrence of single temporary or permanent cysts in our experiments, the severe POD did not occur. Although there was no statistical evidence, treatment with sustained release G [6-D-Phe] and the occurrence of cysts should be evaluated in a larger group of treated animals. The occurrence of follicular cysts is reported to be a severe disorder followed by infertility [40]. In literature, the treatment with LHRH-analogues is indicated in the presence of ovarian cysts in dairy cows also in combination with cloprostenol to treat these abnormal follicles [53] [57]. A possible explanation for this contradictory effect might be, that a treatment with gonadotropins possibly changes LH:FSH ratio, necessary for normal follicles as stated by Breen and Knox [58]. Also in human medicine, an increased development of follicular and luteal cysts was reported as complication during preparation for in vitro fertilization by the application of GnRH-analogues [55] [56] [59].

7.5 Conclusion

Our results showed that it was possible to achieve a cycle blockage with a sustained release formulation of G [6-D-Phe] in swine. Spray congealed solid lipid microparticles are a suitable

delivery system to influence and control the estrus. Particles were prepared without using organic solvents and were evaluated to be well tolerated and highly biocompatible in both in vivo studies.

Reconstitution and application of the microparticular suspension was difficult with the use of a Na CMC-based medium. Especially when high particle doses (3750 µg/animal) needed to be administered, foam formation and attachment of particles to the liquid/air interface occurred. These drawbacks could be avoided using the optimized, PVP-based medium with higher emulsifier concentration, facilitating reconstitution and application.

The first study confirmed the successful estrus control with the use of 750 µg/animal G [6-D-Phe] resulting in a mean cycle blockage of 10 to 16 d with an onset of follicular growth after 15 to 17 d. These values came very close to the desired cycle blockage of 15 d. Here, a treatment with D116 in combination with 10 % GMS resulted in the highest synchronicity within the group. Compared to the placebo treatment, a significant reduction in ovulation was observed after the application of the higher dose (3750 µg/animal) and without statistical evidence after treatment with 750 µg/animal. Three out of four treated groups showed development of temporary and permanent follicular cysts without statistic relevance compared to controls.

Having proven our concept with the first pre-clinical study, **the second study** focused on the application of 750 µg/animal via four different formulations to narrow the time window to the desired 15 d with a higher synchronicity. Best results were achieved with the formulation D114 + 5 % Span 40, where a duration of 6 d and the highest synchronicity in all trials could be observed. Follicular growth was setting in between 9 and 13 d after application. No significant differences in ovulation behavior between treatment and control was observed. All treatment groups showed development of ovarian cysts indicating a correlation between treatment with G [6-D-Phe] and the occurrence of these structures, without statistical evidence.

In summary, it is possible to achieve a cycle blockage in variable time frames with the application of G [6-D-Phe]-loaded microparticles by variating the type of emulsifier, as well as its concentration. Furthermore, we confirmed, which is economically favorable, the successful control of estrus cycle with the low dose G [6-D-Phe].

7.6 References

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8 LONG-TERM STABILITY STUDY OF G [6-D-PHE]-LOADED MICROPARTICLES

Parts of this chapter are intended for publication

HPLC analysis for determination of drug content was performed by ***BFC-Feinchemie***, Weinbergen Grabe.

8.1 Abstract

The lead candidate from the first clinical study was investigated concerning long-term stability. Microparticles were stored at 2-8, 25 and 40 °C and samples were taken after 0, 1, 3, 6, 9 and 12 months. Analysis of particle size, reconstitution properties, lipid polymorph, peptide content and release behavior was performed. Formulations showed particle agglomeration upon storage, which was minor at 2-8 °C and most pronounced when stored at 40 °C. Reconstitution to a homogenous suspension was possible with samples stored at 2-8 °C and 25 °C but limited after 40 °C storage. Drug content was not affected in any sample by storage even after 12 months at 40 °C and no degradation products were detected. The initially observed release over two weeks was preserved when microparticles were stored at 2-8 °C. Storage at higher temperatures led to a higher burst and a more complete release. Thermal analysis revealed the transformation of the initially obtained α-polymorph to the thermodynamically more stable β-form after storage at 40 °C. Upon storage at 2-8 °C, the α-modification was maintained over 12 months, whereas XRPD of samples stored at 25 °C already indicated ongoing transitions. Thus, storage at 2-8 °C was highly recommended to assure stability with a reliable release profile accompanied by good reconstitution behavior and peptide integrity.

8.2 Introduction

Lipid microparticles are a promising tool for the sustained release of peptides and proteins [1]–[5]. We developed microparticles loaded with G [6-D-Phe] which showed drug release over two weeks in vitro as well as efficacy in vivo [6]. Consequently, the stability of these microparticles was tested at different temperatures over 12 months. Ideally, the product should be stable at ambient temperature to enable easy handling by veterinarians and farmers. It should further guarantee a continuous release in a two-week time window. Furthermore, particle size and lipid polymorph must be preserved [7] [8]. Another major aspect is the integrity of the incorporated peptide.

8.3 Materials and Methods

8.3.1 Materials

Gonadorelin [6-D-Phe] (G [6-D-Phe]) was donated by Veyx Pharma GmbH (Schwarzenborn, Germany). The triglyceride Dynasan 118 (tristearin, D118) was kindly provided from Cremer Oleo (Witten, Germany). Glycerol monostearate (GMS) with a monoester content of 40-55 % was purchased from Caelo (Caesar & Loretz, Hilden Germany). Ultrapure deionized water with a conductivity of less than 0.055 µS/cm (Milli-Q Water systems, Millipore, MA, USA) was used for preparation of buffers and mobile phases in this study. Visking dialysis tubings with a diameter of 1.6 mm and a cut-off of 12-14 kDa for performance of release studies were sourced from Serva Electrophoresis GmbH (Heidelberg, Germany). Centrifuge tubes were purchased from VWR International GmbH (Darmstadt, Germany). The reconstitution medium consisted of 1 % Tylopur® C 300 (sodium carboxymethylcellulose, Na CMC, Clariant, Muttenz, Switzerland) and 0.02 % polysorbate 20 (PS 20, Merck KGaA, Darmstadt, Germany) in distilled water (Wagner und Munz, München, Germany). All other chemicals were of analytical grade.

8.3.2 Production of Gonadorelin [6-D-Phe]-loaded microparticles

Lyophilized G [6-D-Phe] was milled using a Retsch® Cryomill (Retsch Technology, Haan, Germany) using two stainless steel balls (10 mm diameter) with a precooling time of 10 min at 5 Hz followed by the actual milling step of 4 min at 20 Hz. The obtained powder was aliquoted under nitrogen atmosphere. 10.6 g of D118 and 1.2 g of GMS were melted on a hot plate at approximately 90 °C. 200 mg of peptide was added and homogenized using a T-10 basic Ultraturrax (IKA Laboratory Technology, Staufen, Germany) for 2 min. The lipid dispersion was poured into the sample container of a B-290 Mini spray dryer (Büchi, Flawil, Switzerland) with additional spray congealing equipment, which was pre-conditioned for 1.5 h (sample container 110 °C, inlet temperature 13-15 °C) prior to the production step. The additional equipment consisted of a sample container, surrounded by a PEG 400 bath, whereas the sample container was connected to the nozzle via a silicon tubing heated with an external resistance wire-based heating to approximately 70 °C. The melt was sprayed using nitrogen as spray gas at 6 bar spray pressure resulting in a spray flow of 414 l/h and with aspirator power set at 100 %.

8.3.3 Storage conditions and study set-up

For each sampling point, samples of approximately 350 mg were stored at 2-8, 25 and 40 °C in two glass vials. Microparticles were weighed under LAF conditions using a HeraSafe HSP 12 (Heraeus, Hanau, Germany), closed with rubber stoppers and crimped. Analysis was performed after 0, 1, 3, 6, 9 and 12 months.

8.3.4 Macroscopic inspection of the microparticles

Microparticles were visually inspected concerning appearance and aggregation behavior. Documentation was performed using a Nikon D5300 (Nikon, Tokyo, Japan) equipped with a macro-objective.

8.3.5 Observation of surface morphology by scanning electron microscopy (SEM)

A Jeol JSM 6500-F SEM (Jeol, Tokyo, Japan) was used for SEM images at a voltage of 2 kV using a magnification of 150, 900 and 1500 for each sample. Microparticles were fixed on aluminum sample holders with black, double adhesive tape (Plano GmbH, Wetzlar, Germany) and were analyzed without additional graphite coating.

8.3.6 Stability of the suspension

Approximately 200 mg of stored microparticles were shortly shaken in a glass vial and 2 ml of reconstitution medium were added via a syringe through the rubber stopper. The dispersion was vigorously shaken by hand for approximately 20 s to suspend the particles in the surrounding medium. Photographic images were taken immediately after reconstitution and after standing times of 2, 5, and 10 min.

8.3.7 Particle size analysis using laser diffraction

Approximately 20 mg of microparticles were suspended in an aqueous 0.02 % PS 20 solution and analyzed using the laser diffraction system Horiba Partica-LA 950 (Horiba, Kyoto, Japan).

8.3.8 Determination of polymorphic behavior by differential scanning calorimetry (DSC)

Samples of 5-15 mg microparticles in aluminum crucibles were analyzed using a Mettler DSC 821e (Mettler Toledo, Columbus, OH, USA) at a heating and cooling rate of 10 K/min from 0 °C to 110 °C in two cycles against an empty crucible as reference.

8.3.9 X-Ray powder diffraction (XRPD) measurements

A PANalytical Empyrean (PANalytical, Almelo, the Netherlands) equipped with a copper anode (45 kV, 40 mA, Kα1 emission, λ = 0.154 nm) and a PIXcel3D detector between 5-50 ° 2-Theta with a step of 0.039 ° was used for XRPD measurements.

8.3.10 Drug content analyzed via RP-HPLC

Approximately 10 mg microparticles were weighed into centrifuge tubes and dissolved in 2.0 ml of methylene chloride. 2.0 ml of HPLC gradient grade water (Fisher Chemical, Loughborough, UK) were added and the tubes were placed in a Stuart tube rotator SB 3 at a

rotation speed of 10 rpm (Bibby Scientific Limited, Stone, UK) at ambient temperature for 45 min. Samples of 1 ml were taken from the water phase and analyzed using RP-HPLC. Each stored microparticle sample was extracted in duplicate. RP-HPLC analysis was performed using a Varian RP-HPLC system (Varian Inc., Palo Alto, CA, USA), supplied with a Luna C-8-column (Phenomenex, Aschaffenburg, Germany) and the guard column SecurityGuard (Phenomenex, Aschaffenburg, Germany). Columns were maintained at 40 °C and using the following gradient:

Time [min]	A [%]	B [%]
0	65	35
1	65	35
21	65	35
22	10	90
26	10	90
35	65	35

Mobile Phase A: 1000 ml highly purified water + 1 ml trifluoracetic acid

Mobile Phase B: 800 g Acetonitrile + 200 g water + 1.2 ml trifluoracetic acid

An injection volume of 20 µl was used for each sample and calibration standards. The actual drug content was normalized to a theoretical initial weight of 10.0 mg. This analysis was performed by Biopept-Feinchemie GmbH, Weinbergen/Grabe.

8.3.11 Determination of release behavior

50 mg of microparticles were transferred into dialysis tubings, which were previously closed at one side with a cotton string. 2.0 ml phosphate-buffered saline (PBS) pH 7.4 preserved with 0.05 % NaN_3 and five glass balls (0.2 mm diameter) for additional weight were added to suspend the microparticles. The closed tubings were placed in 50 ml-centrifuge tubes, 15 ml PBS were added and the samples incubated on a horizontal shaking incubator GFL 3031 (Gesellschaft für Labortechnik, Burgwedel, Germany) at 39 °C and 60 rpm. At predetermined time points, samples of 1 ml volume were taken, replaced by fresh PBS and stored at 2-8 °C until HPLC analysis was performed. An Agilent HPLC system (Agilent, Santa Clara, CA, USA) was used using the setup mentioned in *8.3.10.*

8.4 Results and Discussion

8.4.1 Influence of storage time and -conditions on particle morphology, -size and polymorphic behavior

G [6-D-Phe]-containing, freshly prepared microparticles appeared as fine powder without agglomerates (*Figure 8-1*). Manual shaking of the vial did not lead to adherence of the particles to the chamber wall, which indicated little electrostatic charging, as frequently observed for spray dried products [9] [10]. SEM images revealed a smooth and regular surface [11]–[13]. The smooth surface obtained after production of lipid microparticles by spray congealing frequently comes with the presence of metastable polymorphs [13]–[16]. The α-form with the lowest melting point obtains very small crystals and a hexagonal orientation of the carbon chains, which enable less chain-chain-interaction and a loose packaging [17]–[19]. The α-polymorph was confirmed by DSC and XRPD (*see 8.4.1*).

0 M

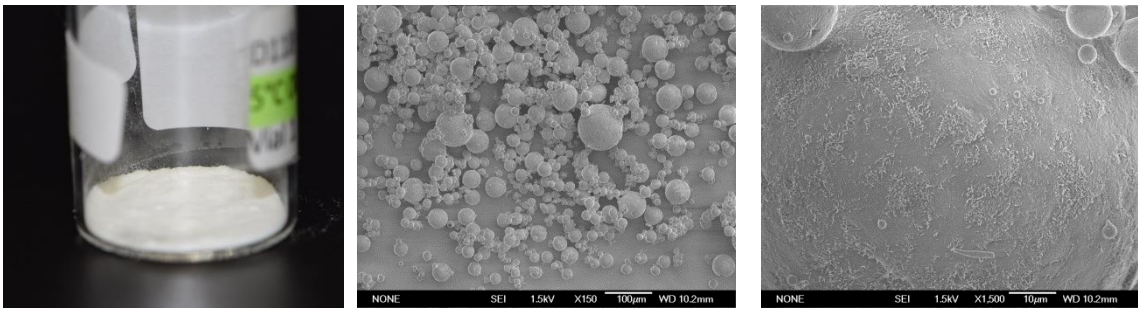

Figure 8-1: Appearance of microparticles directly after production

After storage time of one month at 2-8 °C, particles still appeared as a fine powder. Reconstitution could be performed easily in the reconstitution medium resulting in a stable suspension over the monitored time of 10 min. Neither irregularities nor an increased roughness were observed on the microsphere´s surface. The 25 °C stored samples showed a tendency towards agglomeration (*Figure 8-2*). Upon shaking, the larger agglomerates could be easily separated and reconstitution could be performed without extending the reconstitution time. Samples stored at 40 °C formed a sintered cake already after one month. Complete disintegration could not be achieved by vigorous shaking after adding the suspension medium. The formation of larger agglomerates was also observed in the SEM, where non-spherical, large particles with a rougher and more irregular surface were monitored.

For this reason, analysis after reconstitution was stopped after one month and only polymorph and peptide analysis were performed at the later time points.

The mean particle size of 25 µm at T_0 was increased to 50 µm after one month at 2-8 °C, 60 µm at 25 °C and 58 µm at 40 °C (*Figure 8-4).* Samples analyzed at 40 °C were not representative

since the large sintered clumps were not removed from the sample prior to analysis. In the samples stored at 2-8 °C and 25 °C no increase in particle size can be found in SEM. Thus, the increased particle size obtained in laser diffraction can be referred to larger agglomerates.

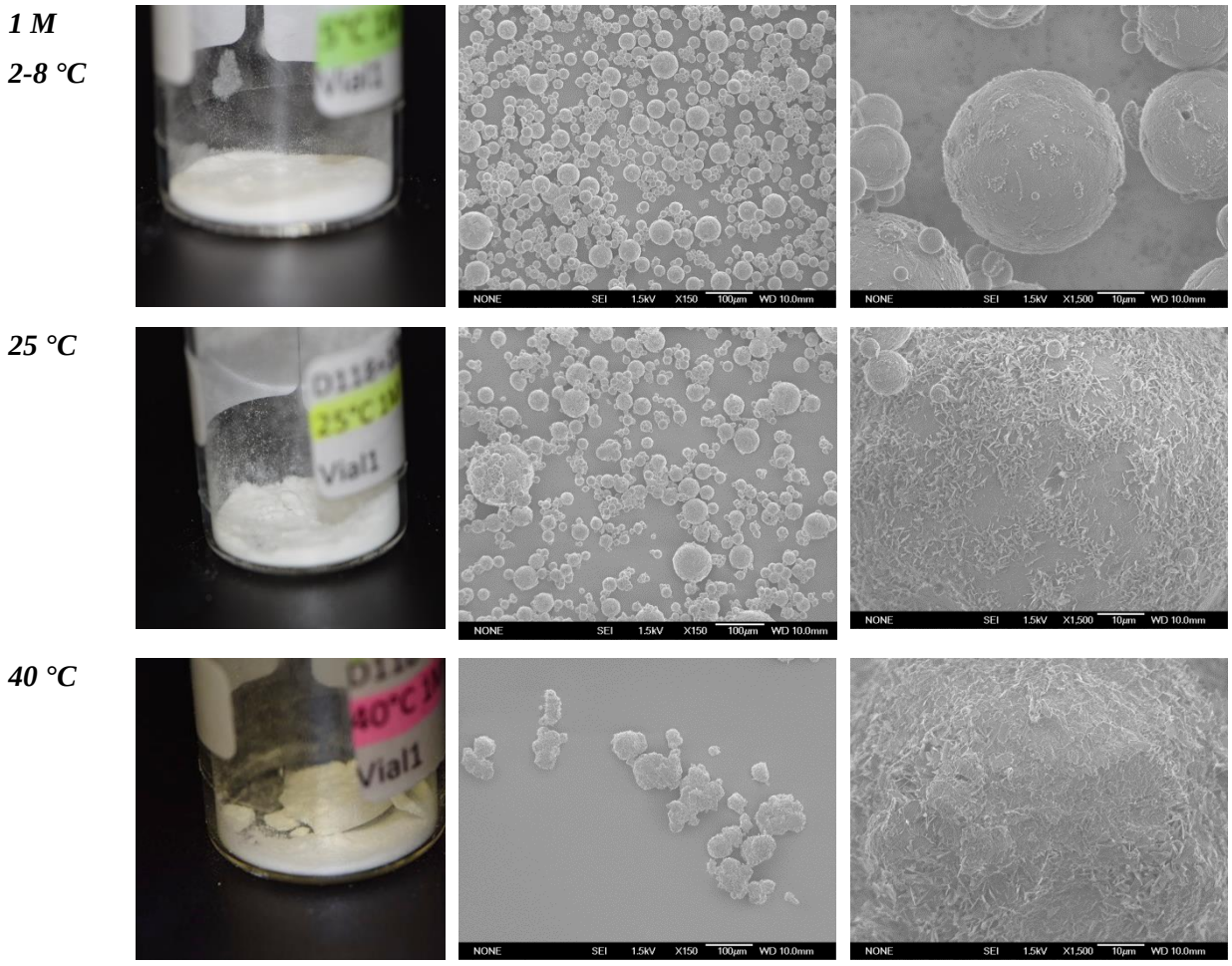

Figure 8-2: Appearance of microparticles after 1 M storage at 2-8, 25 and 40 °C

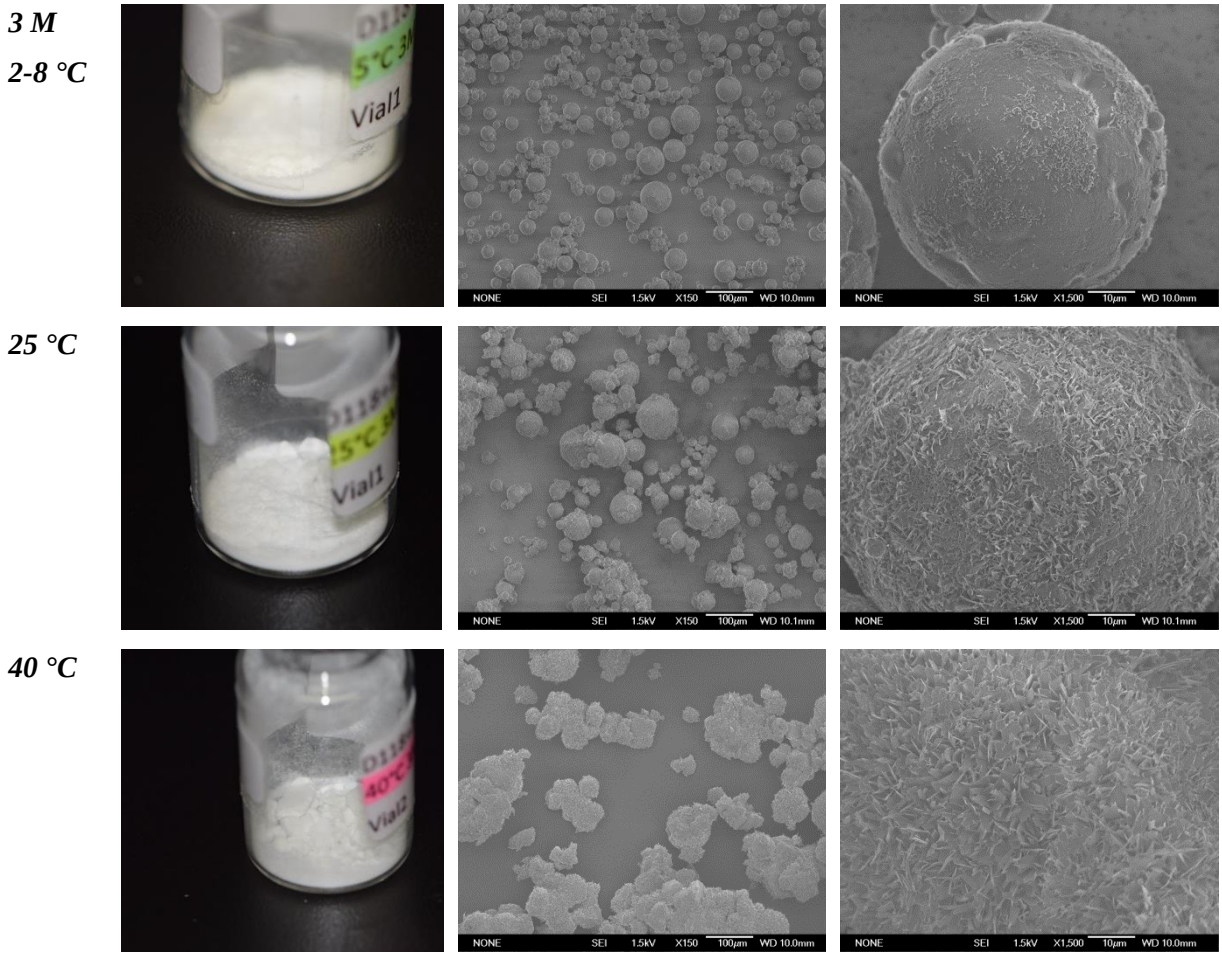

Figure 8-3: Appearance of microparticles after 3 M storage at 2-8, 25 and 40 °C

Further storage for three months at 2-8 °C did not affect the shape and morphology of particles (*Figure 8-3*). Particles still showed a smooth surface without irregularities. Particle size was similar to the one-month time point. Agglomerates could be seen macroscopically after three months at 25 °C which could easily be separated by manual shaking. SEM images revealed the formation of larger particle agglomerates. A closer look on the surface showed, that the particles exhibited a rougher surface indicating lipid crystallization [13] [18]. This effect was even more pronounced in the samples stored at 40 °C with large sintered agglomerates in SEM and flaky crystal structures on the surface. This "blooming" and growth of larger flake- or needle-like structures corresponds to the conversion into the more stable β-polymorph (see *Figure 8-7* and *Figure 8-8*) [18] [20]. Laser diffraction demonstrated further increase in particle size to 90 µm after three months at 25 °C (*Figure 8-4*).

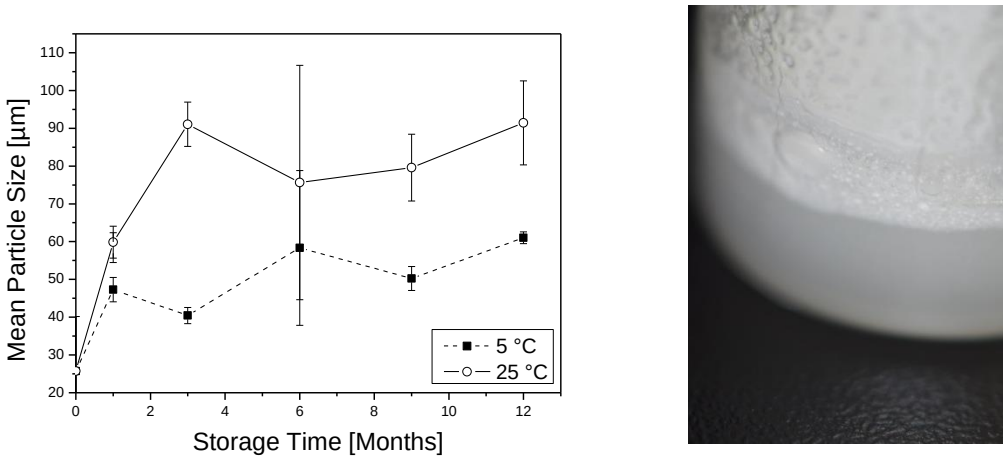

Figure 8-4: Mean particle size measurement using laser diffraction over a 12-month storage time at 2-8, 25 and 40 °C (Mean and SD, n = 3, left); Reconstitution of 2-8 °C-stored samples after 6 months exemplarily for acceptable reconstitution properties (right)

Comparable results were obtained for samples stored for six and nine months. At 2-8 °C particles kept their shape without changes in surface morphology, whereas particles stored at 25 and 40 °C showed a high tendency to agglomeration. *Figure 8-4 (right)* shows exemplarily the suspension of reconstituted microparticles stored at 2-8 °C in the medium, giving evidence to good reconstitution properties. At 40 °C, the microparticles had formed a dense cake which needed to be crushed prior to SEM analysis and could not be analyzed providing meaningful data. Laser diffraction results were comparable to three-month data.

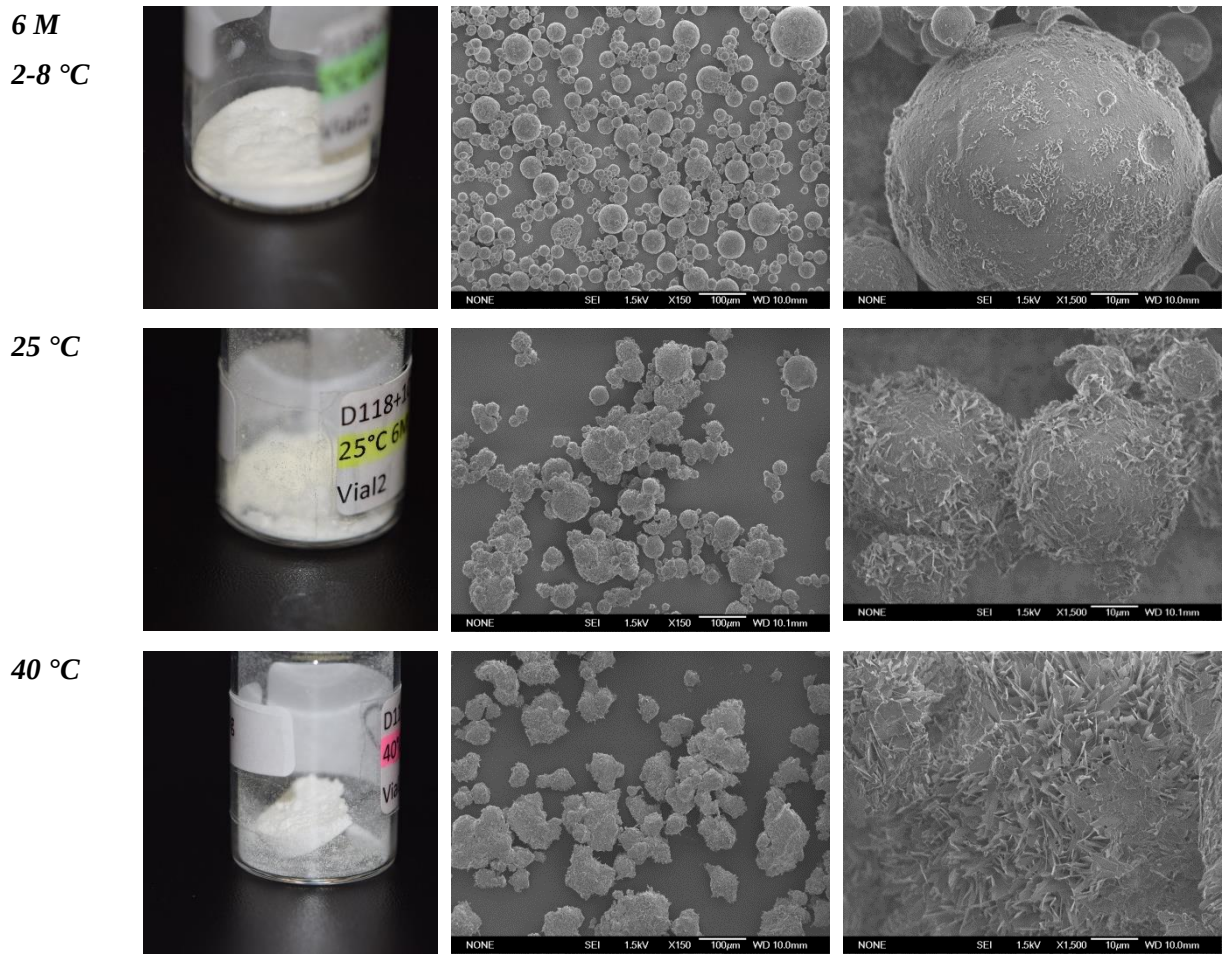

Figure 8-5: Appearance of microparticles after 6 M storage at 2-8, 25 and 40 °C

After twelve months, no further changes were observed for microparticles stored at 2-8 °C (*Figure 8-6*). Compared to previous time points the results after twelve months did not significantly change regarding particle appearance, morphology, size and agglomeration. Overall, storage temperature had a major influence on particle agglomeration and surface roughness. SEM and particle size measurements indicated crystallization phenomena as well as agglomeration in the samples stored at 25 and 40 °C [21]. Samples stored at 2-8 °C only showed a slight tendency to formation of agglomerates.

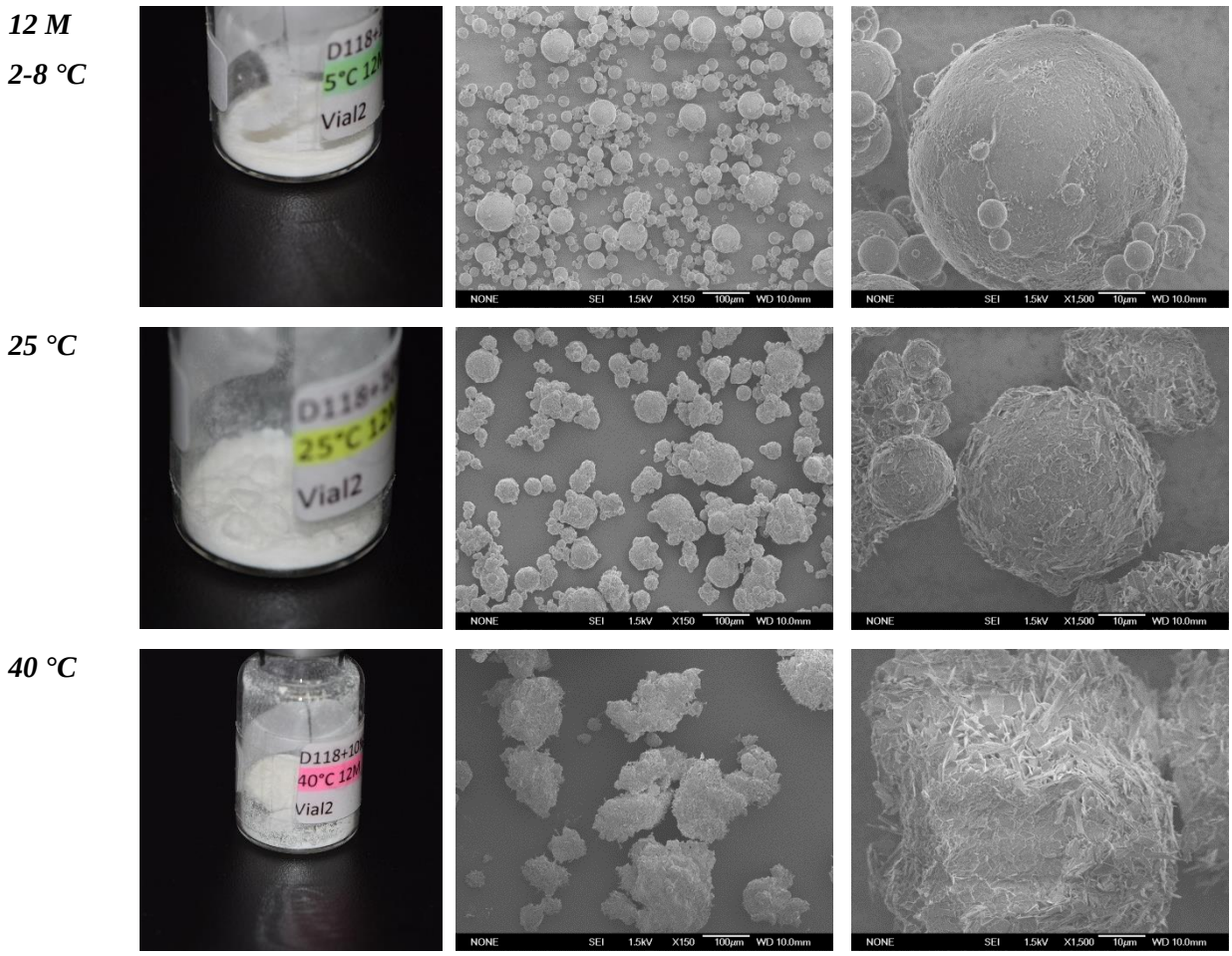

Figure 8-6: Appearance of microparticles after 12 M storage at 2-8, 25 and 40 °C

Polymorphic behavior of lipids has been discussed as a stability problem [22]–[26]. To investigate polymorphic transitions, DSC-thermograms were recorded, which proved the presence of the lower melting α-polymorph within the particles directly after production (*Figure 8-7*). A melting endotherm of 55 °C indicates the α-form, whereas the β′- and β-modification melt at higher temperatures of approx. 63 and 73 °C [27] [28]. The polymorph did not change over a twelve-month storage at 2-8 °C. This observation was in good agreement with literature that the α-polymorph is stable at low storage temperatures [29]. As polymorphism or recrystallization are known to influence release kinetics, a conservation of the initially observed polymorph is important [8] [30] [31]. At 25 °C, same effects are visible. Upon storage at 40 °C, formation of the higher melting β-polymorph was already completed at least after one month. This observed effect corresponds to the transition obtained in *Chapter two* for microparticles and for tempering of triglyceride-based implants to form the stable β-polymorph as described by Kreye et al. [8]. Whereas addition of emulsifiers like sorbitan esters may stabilize the α-polymorph, others are reported to enhance α-β transition [24] [25] [32]–[34].

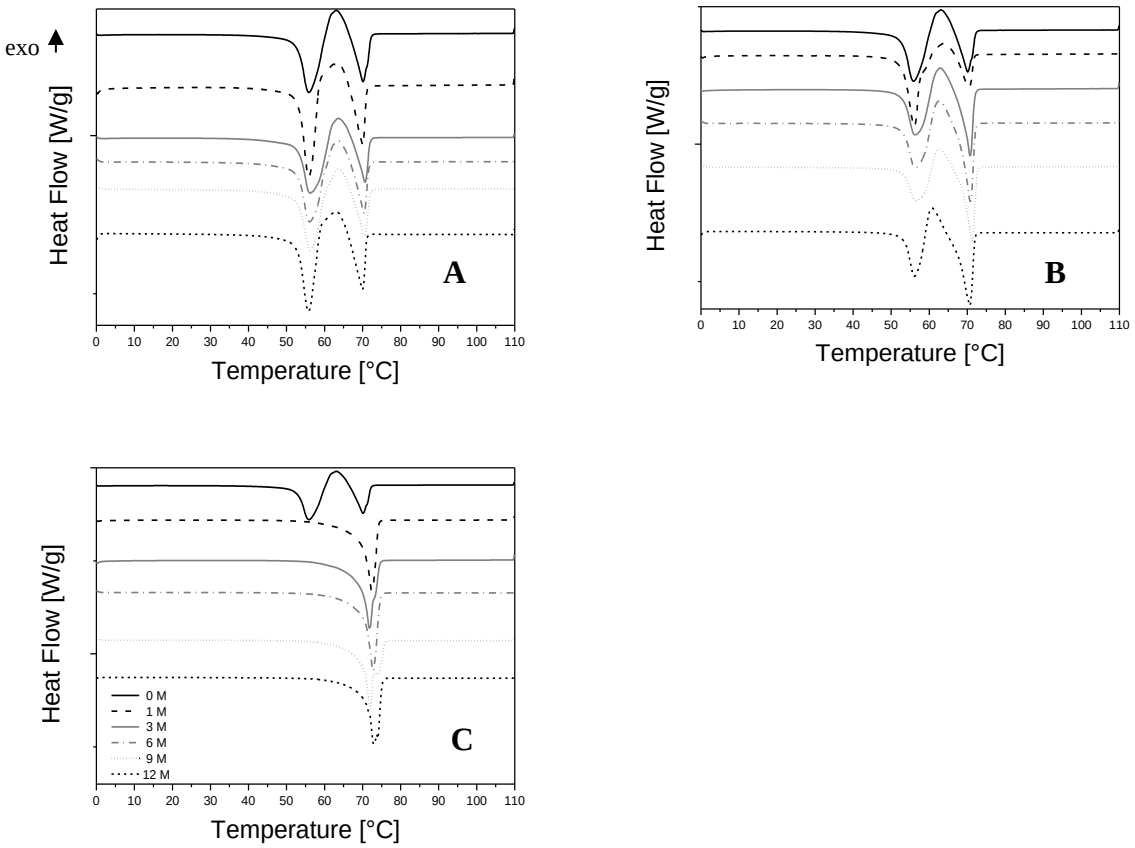

Figure 8-7: Representative DSC thermograms of microparticles stored at (A) 2-8 °C; (B) 25 °C; (C) 40 °C (n = 2); sampling was performed after 0, 1, 3, 6, 9 and 12 months

These findings were confirmed by XRPD measurements of the 12 M sample (*Figure 8-8*). After one year at 2-8 °C microparticles were present in the α-polymorph (one single peak at 21 ° characteristic for the α-modification) [19] [35]. Storage at 25 °C resulted in the presence of both α- and β-polymorph with a more α-polymorph pronounced pattern [19] [15]. In contrast, the sample stored at 40 °C showed the characteristic pattern of the β-modification with three peaks at 18, 23 and 25 ° [19]. These observations confirmed the data obtained by DSC measurements and underline that a storage at 2-8 °C is mandatory in terms of crystallization of the lipid matrix. Overall the storage effects are typical for triglycerides, whereas the presence of additives [23] [36] [37] and drug types influence crystallization behavior [18]. Due to rapid cooling during the spray congealing process, the crystallization of the triglyceride D118 in the lower melting α-polymorph is enabled. Several tempering steps, additionally performed directly after production, are reported for implants, to allow an accelerated crystallization to the β-polymorph [8] [38].

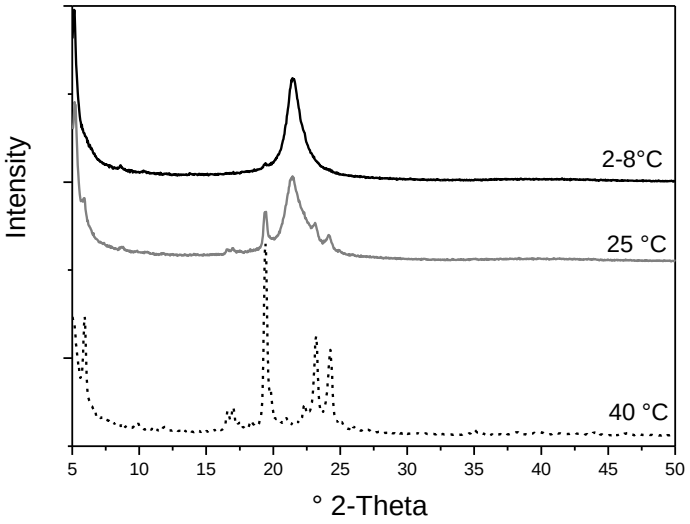

Figure 8-8: Representative XRPD measurement of the 12 M-stored samples at 2-8, 25 and 40 °C. Single graphs were displaced along the ordinate for better visualization (n = 2)

8.4.2 **Drug content and release behavior as a function of storage time and temperature**

The drug content in the lipid matrix did not change significantly over one year at 2-8, 25 and 40 °C *(Figure 8-9)*. Stability was even reported for proteins, like BSA directly after encapsulation into lipid microparticles produced by spray congealing [5]. The minor variability in drug content was due to the used extraction method based on methylene chloride. Furthermore, no degradation peaks were detected in HPLC. Encapsulated G [6-D-Phe] was not affected by higher storage temperature and the process itself, as reported for other peptide drugs [13].

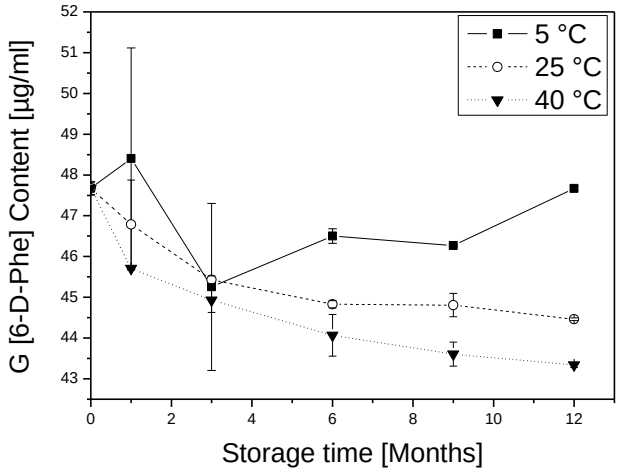

Figure 8-9: G [6-D-Phe] content in D118 + 10 % GMS microparticles during a 12-month storage time at 2-8, 25 and 40 °C (mean and SD, n = 3)

The most important characteristic for the developed drug delivery system was to guarantee the required release profile. Particle size and morphology, as well as an integer matrix with the same polymorphic modification have a major influence on release behavior.

Freshly prepared microparticles showed a burst of approximately 45 % followed by a continuous release until day 21 releasing 70 % of incorporated drug. Regarding a storage at 2-8 °C, the release profile did not significantly change over a one year storage.

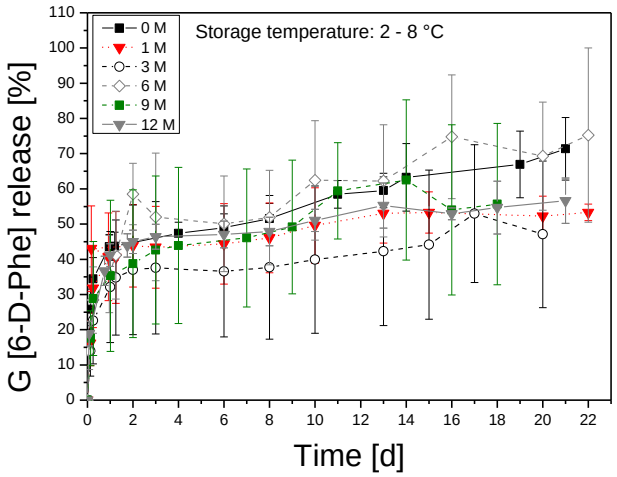

Figure 8-10: Release profile of G [6-D-Phe] microparticles consisting of D118 + 10 % GMS after a storage at 2-8 °C (mean and SD, n = 3)

Monitoring release behavior after a one year storage at 25 °C, the profile differed considerably from the original formulation. Already after one month, the burst was remarkably increased without a sustained drug release following. Enhanced release profiles could also be observed for triglyceride implants after thermal treatment and applying higher extrusion temperatures [8] [31].

Higher storage temperatures are reported to massively influence release rates from lipid-based microspheres, due to change in microstructure of the lipid matrix [39] as well as the polymorph [18]. After one month at 40 °C, the initial burst increased to 80 % without following sustained release. Overall, an amount of 95 % was released. The effect became more pronounced over one year storage. This change in release profile may be attributed to the polymorph change and the sintering of the microparticles corresponding to change in the surface area and the inner structure [30] [34] [40].

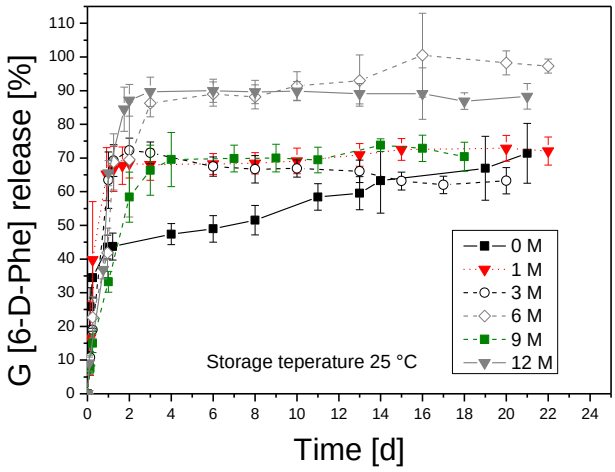

Figure 8-11: Release profile of G [6-D-Phe]-loaded microparticles consisting of D118 + 10 % GMS after a storage at 25 °C (mean and SD, n = 3)

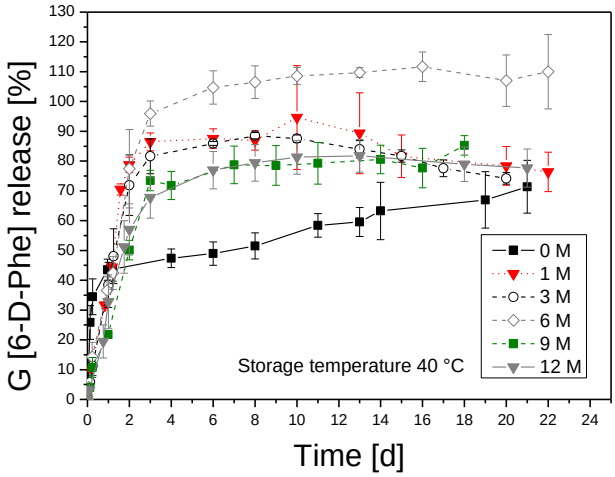

Figure 8-12: Release profile of G [6-D-Phe]-loaded microparticles consisting of D118 + 10 % GMS after a storage at 40 °C (mean and SD, n = 3)

Thus, the G-[6-D-Phe]-loaded D118/GMS microparticles need to be stored at 2-8 °C to assure the preferred release profile with a continuous in vitro release of two weeks. Peptide stability was not impaired by storage at higher temperature. Furthermore, particle agglomeration could be minimized at 2-8 °C and the initial polymorphic structure could be kept upon further storage. In contrast, storage at 25 and 40 °C did lead to transition to the thermodynamically stable β-polymorph and particle sintering.

8.5 Conclusion

G [6-D-Phe] in D118/GMS microparticles were stored over one year at 2-8, 25 and 40 °C and analyzed for particle morphology, size, agglomeration, lipid polymorph, drug content and resulting release behavior. Storage at 2-8 °C was mandatory to ensure minimal particle agglomeration and to guarantee the presence of the initially observed α-polymorph. Polymorphic transition, accompanied with blooming on the surface occurred upon storage at 25 and 40 °C: Release behavior could only be maintained at a storage temperature of 2-8 °C. Furthermore, reconstitution behavior was not affected upon one year storage at 2-8 °C. The study confirmed the stability of G [6-D-Phe] after encapsulation into lipid-based microparticles at all storage temperatures without peptide degradation.

8.6 References

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9 Summary and Future Work

The main goal of this work was the development of a microparticular, sustained release delivery system for the peptide drug gonadorelin [6-D-Phe], which is currently used in combination with other substances to influence the reproductive cycle in swine [1] [2]. Our final product should provide an environmentally friendly alternative for estrus synchronization in swine. Due to harmful effects on fish with detrimental effects on their reproduction, it is necessary to avoid the pollution of ground water with higher amounts of gestagens and their active metabolites [3]–[6]. Commonly used gestagens are usually fed in amounts of 15-20 mg/d over a time period of 10 to 18 days [7]–[9]. The peptide drug G [6-D-Phe] is a promising, rapidly metabolized alternative.

Chapter One reviews the state of the art of polymer-based delivery systems with special focus on the delivery of peptides and proteins. Manufacturing techniques, especially for lipid microparticles are discussed. Furthermore, different regimen and drugs currently used for estrus synchronization in livestock animals are introduced. Due to good physiological tolerance, lack of side effects and the low costs, solid lipids were of special interest for the development of a sustained release drug delivery system. For the injectable, controlled release system for G [6-D-Phe] for an intended release over two weeks, microparticles represent the ideal system.

The spray congealing process was investigated in **Chapter Two** to establish suitable process settings with a focus on yield and particle size. Microparticles could be produced using the triglycerides D116, D118 and D120 with a round shape and smooth surface. The placebo particles were only marginally influenced by the applied spray flow and product flow, with an overall stable process. The achieved particle size was between 30 and 70 µm. The triglyceride particles exhibited the low melting α-polymorph, which can be transformed to the β-form upon tempering for 24 h at 45 °C.

Chapter Three dealt with the incorporation of hydrophilic model substances, specifically tryptophan and aspartame. A suitable system for the determination of drug release was developed. Pure triglycerides did not provide a sustained release profile. Addition of surfactants like GMS led to an increased burst release and a more complete drug release of tryptophan. The release could be controlled between 8 (5 %) and 24 d (10 % GMS). The addition of lipase to the release medium enhanced drug release. For aspartame, the addition of 5 and 10 % Span 40 was most promising for the release duration, which reached 12 days.

Since the product yield upon spray congealing was still low, the manufacturing process was to be optimized before production of G [6-D-Phe] microparticles in **Chapter Four.** High material

losses occurred in the spray tower and the sample container. Yield could be improved by increasing the spray flow, reducing the melt temperature and by installation of a vibrating stirrer. The encapsulation efficiency and particle size could be improved by a reduction of the spray flow. Additionally, a reduction of melt temperature followed by an increased viscosity had a positive effect on encapsulation efficiency.

The peptide drug G [6-D-Phe] could successfully be incorporated into spray congealed lipid microparticles at a theoretical concentration of 1.8 %, which was shown in **Chapter Five**. A broad screening revealed formulations with a high potential for a sustained release over two weeks. Most promising release profiles could be obtained by the addition of 5 and 10 % GMS and Span 40, with a continuous release up to 22 d. Burst release could be reduced by the suspension of lipid microparticles in thermosensitive poloxamer gels. Moreover, polymorphic behavior was evaluated using XRPD and DSC measurements showing that the added surfactant influenced crystallization behavior. Additionally, the triglyceride-emulsifier composition also influenced the water uptake upon incubation, which could be confirmed by the performance of contact angle measurements.

In **Chapter Six** the distribution behavior of lipid microparticles in suspension after injection into the neck and caudal thigh muscles ex vivo was evaluated. Therefore, dye-loaded particles were injected and distribution of the reconstitution medium, as well as the particles monitored by the means of surgery and ultrasound. Particles stayed at the injection site and a hydrophilic drug could diffuse out and distribute along the muscle fibers.

The estrus synchronization was evaluated in **Chapter Seven.** The first pre-clinical study investigated the delivery of 750 and 3750 µg/animal in two different formulations containing triglycerides and 10 % GMS. The lower dose was proved to be sufficient to achieve estrus synchronization. Using 3750 µg/animal, neither higher synchronicity nor longer duration of action could be found, but the percentage of gilts that ovulated decreased significantly. The second study focused on the application of 750 µg/animal in formulations containing triglycerides, GMS and Span 40. The drug-containing system did not have statistically more adverse reactions and was generally well tolerated. The longest duration of cycle blockage, 15.8 ± 4.2 days, was observed after the application of microparticles composed of D118 + 10 % GMS 3750 µg/animal G [6-D-Phe]. The highest synchronicity could be found after injection of D114 + 5 % Span 40 microparticles, where 5 out of 5 animals showed a cycle blockage of 6.4 ± 0.2 d. A statistically relevant delay in onset of follicular growth (after

13.4 ± 0.2 d) could also be observed when using D114 + 5 % Span 40 microparticles, again with the highest synchronicity observed.

The long-term stability of the peptide and drug delivery system was evaluated in **Chapter Eight** over a period of twelve months. Particles were tested in terms of particle size, reconstitution properties, particle morphology, polymorphic forms, drug content and release behavior. Storage at 2-8 °C should be preferred to a storage at 25 °C, although the drug stability was not affected. Regarding particle size, polymorphic transition and release behavior, a storage of 2-8 °C could be guaranteed over the monitored time. Degradation of the peptide did not occur over 12-month storage.

Several milestones in the development of a sustained delivery system for G [6-D-Phe] have been achieved during the work on the current thesis, foremost the incorporation of the peptide into spray congealed microparticles and the variation of release duration in vitro. Additionally, this approach was identified as a possible way for estrus synchronization. The efficacy of the product was confirmed in the pre-clinical studies, revealing a high tolerance and an adequate degree of synchronicity during the follow-up study. Storage stability was confirmed over at least one year.

Future work should focus on the optimization of formulation aspects to keep the synchronous onset of estrus as achieved in the formulation containing G [6-D-Phe] 750 µg/animal D114 + 5 % Span 40, but to prolong the duration of action to 14 days in vivo as achieved in formulations containing 750 µg/animal G [6-D-Phe] in D118 + 10 % GMS. It should be evaluated whether a higher drug load could fasten the release, provide an extended duration of the estrus control and facilitate application as less microparticles need to be injected to achieve the same effective dose. The in vivo effects should be confirmed in further clinical studies or larger herds to evaluate the role of non-responders, adverse events and occurrence of ovulation. Moreover, the application of sustained release G [6-D-Phe] may be connected to the occurrence of permanent ovarian cysts, although we did not find statistical evidence. Since the developed drug delivery system was only tested in gilts, it would be of high interest if the effect is also present in sows. Additionally, a sterilization protocol should be developed and the influence of an irradiation sterilization should be evaluated.

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