Lipid Implants for Controlled Release of Proteins

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Meiner Familie

in Liebe und Dankbarkeit gewidmet

‚Wer recht erkennen will, muss zuvor in richtiger Weise gezweifelt haben.'

Aristoteles

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Chapter 1

Introduction

and

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Lipid implants for controlled release of protein drugs

Why protein therapeutics.....the 'druggable' genome

Biotechnology industry has grown substantially during the past 30 years and is now an integral part of the healthcare system [1]. Beginning in the early 1970s, advances in molecular biology and genetic engineering have laid the foundations for an enormous progress in understanding the biomolecular roots of human disease. The development of methods to manipulate DNA opened the door to virtually unlimited possibilities for recombinant protein production [2]. Proteins are integral parts of the body as they carry out all important physiological, biological processes like ligands for signaling, enzymes for biotransformation reactions, receptors for pharmacological response, antibodies in immune system interactions and genomic transcription and translation [3].

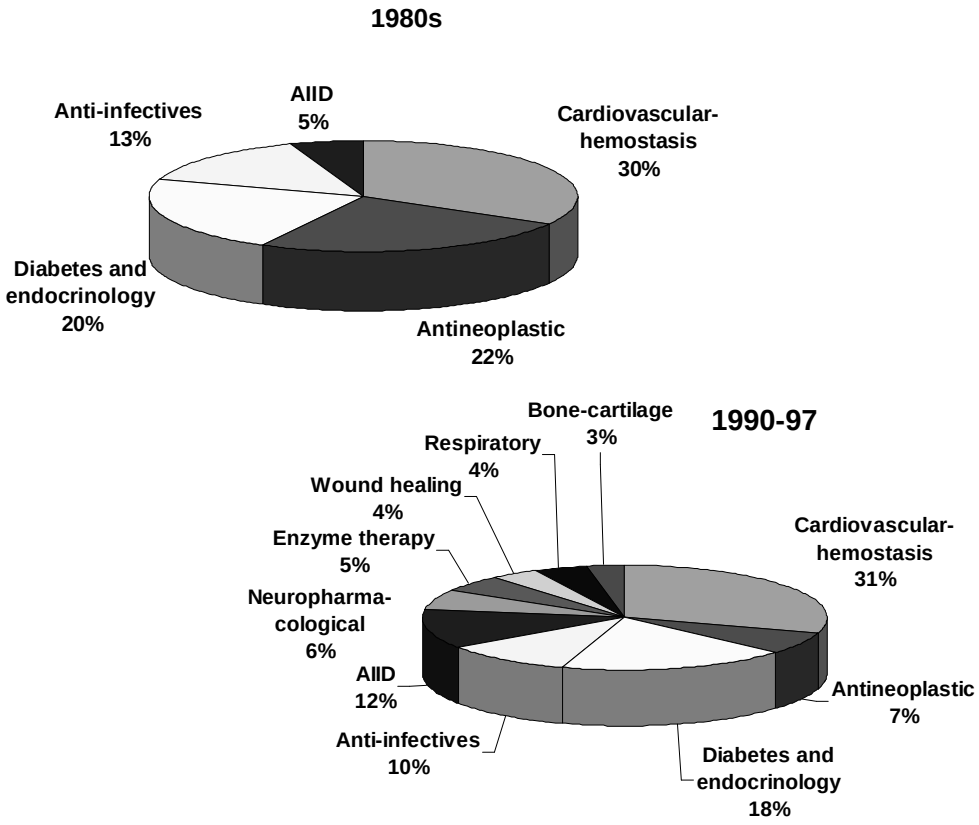

Fig. 1: Therapeutic categories of rDNA products studied in clinical trials. (AIID Arthritis, inflammation and immune disorders) [2].

Recombinant DNA (rDNA) products that entered clinical studies in the 1980s were studied in five major therapeutic categories (fig. 1), however interferons and interleukins accounted for more than 30% of protein therapeutics tested [2]. As molecular biology and medical knowledge advanced in the 1990s, the number and variety of rDNA therapeutics entering clinical study increased and a wider therapeutic focus was envisaged (fig. 1). The variety of products in the period since 1997 is similar to that of 1990-97, while the average number that entered clinical studies per year is somewhat lower. Today, biopharmaceuticals (recombinant therapeutic proteins, monoclonal antibody-based products used for *in vivo* medical purposes and nucleic acid based medicinal products) represent one in every four genuinely new pharmaceuticals coming on the market [4].

The knowledge of genomic information including the recent sequencing of the genome [5, 6] has greatly increased the interest in discovering new proteins, both as therapeutic agents and as potential targets for disease management. The human genome comprises about 30,000 genes, of which 3,000 to 10,000 are estimated to be disease related genes, however, with the current therapeutical strategies, only about 600 of them are considered to be 'druggable', that is, constituting potential targets for therapies [7]. While globally well in excess of 500 candidate biopharmaceuticals are undergoing mid- to late stage clinical trials (fig. 2), the number of new targets addressed comes down to an average of four each year [7].

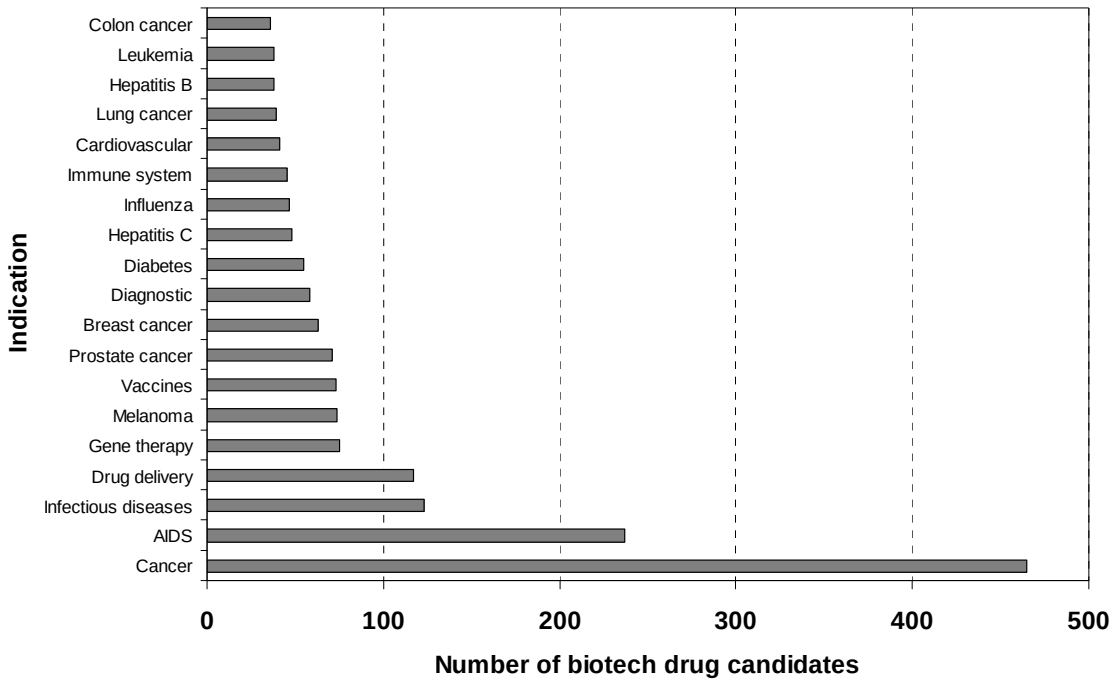

Fig. 2: Investigational biotech drugs by indication in December 2004 [8].

There is a broad consensus among experts in this field that the development of alternative methods of product delivery such as non-oral and sustained release delivery systems will expand the range of potential drug targets [7] and allow to fully profit of the opportunities presented by a combined knowledge of genomic information and the understanding of protein structure-functional relationships [2, 4, 9].

Why implants ..... the issue of parenteral delivery

Nature did not evolve proteins for manufacture ex vivo [10]. For this reason, many human proteins produced in recombinant form are difficult to manufacture and even more challenging in delivery to their site of action in the right time frame. The main obstacles to the successful delivery of proteins are enzymatic barriers and absorption barriers [3]. Because of their large molecule size and hydrophilic nature, proteins show extremely poor intrinsic permeabilities across biological membranes, being a major drawback for oral as well as nasal, ocular, transdermal, rectal and pulmonary delivery [11]. In addition, the oral bioavailability is restricted to less than 1-2% as proteins are easily degraded by proteolytic enzymes in the gastrointestinal tract (e.g. pepsin, intestinal pancreatic proteases like trypsin, elastases, brush border proteases like aminopeptidases, carboxypeptidases) [12]. Thus, the major route of administration is parenteral delivery via injections or infusions. As proteins are rapidly cleared from the blood stream and therefore display short *in vivo* half-lives (<30 min) [12], frequent application is required [10] resulting in the need for intensive medical care and representing a considerable burden for the patient.

In this context, the development of sustained release formulations becomes highly desirable, promising triple benefit: for the patient, the therapy and also the protein drug itself. While the patient is relieved of frequent injections, the need for close medical supervision is obviated, and therapy costs are reduced. The use of a controlled release device provides a steady release of drug at a desired rate, thereby maximizing the efficacy-dose relationship [13]. Proteins can be specifically targeted to their site of action, which allows for higher local drug concentration while minimizing systemic exposure and associated side effects. Apart from that, incorporation in a controlled release formulation can protect the highly sensitive protein drugs from detrimental influences during storage and delivery.

For example, delivery to the central nervous system (CNS) is an area with an urgent need for improvement [9, 14-16]. Major achievements have been made in understanding the molecular mechanisms responsible for neurodegenerative diseases and simultaneously,

numerous proteins such as neurotrophic factors, anti-apoptotic or anti-oxidant agents have been identified as potential therapeutics. However, their efficacy when tested *in vivo* proved to be low, and inadequate protein delivery is believed to be part of the problem [9]. While the continuous infusion of drugs via pump systems was commonly employed in the investigations of drug efficacy [14, 17], the permanent risk of infection and the often low stability of protein drugs within the reservoir of the infusion liquid demonstrate the need for improvement. As protein drugs cannot pass the blood brain barrier [18], the development of a controlled release system, which could be placed at the desired site of action by stereotactic implantation would be of extreme value [19, 20].

Why lipids.....polymers, the big draw with a small flaw

The history of controlled release dates back to Folkman and Long who developed digoxine-releasing silicon rubbers in 1964 [21]. Foundations for the delivery of protein and peptide drugs were laid in 1976 by Folkman and Langer with the release of BSA from poly(ethylene-co-vinylacetate) (EVAc) [22]. While these systems for the first time guaranteed a release of more than 100 days, a major drawback was seen in the fact, that they did not degrade *in vivo* and therefore necessitated surgical removal [23]. Therefore, a variety of synthetic and naturally occurring biodegradable materials have been studied as delivery systems over the past 30 years (tab. 1) in the form of nano- and microparticles, cylindrical implants or scaffolds for tissue engineering, to name a few.

Amongst them, degradable polyesters have found the most widespread use, probably due to their long safety history for their application in medical products and FDA approval. Currently, controlled release formulations of peptide and protein drugs on the market exclusively consist of poly(lactic-co-glycolic acid) (PLGA) microsphere formulations [3]. While having many advantages, over the past few years major shortcomings of these polymers have been identified with regards to protein stability during release. The microenvironment within degrading matrices can change significantly resulting in low pH and increase in osmotic pressure beyond physiological values [24-26]. Under these conditions, hydrolytic degradation of proteins and formation of aggregates as well as chemical reactions between polymer degradation products and peptides have been proven [27, 28].

While natural polymers as an alternative are readily available, relatively inexpensive and allow for a multitude of chemical modifications, they also come with poor geometrical stability, once processed to a drug carrier, due to swelling and poor *in vivo* mechanical

strength, as well as possible antigenic response and tissue irritation due to residual aldehyde crosslinking reagents. Drugs are very often prone to a burst release phase followed by faster release than from synthetic polymeric systems [29].

Synthetic materials	**Natural materials**	
Polyorthoesters	Proteins:	Lipids:
Polyanhydrides	Albumin	Cholesterol
Polyamides	Collagen	Fatty acids /-anhydrides
Polyalkylcyanoacrylates	Gelatin	Lecithin
Polyesters:	Casein	Dipalmitoylphospha-tidylcholin
Lactides/ glycolides	Globulin	Monoglycerides
Polycaprolactones	Fibrin	Triglycerides
Polyphosphazanes	Polysaccharides:	Polyglycerol esters of fatty acids
Pseudo-polyamino acids	Starch	Mixtures of glycerides and fatty acid esters (Gelucire®)
	Cellulose	Waxes
	Chitosan	
	Dextran	
	Alginic acid	

Tab. 1: Biodegradable matrix materials investigated for the controlled release of proteins ([3], references for lipids see table 2).

Lipids have gained increasing attention in this context as they offer some advantages: being physiological substances, they show good biocompatibility [30, 31]. They are less expensive compared to polymers, but also offer a high variability due to different degrees of esterification and chain lengths or even mixtures of lipid components (e.g. Gelucire®) [32, 33]. Their high compactibility allows for the fabrication of matrices by compression, which has already been used in several investigations of lipid cylinders for delivery purposes (tab. 2). Cylindrical matrices, on the one hand, represent a good model delivery system with well-defined geometry, which allows for the investigation of material properties and later transfer to microparticulate carriers, being more complicate in manufacture. On the other hand, mini-cylinders themselves can be implanted *in vivo* as a drug delivery system, allowing for higher dosage and probably longer release periods than microparticles due to the more compact dimensions. However, the systems developed so far only have model character, being restricted to subcutaneous application in animal models due to their large size (tab. 2). Apart from that, the resulting release profiles do not allow for a long-term investigation of protein drugs as they are mainly limited to a period of one month. Lipid microparticles as an alternative would be small enough for other

targets such as CNS delivery, however, the release periods reported so far were even limited to less than two weeks. While for polymeric systems a plethora of models exists, describing the release mechanism from different kinds of materials and geometries, knowledge is sparse for lipid matrices. Consequently, there is a distinct gap between the proclamation of lipids as an alternative to polymeric delivery systems and the actual state of the art in formulation strategies and matrix performance. It was the aim of this thesis to address these problems and boost the competitiveness of triglyceride formulations for the delivery of protein drugs.

Lipid	Excipients	Peptide/ protein drug	Manufacturing method	Dimensions in mm (diameter × height)	Release period (days)	Ref.
Cylinders						
Cholesterol		Insulin	Powder mixture, compression	13 × 1.5	> 40	[34]
Fatty acids, fatty acid anhydrides triglycerides		Insulin	Powder mixture, compression	13 × 1.5	30	[35]
Cholesterol, palmitic acid		Insulin, somatotropin	Powder mixture, compression	13 × 1.5	40	[36]
Cholesterol, lecithin		BSA	Powder mixture, compression	5.5 (27 mg)	10	[37]
Stearic acid		BSA	Powder mixture, compression	5 (30 mg)	3	[38]
Polyglycerol esters of fatty acids		Interferon α	Heat extrusion	1.2 × 10	14	[39]
Glyceryl trimyristate	Gelatin	TAMRA-BSA, hyaluronidase	Powder mixture, compression	2 × 1.7	2 - 10	[40]
Glyceryl tristearate	Trehalose, cyclodextrin, PEG 6000	Interferon α-2a	Powder mixture, compression	5 × 2.3	10 - 30	[41]
Glyceryl palmito-stearate	PEG 4000, Gelucire 50/13	Lysozyme	Powder mixture, compression/ melting	2.5 × 4	4	[42]
Cholesterol	Phosphatidyl-choline, Quil A	FITC-ovalbumin	Powder mixture/ Freeze-drying aqueous dispersion, compression	2 (5 mg)	2 - 10	[43]
Glyceryl tripalmitate		Insulin	Powder mixture, compression	2 × 2	12	[44]
Microparticles						
Dipalmitoylphospha-tidylcholine	Hydroxylethyl-starch	Human Immuno-globulin	Spray drying	4.5 µm	1	[45]
Glyceryl tripalmitate		Thymocartin, insulin	Solvent evaporation melt dispersion	20 - 150 µm	5	[46]
Glyceryl tripalmitate		Somatostatin	Solvent evaporation melt dispersion	50-100 µm	12	[47]
Dipalmitoylphospha-tidylcholine	Chondroitin sulfate	FITC-BSA	Spray drying	6 µm	n.d.	[48]
Glyceryl trimyristate Gelucire® 50-02		BSA	Coating by super-critical fluid technology	500 µm	1 - 24 hours	[49]
Glyceryl tripalmitate	Lecithin, Poloxamer 188, PEG stearate	Insulin	W/O/W multiple emulsion	0.2-0.4 µm	1	[50]
Glyceryl monobehe-nate, -monosterate		GNRH antagonist (Antide)	Co-melting/ solvent stripping	30 µm	10	[51]
Glyceryl tripalmitate		Insulin	Spray congealing	230 µm	n.d.	[52]

Tab. 2: Overview of the use of lipids as matrix material for controlled release of proteins (n.d. not determined).

Goals of the thesis

In order to achieve a better understanding of lipid matrices, triglyceride cylinders were investigated both from an application-based and a mechanistic point of view.

In **chapter 2** an overview is given on general stability problems occurring during processing of protein drugs, exemplified for lipospheres.

An important prerequisite for the success of a controlled release formulation is the identification of a suitable manufacturing strategy, both preserving protein stability and ensuring long-term release. Thereby, it was of special interest to investigate, whether an understanding of the internal matrix structure by the help of confocal microscopy could assist in choosing the best preparation procedure (**chapter 3**) for lysozyme loaded matrices. Besides, it was the aim of this investigation, to allow for a further size reduction of the cylinders to 1 mm diameter and 1 mm length without compromising their potential for long-term protein release. These dimensions were seen as a prerequisite for *in vivo* biocompatibility testing of the matrices in the rat brain and the application of the procedure to brain derived neurotrophic factor (BDNF), a potential therapeutic for Huntington's disease in the CNS.

In **chapter 4**, a PEG co-lyophilization technique, the manufacturing strategy successfully identified before, was tested on interleukin 18, a cytokine with the potential for treating glioblastomas, for which no suitable delivery strategy had been devised so far. To this purpose, release in the nanogram-range and bioactivity of released cytokine were monitored by radioactivity labeling and a splenocyte based bioassay.

Stability problems encountered during incubation of controlled release matrices can only be addressed properly, if the underlying mass transport mechanisms are known. Therefore, it was the goal of our investigations in **chapter 5,** to identify the mechanisms governing release from triglyceride matrices by diffusion studies with fluorescently labeled model drugs visualized by confocal microscopy. In addition, the behavior of matrices at different loadings and in the presence of PEG 6,000 as a release modifier were examined.

As buffer penetration and drug diffusion in water filled pores were of major interest in this context, it was highly interesting to characterize the effect of matrix and drug properties on the two crucial processes during release. Wettability was investigated in **chapter 6** for its influence on buffer penetration and the possibility to tailor this process by matrix lipophilicity and surface activity of the drug.

In **chapter 7,** it was assessed to characterize the dependence of drug diffusion from lipid matrices on molecular weight of model substances with and without inherent surface activity: proteins (lysozyme, trypsin, ovalbumin, bovine serum albumin, alcohol dehydrogenase, catalase and thyroglobulin) and FITC-dextrans (4 to 2,000 kDa) respectively.

Taking these investigations together, an approach to comprehensively covering the most important aspects of controlled release device design was attempted. Thus, it was endeavored to take a decisive step towards establishing triglycerides as a competitive matrix material for protein delivery.

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Chapter 2

Liposphere as Delivery Systems for Peptides and Proteins

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In: C. Nastruzzi, (Ed.), Lipospheres in Drug Targets and Delivery: Approaches, Methods, and Applications, CRC Press (2005), 67-86

1. Introduction

Delivery systems are designed to protect an incorporated drug from the environment during delivery and to provide a controlled release. The goal may either be to deliver a drug locally to specific sites in the body or to prepare a drug carrier system that acts as a reservoir at the site of injection over a certain time period [1].

In recent years, a growing number of potential peptide and protein drugs has been discovered due to progress in biotechnology and genetic engineering. Unfortunately, protein drugs are subject to numerous chemical and physical instability mechanisms and rapid enzymatic degradation;, therefore, they often show low bioavailabilities and have short *in vivo* half lives thus necessitating parenteral delivery [2]. To sustain therapeutic effects, they have to be administered by infusion or frequent injections. It is obvious, that there is an urgent need for suitable delivery systems capable of preserving protein stability and improving administration frequencies, and thus lessening the strain on patients.

Particulate drug carriers, which have been investigated for this purpose are o/w emulsions, liposomes, microparticles and nanoparticles based on synthetic polymers or natural macromolecules [3]. Successful long-term delivery of peptide and protein drugs has been achieved by using biodegradable polymers such as copolymers of lactide and glycolide [4, 5]. Use of synthetic materials, however, often goes along with biocompatibility problems, residual solvents and detrimental effects on incorporated drug during manufacturing procedure or during polymer degradation after application [6]. Therefore, alternative carrier substances have been investigated in recent years. Among them, lipidic materials have gained growing attention. Successful peptide or protein incorporation and delivery has been reported for liposomes [7], multivesicular liposome preparations [8], cubic phase gels [9], hollow lipid microparticles [10] and hollow lipid microcylinders [11], microparticles [12, 13] and solid lipid nanoparticles (SLN) for intravenous applications [14, 15].

Lipospheres have first been reported by Domb, who describes them as water-dispersible solid microparticles of a particle size between 0.2 to 100 µm in diameter, composed of a solid hydrophobic fat core stabilized by one monolayer of phospholipid molecules embedded in the microparticles' surface [1]. Using this definition, liposphere size also ranges in the nanometer scale. Usually, nanoscale particles consisting of a solid lipid core are termed SLN [16], though sometimes inconsistent nomenclature can be found. Unlike SLN, lipospheres are restricted to the stabilizing material of a phospholipid layer because

of their definition [1]. This chapter focuses on research results obtained for peptide and protein formulations termed lipospheres, and it does not consider SLN literature at large.

Lipospheres have successfully been used to incorporate and deliver a variety of substances, including anti-inflammatory compounds [17], local anesthetics [18], antibiotics [1], insect repellants [19], vaccines and adjuvants [20]. The number of publications concerning protein delivery, though, is still limited. To the best of our knowledge, only few peptide and protein drugs have been incorporated into lipospheres and characterized for release behavior to date (tab. 1). Prerequisites for the use of any carrier for drug delivery are sufficient drug load, physical stability of the aqueous dispersion and optimized drug release profiles [21]. This chapter will try to point out the special demands and difficulties associated with peptide and protein drugs when aiming at the realization of these prerequisites. Discussion of issues such as particle characterization and biocompatibility can be found elsewhere in this publication.

Proteins are challenging substances to formulate because of their many instabilities and, most often, high hydrophilicity [22]. The latter is one of the main obstacles encountered when designing delivery systems, as potential carriers most often consist of lipophilic materials, thus complicating preparation procedures and impeding high drug loadings. Often, proteins are exposed to detrimental conditions in the manufacturing procedure, and there are several publications dealing with stability issues during microparticle formulation [6, 23]. We give an overview of protein stability issues before discussing preparation procedures for peptide and protein loaded lipospheres.

2. Protein stability

2.1. General considerations

Peptide and protein stability is highly dependent on amino acid composition and sequence and, for proteins, on the formation of higher-order structures, which means that every protein has to be considered as a special case. Given a certain sequence, also external factors such as pH, ionic strength, temperature, pressure and the existence of interfaces can have a tremendous impact on peptide and protein integrity [24].

There are two main degradation pathways: physical or non-covalent degradation, which leads to changes in secondary and tertiary structures and chemical inactivation, which results from changes in primary structure [6].

The term 'stability' can have different meanings in the context of protein formulations. A stable pharmaceutical product according to the U.S. Food and Drug Administration (FDA) definition is one that deteriorates no more than 10% in 2 years [25]. Conformational and physical stability of a protein is defined as the ability of the protein to retain its tertiary structure [6]. Noncovalent degradation is relevant mainly for proteins having higher-order structures, rather than peptides. Native structure is maintained by a balance of noncovalent interactions such as hydrogen bonds, van der Waals interactions, salt bridges and hydrophobic interactions [26]. Classic conditions leading to loss of conformational stability, called denaturation, are elevated temperature, extremes of pH, denaturants and adsorption to hydrophobic surfaces [6]. Proteins can unfold locally and globally, which may lead to inactive forms. In biochemistry, this is expressed by the magnitude of the change in Gibbs free energy between the folded and the unfolded state of the protein. The larger the free energy change, the more stable the protein. For most proteins, the unfolded state is insoluble and favors aggregation [24].

Considering chemical stability, even alterations at single amino acids or the peptide bond can be detrimental [6]. Chemical reactions having an impact on protein stability include hydrolysis of the peptide bond, deamidation, oxidation, β-elimination, isomerization or disulfide bond breakage and formation. The extent to which they occur is mainly influenced by the temperature and pH value of the solution [24].

Bearing in mind that proteins react sensitively to the above-mentioned environmental conditions, preparation procedures for protein pharmaceuticals have to be chosen very carefully in order to preserve protein integrity and functionality.

2.2. Protein stability during formulation procedures

Protein stability during encapsulation in biodegradable polymer microparticles has been reviewed in detail [6, 23, 27]. In comparison, little information is available on lipid materials. However, conditions causing stability problems are not specific for polymer microparticle formulations. Lipids, being a hydrophobic material like many biodegradable polymers, may involve similar processing parameters [22, 28].

When formulating lipophilic materials, techniques often involve the use of organic solvents, interfaces with aqueous solutions and high shear forces [6]. One of the most often used techniques to encapsulate proteins is the so-called water/oil/water (w/o/w) double-emulsion solvent evaporation technique, in which an aqueous protein solution is emulsified into an organic solution of the matrix material. This primary emulsion is added to an outer aqueous phase, in which particles start to harden as the organic solvent evaporates. Alternatively, the solid protein can be added directly to the organic solution in a solid/oil/water (s/o/w) emulsion method [23].

Upon contact of an organic solvent with an aqueous protein solution, the solvent can diffuse into the water phase, alter its ionic strength or bind directly to the protein, all favoring the exposure of the protein's hydrophobic regions, which can lead to formation of soluble and insoluble aggregates [6]. Some organic solvents are capable of solubilizing lyophilized proteins without denaturing them. They are generally protic and hydrophilic [6]. An important factor in influencing protein solubility is the pH of the aqueous solution before lyophilization [29].

Upon addition of proteins to aprotic, hydrophobic solvents increased intramolecular interactions of the lyophilized protein result in restricted conformational mobility of the protein, thus restricting activity [30]. However, proteins display increased thermostability in anhydrous organic solvents due to the reduced conformational mobility [31], and water-free methods may help avoid aggregation processes that occur when using the double-emulsion technique, in which the protein is conformationally mobile.

During emulsification, a large, hydrophobic surface is formed. Exposure to air, which has a high hydrophobicity that favors unfolding, is considered a main cause of protein inactivation during the emulsification processes [6]. Proteins can adsorb strongly to both hydrophilic and hydrophobic materials. Whereas the former adsorption is typically reversible, the latter results in irreversible conformational changes. Adsorption is strongest at the isoelectric point of the protein [6]. Methods employed for emulsification, such as

homogenization or ultrasonication, will introduce large pressure gradients, shear forces and heat development in the emulsion, thus speeding up unfolding and denaturation [28]. In addition, ultrasound has been proven to produce free radicals that can initiate chemical reactions [32].

Before evaluating protein stability during liposphere preparation, a summary of the different approaches for peptide and protein encapsulation will be given. Table 1 shows an overview of relevant publications arranged according to their publication dates.

Peptide/Protein drug	**Matrix material**	**Preparation method**	**Author**	**Publication date**	**Ref.**
Antigen	Waxes, fatty alcohols, paraffins, hard fat	Melt method, Solvent technique	Domb	1990	[1]
[D-Trp-6]-LHRH	Stearic acid	W/O/W multiple microemulsion	Morel	1994	[38]
Thymopentin	Stearic acid	W/O/W multiple microemulsion	Morel	1996	[39]
		O/W multiple microemulsion			
R32NS1 Malaria antigen	Tristearin	Melt dispersion	Amselem	1996	[20]
	Polylactide				
	Polycaprolactone				
Somatostatin	Triglycerides	Cosolvent-solvent evaporation	Reithmeier	1999	[37]
Triptorelin	L-PLA	Cosolvent-solvent evaporation	Rasiel	2002	[35]
Leuprolide	PLGA 50:50				
	PLGA 75:25				
Hydrophilic model drug	Triglycerides	Melt dispersion	Cortesi	2002	[36]
	PLA	Solvent evaporation			
	Eudragit RS 100	W/O/W double emulsion			

Tab. 1: Overview of different approaches reported for peptide and protein encapsulation into lipospheres.

3. Preparation of peptide and protein loaded lipospheres

3.1. Preparation methods

Lipospheres can contain biologically active agent in the core, in the phospholipid, adhered to the phospholipid, or a combination of the two [1]. Since the emergence of lipospheres, a number of research teams have conducted studies to investigate relevant production parameters such as the effects of different compositions, ratio of ingredients, drugs, and preparation procedures on encapsulation efficiency, size distribution and release characteristics [20, 33-36]. Within this chapter, only results relating to peptide and protein drugs shall be considered, and the reader is referred to the literature and the other chapters in this book for a complete overview.

Two preparation methods for drug-loaded lipospheres can be used: a solvent technique or a melt technique [1]. For the solvent technique, organic solvents are employed to dissolve the active agent, the solid carrier and the phospholipid component. After evaporating the solvent, warm buffer solution is mixed with the resulting solid until a homogeneous dispersion of lipospheres is obtained.

In contrast, the melt method, where the lipophilic agent is melted together with the lipid core material or dissolved in melted core material, is described as the preferred technique. The phospholipid, together with warm aqueous medium, is added as a solid, followed by mixing (mechanical shaking or stirring, fine mixing using homogenization and sonication) and rapidly cooling the preparation to solidify the liquid core.

It has been suggested that hydrophilic antigens should be dissolved in aqueous buffer and added to the molten mixture of vehicle and phospholipid [1]. For the preparation of R32NS1 malaria antigen lipospheres, the lipid components at a 1:1 molar ratio were dissolved in chloroform in a round–bottom flask. After evaporation of the organic solvent, the lipid mixture was heated from 40 to 80°C to melt the fat. Warm phosphate-buffered saline (PBS) containing the antigen was added, and the formulation was mixed until a homogeneous dispersion was obtained. Cooling was performed by immersion of the flask in a dry ice-acetone bath for several seconds while shaking. Antigen encapsulation was found to be more than 80% [20].

Although lipospheres are primarily designed for the incorporation of lipophilic substances, Domb suggests approaches for processing a water-soluble agent [1]. Because the inner core of the liposphere is hydrophobic, it is recommended that the water solubility of the agent

be decreased before liposphere preparation. Possible methods suggested are using a water-insoluble salt or base, a complex, or insoluble precursor form of the agent, or preparing an aqueous medium in which the agent is less soluble (e.g. by adjustment of pH or ionic strength or by adding salts or additives).

3.1.1. Preincorporation into lipophilic carriers

Alternatively, the hydrophilic agent can be preincorporated into liposomes or microparticles that can be used as hydrophobic agent particles and incorporated into lipospheres with a matrix having a lower melting point [1]. This was demonstrated for tetracain; however, no example exists for peptide or protein incorporation. Successful reports about model peptide incorporation into lipid microparticles can be found in Reithmeier: a solvent evaporation and a melt dispersion were compared for insulin, somatostatin and thymocartin [12, 13, 37]. For the solvent evaporation method, the peptide drug was added as a solid or an aqueous solution to an organic lipid solution, which was then dispersed in an outer aqueous phase and stirred for evaporation of the organic solvent. For the melt dispersion method, the peptide drug was added as a solid or an aqueous solution to a lipid melt, which was subsequently poured into a cooled outer aqueous phase and stirred until solidification of the particles occurred.

Domb presents an example of liposphere encapsulation of tetracain/ tristearin microparticles having a size of less than 38 µm. The particles were suspended in molten ethyl stearate containing lecithin at 40°C. The melting point of tristearin is 65 to 72°C, so the microparticles remained solid during liposphere preparation. Warm phosphate buffer was added and the formulation was mixed and cooled. The resulting lipospheres had a particle size of 50 µm [1].

Domb further describes a method of incorporating antigens into lipospheres, where the antigen, together with Lipid A, an adjuvant, was first incorporated into multilamellar liposomes. Ethyl stearate and L-alpha-lecithin were heated to 40°C to melt the ethyl stearate. Warm liposome dispersion was then added and the formulation shaken and cooled as described for the melt method before [1].

3.1.2. Multiple microemulsion

A different approach of protein encapsulation is reported by Morel, Gasco and Cavalli [38]. These authors describe a method applying a warm multiple microemulsion, in which the

peptide is dissolved in an aqueous solution and added to a mixture of melted stearic acid, egg lecithin and butyric acid at 70°C. This primary microemulsion is then added at 70°C to an aqueous solution of egg lecithin, butyric acid and taurodeoxycholate sodium salt (TDC). Addition of warm multiple microemulsions to water at 2°C leads to precipitation of the lipid phase, forming solid lipospheres. This method resulted in an encapsulation efficiency of 90% and in particles having an average diameter of 300 nm [38]. Müller reports that large-scale experiments at Vectorpharma in Italy are employing this method [16].

The same group of authors has reported encapsulation of thymopentin, again using the warm w/o/w multiple microemulsion and additionally introducing an o/w method, in which the distribution coefficient of thymopentin is altered by forming a salt with a lipophilic counter ion, sodium hexadecyl phosphate (SHDP) [39]. The peptide was thus contained in a stearic acid melt that was mixed with an aqueous solution of egg phosphatidylcholine, TDC and butanol. TDC, like sodium hexadecyl phosphate, has the potential to act as a counter ion for the peptide. Determination of the distribution coefficient revealed that it only showed a minor effect and even reduced SHDP efficiency. In this preparation though, TDC is supposed to be occupying the interface and thus not interfering with the salt formation between peptide and SHDP.

Particles resulting from the o/w method were found to have a size of 100 nm. After washing, an incorporation of 5,2% peptide was obtained, recovery being 47% compared to 1,7% incorporated peptide and 63% recovery with the W/O/W method; particle size was 200 nm.

Release experiments with lipospheres containing a lipid core have shown sustained release ranging from a few hours to several days. The preferred core material for delayed release, according to Domb, is a polymer such as polylactide [1]. To create lipospheres using polymers, the same melt dispersion as described above has successfully been applied for the formation of antigen-loaded lipospheres using a 1:1 (w/w) ratio for phospholipid and polymer [20].

3.1.3. Cosolvent method

A new approach using a cosolvent-solvent evaporation method for peptide-loaded lipospheres having a polymer core has been described by Rasiel and coworkers [35], who investigated solvents suitable for dissolving the polymers and at the same time mixing with a protein solution in an organic solvent as well. The final preparation consisted of

poly(lactic acid) (PLA) and hydrogenated soybean phosphatidylcholine (HSPC) dissolved in chloroform and mixed with peptide dissolved in N-methylpyrollidone (NMP) to create a clear solution. This solution was then added to 0,25% aqueous PVA solution by vortex mixing, to form the hydrophobic core. After adding this solution to a larger amount of 0,1% poly(vinyl alcohol) (PVA), the system was stirred for 30 minutes.

An attempt was made to prepare peptide-loaded lipospheres acccording to Domb's description of antigen encapsulation [1], where a thin film of polymer, phospholipid and drug is formed after evaporation of organic solvent, and lipospheres are created by adding warm buffer solution and mixing. This resulted only in the formation of large particles at low yield.

Several organic solvents were investigated including dichloromethane, chloroform, ethyl acetate, acetone, methylethylketone, tetrahydrofurane, acetonitrile and mixtures thereof, but only water-insoluble solvents were suited for dissolving polymer and phospholipid in high concentrations and forming spherical particles in good yield.

Polymers with a molecular weight above 50,000 did not form uniform particles, and therefore L-poly(laytic acid) (L-PLA, M_w 2,000 Da), poly(lactic-co-glycolic acid) (PLGA, 75:25, 15,000 Da) and PLGA (50:50, 23,000 Da) were chosen for further investigation. Only L-PLA showed good entrapment efficiencies (80% for triptorelin and >50% for leuprolide), PLGA failed to entrap more than 10% in both cases. In comparison, microspheres were produced that differ from the liposphere preparation only in that the solid hydrophobic core of the lipospheres is stabilized by a monolayer of phospholipid molecules embedded in its surface. All liposphere particle diameters were smaller compared to those of the microspheres.

Another group having done extensive studies on the influence of preparation procedure on liposphere characteristics is Cortesi et al. [36]. Strictly speaking, they were not investigating particles as described by Domb, who states in the patent that phospholipids may be replaced only in part with surfactants such as Tween, Span and PEG surfactants. Steroids cannot function alone but may be incorporated, and amphiphils can be added to the phospholipid coating to alter the surface charge [1]. Cortesi et al. worked completely without phospholipids and used cholesterol, cetyl alcohol, monostearate and oleate as polar lipids in combination with triglycerides as apolar components, but they still termed the resulting particles 'lipospheres'. For the encapsulation of proteins, they suggest a solvent evaporation method to avoid high-temperature exposure during melt method preparation. Consisting of tristearin/glyceryl monostearate 2:1 (w/w), particles proved their poor

mechanical properties, being fragile and having formed an increased number of interparticular bridges as compared to through the melt method. Thus, they investigated mixed matrices constituted of lipids in combination with polymers up to 20%. Both biodegradable (PLA) and non-biodegradable polymers (Eudragit® RS 100) were used, and they allowed an improvement of mechanical characteristics. Unfortunately, there is no data published about the incorporation of proteins in context with this composition. For the hydrophilic model drug sodium chromoglycate (SCG), a melt dispersion and a w/o/w double emulsion were compared for a tristearin/glyceryl monostearate formulation. The melt dispersion resulted in 2% encapsulation efficiency, which could be improved by the double emulsion method by up to 50% encapsulation efficiency.

3.2. Influence of preparation parameters on drug encapsulation

Apart from different preparation procedures, factors determining the loading capacity of a drug in lipid carriers have been found to be the matrix composition and thus the solubility of drug in melted lipid, the miscibility of drug melt and lipid melt, the chemical and physical structure of the solid lipid matrix and the polymorphic state of the lipid material [16].

3.2.1. Preparation method

Melt method procedures are reported to show higher incorporation efficiencies [18, 37]. However, a problem arising from the use of molten lipid phase is a different crystallization behavior than that exhibited during solvent processes. Reithmeier reports about differential scanning calorimetry (DSC) investigations of microparticles prepared by the melt and the solvent evaporation method.

Whereas lipid bulk material and microparticles prepared by solvent evaporation show only one single endothermic peak that results from the melting of the stable crystalline form (β-modification), for microparticles prepared by melt dispersion, three peaks were detected [13]. The first endothermic peak represents the melting of the α-modification, which crystallizes subsequently in β'-modification, resulting in an exothermic peak. The second endothermic peak corresponds to the melting of β'-modification and the third to melting of the stable β-modification (fig. 1).

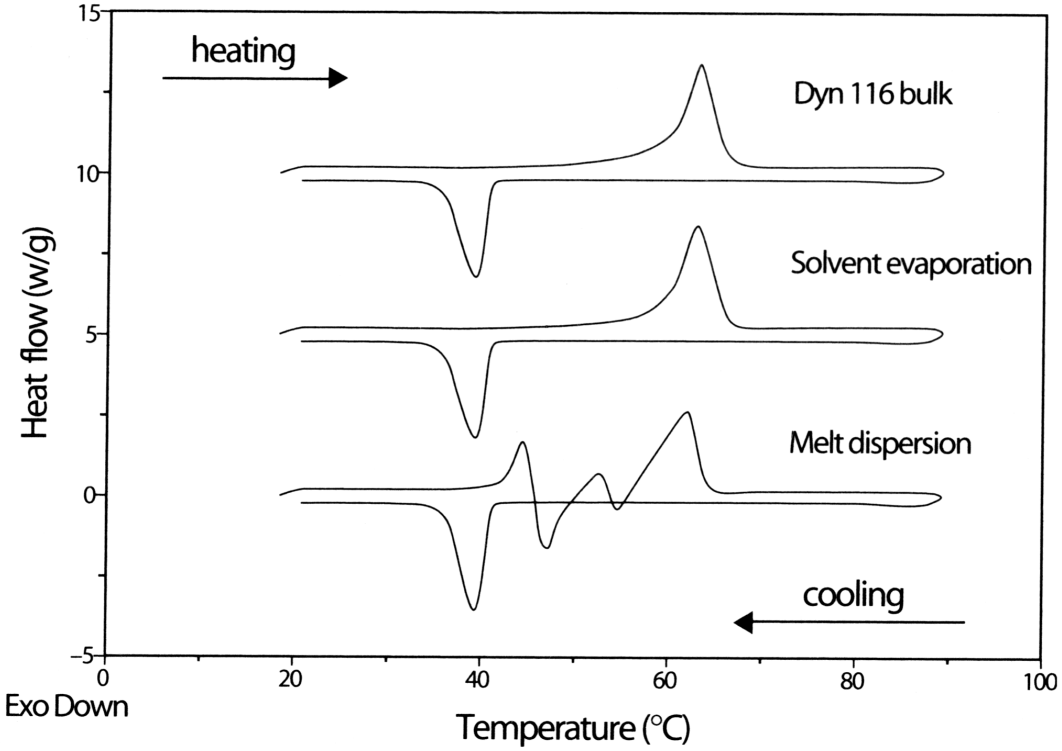

Fig. 1: DSC heating and cooling curves of glyceryl tripalmitate (Dyn 116) bulk material, with microparticles prepared by solvent evaporation and microparticles prepared by melt dispersion 1 day after the preparation. The plots are displaced vertically for better visualization. (adapted from [13] with permission from Elsevier).

Melt dispersion techniques most often comprise a fast congealing step, in which only the instable α-modification is formed, whereas slow diffusion of organic solvent into the outer aqueous phase allows for slow solidification and arrangement of the molecules in stable β-modification [13]. Higher drug-loading capacities have been reported for unstable modifications with lower crystalline order [40], as less perfect crystals with many imperfections offer more space to accommodate drugs. During storage, however, a transformation of unstable modifications takes place and the formation of more stable modifications has shown to promote drug expulsion, which can result in burst release behavior [41].

The presence of surfactant is also reported to lead to reduced crystallinity [16] being another possible reason – apart from drug solubilization – for higher incorporation efficiencies into lipid carriers.

3.2.2. Phospholipid content

The influence of phospholipid content on drug encapsulation and release has been examined both for classic lipospheres having a lipid core and polymer lipospheres having a polymer core [35, 37].

Reithmeier investigated the influence of fat/phospholipid ratio in order to improve drug encapsulation efficiency into microparticles prepared by the solvent evaporation method [37]. A cosolvent-solvent evaporation method like the one described above for the preparation of polymer lipospheres [35] was used. Here, somatostatin as a model peptide was dissolved in methanol and added to a solution of the lipid components in hexane.[37]. Above a phospholipid content of 6%, the encapsulation efficiency showed a high increase (fig. 2).

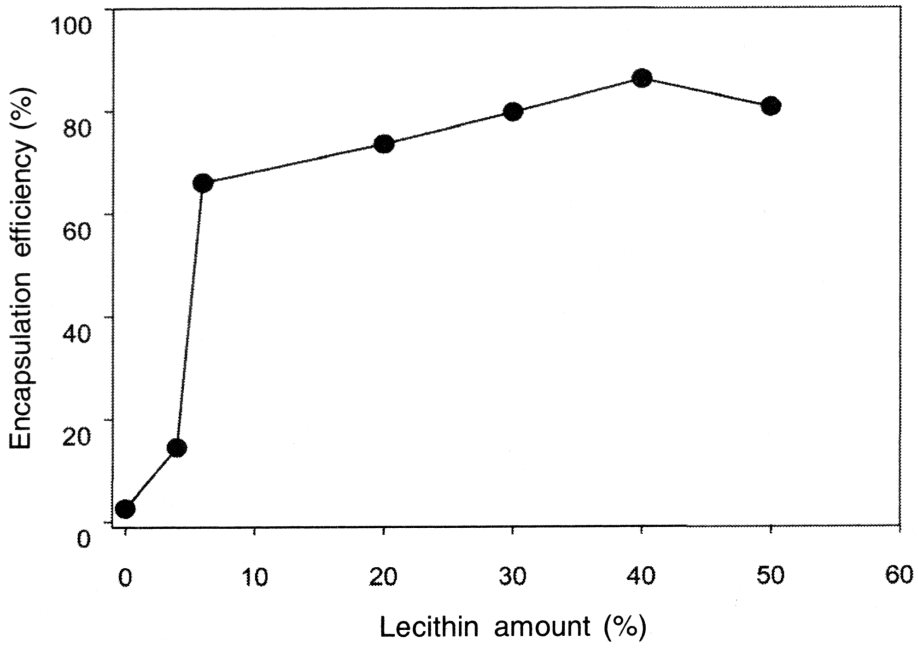

Fig. 2: Influence of added lecithin amount on encapsulation efficiency of somatostatin. Solvent evaporation method: solvent hexane, cosolvent methanol, theoretical loading 2%. (Adapted from [37] with permission from author).

Reithmeier suggests that increased stability of the primary emulsion or electrostatic interactions between peptide and lecithin are possible reasons for this increase [37]. Rasiel compared different phospholipids in varying concentrations [35]. Unlike in Reithmeier's experiments, phospholipids did not stabilize the polymer emulsion, and PVA had to be added to the formulation as a further surfactant. The phospholipids were judged for their ability to interact with polymers regarding free phospholipid content in the supernatant.

Strong phospholipid-polymer interactions were found to result in decreased particle size (HSPC) compared with weaker interactions (egg phosphatidylcholine [EPC]). A ratio of 1:6 was suggested to be most favorable because of an optimal liposphere shape. Different phospholipid/polymer ratios were assessed for their release behavior, which will be discussed later; no effects of phospholipid content on drug-loading capacity were discussed. For the melt method, a phospholipid/triglyceride ratio of 1:4 was found to result in the best yield of drug-free lipospheres when compared with ratios of 1:2, 1:3 and 1:6 [33].

Domb [42] investigated different phospholipid/fat ratios with respect to the phospholipid content on liposphere surface. At a phospholipid/triglyceride ratio of 1:2 to 1:4 70 to 90% of the phospholipid was located at the liposphere surface. Increasing the phospholipid content resulted in the formation of other phospholipid structures, such as liposomes.

The aspect of by-products, which could function as alternative drug incorporation sites is most often neglected in liposphere experiments. Domb observed unincorporated bupivacaine in tristearin formulations in form of dispersible microparticles composed of the solid drug and of phospholipids [42].

Mehnert implicates micelles, mixed micelles, liposomes and drug-nanoparticles, depending on composition, as possible structures resulting from SLN preparation methods, apart from the main particulate carrier. He calls for control samples such as a liposome formulation prepared under identical conditions [40]. Often, liposphere preparation procedures include a washing step with PBS to remove unencapsulated drug, which possibly partly removes by-products as well. Reithmeier reports about a decrease in drug loading of microparticles after a washing step. When washed particles were compared with non-washed particles, a significant decrease in burst release phenomena could also be found (fig. 3). This was explained by removal of surface-located drug crystals that formed during solidification of the lipid carrier [12].

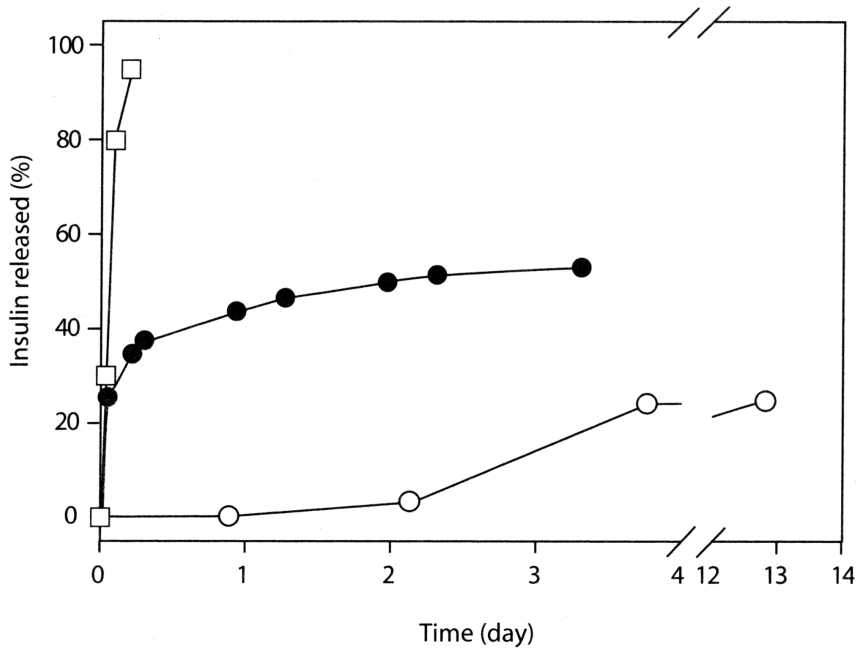

Fig. 3: In vitro release of insulin (release medium: PBS buffer, pH 7.4, 10 mmol, assessment of the residual insulin in the microparticles), (●) microparticles washed with water (drug loading 2.3%), (○) microparticles washed with 0.01 M HCl, (□) control (insulin powder). (Adapted from [12] with permission from Elsevier).

3.3. Stability of protein drugs during preparation

To our knowledge, no explicit studies of protein stability during liposphere preparation and release have been conducted, and protein stability has to be estimated considering what is generally known about detrimental effects during preparation procedures, as described above. Domb suggests that the carrier have a low melting temperature to avoid antigen exposition to high temperatures to preserve the antigenicity during preparation [1]. Antigen functionality was indirectly assessed by immunization of test animals and monitoring of IgG production using an enzyme-linked immunosorbent assay. An immune response comparable to liposome carriers (and better) could be detected [20].

With regards to microemulsions, it should be pointed out that there are different opinions about the structure of these systems [40]. Microemulsions are defined as clear, thermodynamically stable dispersions obtained by mixing surfactant, cosurfactant, oil and water [34]. Gasco, in agreement with other scientists, understand them as two-phase systems composed of an inner and outer phase. Microemulsion proved to be more stable than emulsions [38], sometimes termed "critical solutions" (see [16]), thus obviating the need for high-shear emulsification methods that could exert detrimental effects on a protein

drug. Still, it is desirable that microemulsions be further characterized in terms of phasing, to have a better understanding of the organization of a microemulsion system and, thus, critical parameters for protein stability.

To investigate whether the high temperature needed for melting the lipid components was harmful, Morel assessed thymopentin stability by observation in water heated to 70°C for 1.5 hours (three times as long as it takes for microemulsion preparation) without detecting degradation products [39]. It has been found, however, that the thermal stability of proteins in microemulsions can differ from their stability in water. Although in some cases it was found that protein micellar solutions were stable, physicochemical properties of proteins and thermal protein stability are described as being highly dependent on the water content of a microemulsion system [43].

Rasiel claims that liposphere preparation with the use of N-methylpyrrolidone can no longer be considered to be a double emulsion formulation because there is no use of aqueous inner phase to dissolve the drug. Instead, this preparation is considered to be an o/w emulsion, which is less sensitive to stability problems [35].

Several research groups employ high-performance liquid chromatography (HPLC) analytics to monitor release [35, 38, 39]. Possible degradation products could result in altered retention behavior, but no such observations have been published for peptides or proteins released from lipospheres.

4. Release of peptide and protein drugs from lipospheres

Apart from a sufficient drug load and formulation stability, which have been discussed above, an optimized drug release profile is another prerequisite for a drug delivery system [21]. Drug release of a hydrophilic substance from a lipophilic matrix material can depend on several factors, such as matrix material composition [44], properties of the incorporated drug (solubility in lipid and aqueous medium, molecular weight, interactions with the carrier) [41, 45], drug loading [46, 47], presence of surfactants [37], particle size [48] and preparation method [49], which will be discussed in the following section.

4.1. Classic lipospheres

Domb claims in his patent that the release rate of incorporated substances is controlled by both the phospholipid coating and the carrier [1]. The first peptide-loaded lipospheres to be investigated, malaria-antigen loaded lipospheres prepared by the melt method, were only characterized *in vivo*, where they induced a superior immune response compared to that evoked by liposomes. No *in vitro* data was presented [20].

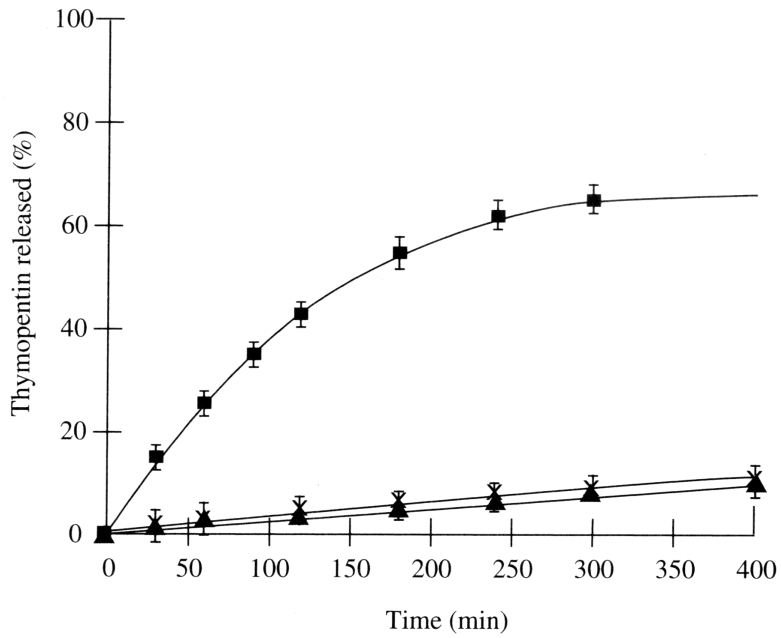

Fig. 4: Percentage release of thymopentin from (▲) lipospheres obtained by o/w microemulsion, from (×) lipospheres obtained by w/o/w microemulsion and diffusion from (■) aqueous solution. (Adapted from [39] with permission from Elsevier).

Liposphere preparations of luteinizing hormone-releasing hormone (LHRH) prepared with the multiple-emulsion method were characterized for release behavior for 8 hours. A pseudo zero-order release of 10% drug loading was observed [38]. Thymopentin lipospheres prepared by the same method released 10% of their loading in 6 hours again following pseudo zero-order kinetics [39]. These results were obtained by placing lipospheres and a blank protein solution for comparison in the donor phases in a multicavity microdialysis cell. This result can only be interpreted taking into account the diffusion data from protein solution, which was 50% in 8 hours for LHRH and 65% in 6 hours for thymopentin (fig. 4). Unfortunately, the experiments were not continued, so the complete release potential can only be estimated. No difference between preparation with counter ion or w/o/w method was found.

Particles in the nanometer size range often show burst release phenomena as a result of large surface area and short diffusion distance of the drug [48]. Zur Mühlen showed a direct correlation between microparticle size range and extent of burst release and the release profile for Compritol microparticles loaded with tetracaine [48]. That is probably one reason why Domb demands that the particles be greater than 1 µm for controlled drug delivery [1].

Reithmeier produced particles below 5 µm [37]. When comparing microparticles obtained with different triglyceride/phospholipid ratios, only particles with a lecithin content below 6% showed a sustained release behavior for 3 days (fig. 5). Higher amounts of lecithin having shown a high increase in encapsulation efficiency led to a burst effect, as depicted for a 1:1 ratio, which was even more pronounced for higher lecithin contents (not shown). A microparticle preparation without the use of lecithin is shown as a comparison. No burst release could be observed; not all peptide was released from the particles though. Incomplete release can result from the loading of drug in amounts that are too small for the formation of a network of pores throughout the whole matrix, through which the drug can diffuse out. Another reason for incomplete release can be the interactions of a released substance with the carrier material as it has been observed for insulin [12].

The accelerating effect of phospholipid on release from lipid microparticles might be even more pronounced *in vivo*, where it is reported that lipid particles degrade faster in the presence of surfactant, which enables the contact with lipases [3].

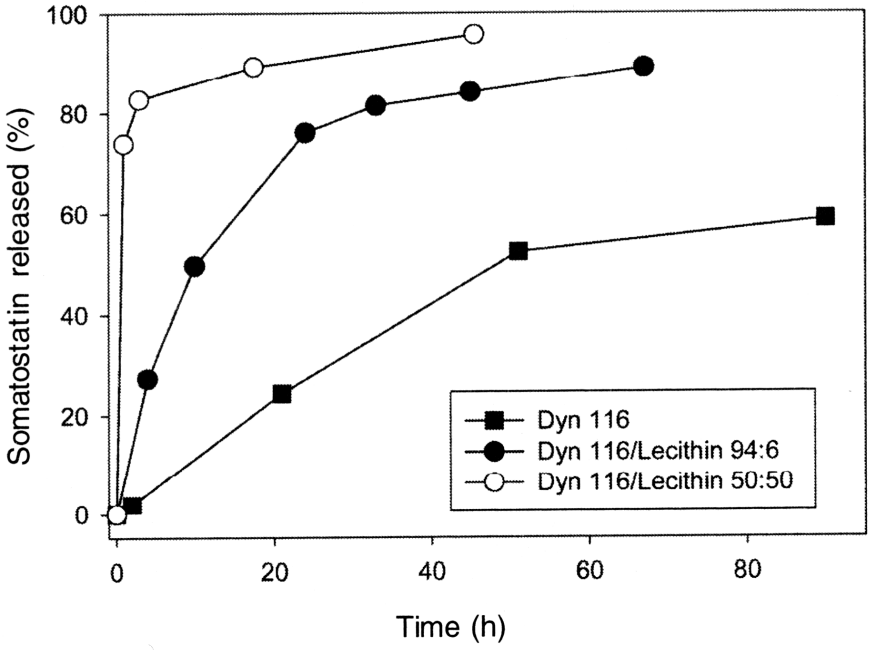

Fig. 5: Influence of lecithin amount added to glyceryl tripalmitate (Dyn 116) on somatostatin release (particles washed with double distilled water). (Adapted from [37] with permission).

4.2. Polymer lipospheres

For matrices made from biodegradable polymers, longer release periods have been reported. When loaded with malaria antigen, one single injection was sufficient to induce an immune response without the help of adjuvant. Polycaprolactone proved to be superior to polylactide, which was explained by polycaprolactone's slower degradation behavior [20]. Polymer matrices represent a powerful tool of controlling release rates. Different profiles can be obtained by varying molecular weight and copolymer composition. For example, PLA matrices made from one single stereoisomer are more resistant to degradation than are racemic polymer. Increasing the amount of glycolic acid in PLGA leads to an increase of degradation rate [50].

Rasiel investigated triptorelin release profiles from lipospheres made from L-PLA, PLGA 50:50 and PLGA 75:25 [35]. While both PLGA polymers showed a burst release within the first 24 hours, L-PLA released the peptide for over 30 days (fig. 6).

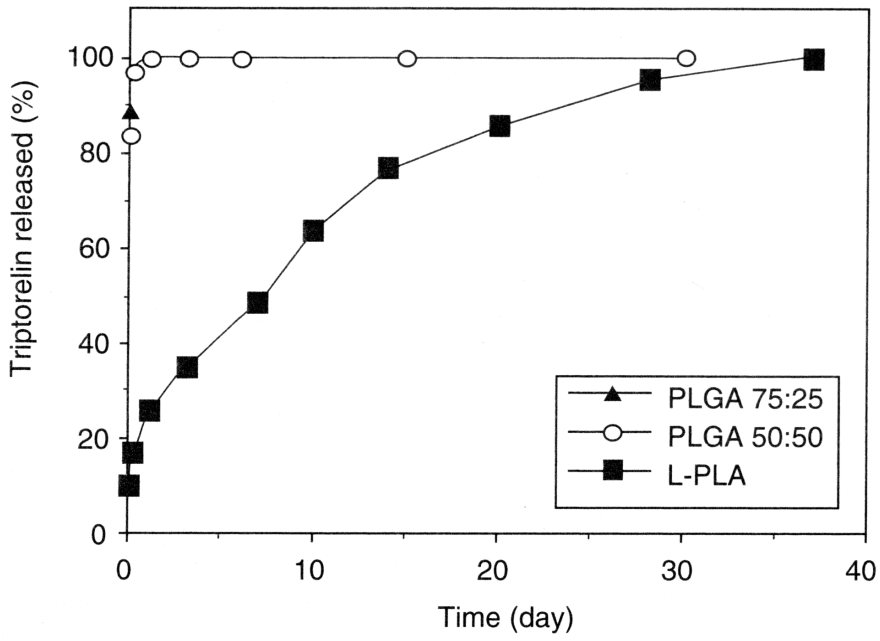

Fig. 6: Effects of polymer type on the cumulative release of triptorelin from lipospheres. Lipospheres were prepared from L-PLA (■), PLGA 50:50 (○) or PLGA 75:25 (▲) with HSPC in a 1:6 phospholipid/polymer ratio. Triptorelin (4 mg) was dissolved in NMP (500 µl) and mixed with a chloroformic solution of L-PLA and HSPC (1 ml). The release experiment was performed in pH 7.4 phosphate buffer, 37°C, and analyzed by HPLC. (Adapted from [35] with permission from Wiley & Sons).

The effect of two different phospholipids in different concentrations was investigated: EPC showed only weak interactions with PLA, and HSPC showed strong interactions. Leuprolide release was fastest from lipospheres prepared from PLA and EPC compared with those prepared from PLA/HSPC and with microspheres prepared without phospholipid (fig. 7).

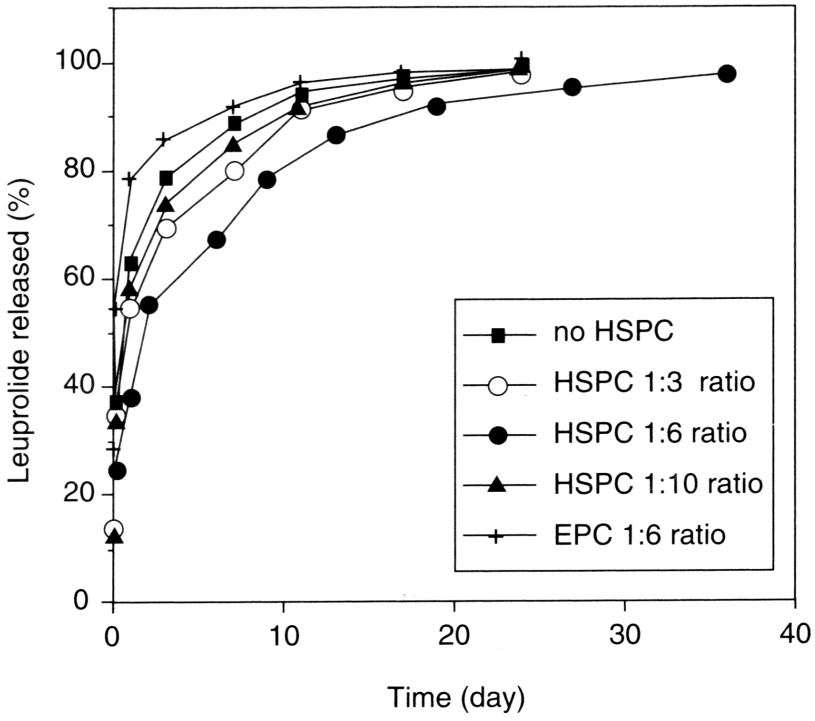

Fig. 7: Effects of phospholipid/polymer ratio on the cumulative release of leuprolide from lipospheres. Lipospheres were prepared from L-PLA and EPC (1:6, +), HSPC (1:3, ○), HSPC (1:6, ●), or HSPC (1:10, ▲) as described in figure 6. Microspheres release data (■) is presented for comparison. (Adapted from [35] with permission from Elsevier).

No difference was found when varying EPC ratios were used, and 80% of the drug was released within the first 48 hours (data not shown), whereas preparations of HSPC/L-PLA showed sustained release for up to 30 days. No clear correlation between phospholipid content and release profile can be deducted, as for lipid lipospheres. Surprisingly, polymer microspheres without the presence of phospholipid showed a faster release profile than lipospheres.

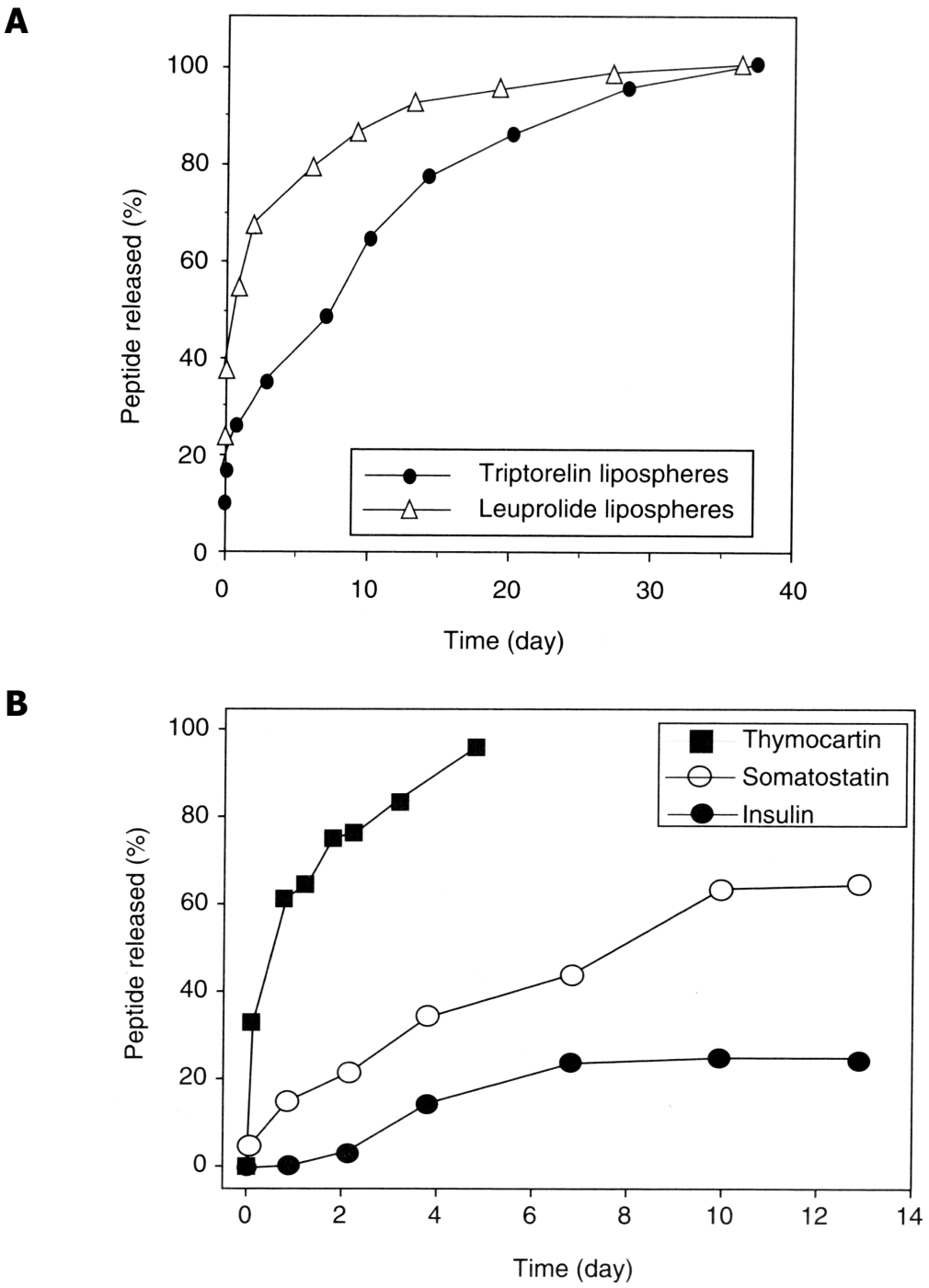

Fig. 8: ***(A)*** *Cumulative release of triptorelin and leuprolide from lipospheres. Lipospheres were prepared from L-PLA and HSPC as described in figure 6. The release experiment was performed in pH 7.4, phosphate buffer, at 37°C and analyzed by HPLC for both formulations. (Adapted from [35] with permission from Elsevier).* ***(B)*** *Comparison of release profiles of thymocartin (loading 9.0%), somatostatin (loading 9.3%) and insulin (loading 6.83%) from glyceryl tripalmitate microparticles. (Adapted from [37] with permission from author).*

Drug release was observed to depend on the kind of protein as well, for both polymer lipospheres (fig. 8A) and lipid microparticles (fig. 8B). As for the peptide and protein release profile, polymer lipospheres are superior to lipid lipospheres if one is aiming at long duration. On degradation and erosion however, the physicochemical environment inside a polymer matrix constantly changes, and peptide or protein drugs may be exposed to detrimental effects including low pH and acylation by degradation products. Triglyceride matrices, in contrast, have been shown to preserve the integrity and bioactivity of encapsulated model peptides and seem to be a promising alternative to polymer matrices [41].

5. Conclusion

Lipospheres have successfully been used to deliver a variety of substances, among them peptide drugs. Lipospheres seem to fulfill the basic requirements of a carrier for drug delivery. A sufficient drug load can be achieved, depending on the incorporation methods. Attempts have been made to investigate procedures limiting detrimental preparation steps for peptide and protein drugs, though there is still a need for further optimization.

Lipospheres are often praised for their good physical stability and dispersibility in aqueous solution [1]. For increased storage stability of protein drugs, they can be freeze-dried and reconstituted prior to use. They show potential for being used to target peptide and protein drugs to the site of action while avoiding systemic side effects. Drug release profiles between days and several weeks can be adjusted by the choice of matrix material; classic lipospheres, however, are more suitable for shorter time periods, necessitating the use of a polymer core for sustained release of more than 3 days.

To estimate the future prospect of lipospheres as a drug delivery system for peptides and proteins, more investigations with a wider peptide and protein spectrum are desirable, as the available data is still restricted to only a few publications. Stability during preparation and long-term storage has not yet been dealt with in detail for peptide and protein drugs, but is one of the prerequisites for successful protein pharmaceutics.

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Chapter 3

Towards Controlled Release of BDNF – Manufacturing Strategies for Protein Loaded Lipid Implants and Biocompatibility Evaluation in the Brain

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Abstract

It was the aim of this study to establish triglyceride matrices as potential carriers for long-term release of brain-derived neurotrophic factor (BDNF), a potential therapeutic for Huntington's disease. First, four different manufacturing strategies were investigated with lysozyme as a model substance: either lyophilized protein was mixed with lipid powder, or suspended in organic solution thereof (s/o). Or else, an aqueous protein solution was dispersed by w/o emulsion in organic lipid solution. Alternatively, a PEG co-lyophilization was performed prior to dispersing solid protein microparticles in organic lipid solution. After removal of the solvent(s), the resulting powder formulations were compressed at 250 N to form mini-cylinders of 2 mm diameter, 2.2 mm height and 7 mg weight. Protein integrity after formulation and release was evaluated from an enzyme activity assay and SDS-PAGE. Confocal microscopy revealed that the resulting distribution of FITC-lysozyme within the matrices depended strongly on the manufacturing method, which had an important impact on matrix performance: matrices with a very fine and homogeneous protein distribution, as provided by PEG co-lyophilization, continually released 60% of the total protein loading in 2 months. The other methods did not guarantee a homogeneous distribution and either failed in sustaining release for more than 1 week (powder mixture), completely liberating the loading (s/o dispersion, more than 75% non-release) or preserving protein activity during manufacturing (w/o emulsion, formation of aggregates and 25% activity loss). Based on these results, miniature-sized implants of 1 mm diameter, 0.8 mm height and 1 mg weight were successfully loaded by the PEG co-lyophilization method with 2% BDNF. Release studies in phosphate buffer pH 7.4 at 4 and 37°C revealed a controlled release of either 20 or 60% intact protein over one month as determined by ELISA. SDS-PAGE detected only minor aggregates in the matrix during release at higher temperature. *In vivo* evaluation of lipid cylinders in the striatum of rat brains revealed a biocompatibility comparable to silicone reference cylinders.

As a conclusion, we suggest lipid implants as a biocompatible, long-term release system with a potential application for protein delivery to the brain.

1. Introduction

In recent years, we have seen significant progress towards understanding the role of proteins in both physiological and pathological processes. Especially neurotrophic factors have increasingly come into focus for the treatment of neurodegenerative diseases [1, 2], however still no satisfying delivery strategies for long-term applications have been established [3-5]. Most proteins possess a hydrophilic character and can therefore be retarded by encapsulation into more hydrophobic matrix materials. Physiological triglycerides have repeatedly shown great promise in this regard, e.g. being formulated to microparticles and cylindrical matrices loaded with diverse peptides and proteins such as insulin [6, 7], somatostatin [8], IFN α-2a [9], BSA and hyaluronidase [10].

However, little effort has been dedicated so far to illuminating the relationship between manufacturing strategies for protein-loaded lipids and resulting matrix performance, such as protein stability, homogeneity of distribution and release behaviour. Most studies involving cylindrical matrices up to know have relied on mixing lyophilized protein and lipid powder in a mortar prior to compression, resulting in relatively short release periods of several days to one month [9-13]. Additionally, most implantable systems that have been described so far, highly exceed the size limit that would allow for a use *in vivo* such as implantation into the brain for research on neurodegenerative diseases in animal models. Thus, it was the aim of this study to develop lipid mini-cylinders being small enough for an *in vivo* application to the rat brain while proving capable of controlling protein release for several months. Special attention was given to understanding the context between formulation strategies and resulting matrix performance on a structural basis.

To this end, lysozyme, a protein of approximately 14 kDa, was chosen as a model substance. Lysozyme is not only one of the best characterized proteins [14, 15] - its size qualifies it for a comparison with many cytokines and growth factors. Being an enzyme, it allows for monitoring activity as a key to stability issues. In a second step, the most successful manufacturing strategy identified with the help of lysozyme was then applied to the formulation of brain derived neurotrophic factor (BDNF) loaded implants. BDNF is a member of the neurotrophin family of growth factors, which have the potential of treating a variety of neurological disorders [3]. The mature protein has a molecular weight of 13.6 kDa and among mammalian species there is a complete identity of its amino acid sequence [16]. It is regarded as a potent cell survival factor [17, 18] and has proven to promote axonal regeneration and angiogenesis in the spinal cord [19]. Hopes have been set

upon its potential use in the treatment of Huntington's disease, where a direct application to the striatum would be required [2]. As biocompatibility for triglycerides so far has only been confirmed for subcutaneous application, additionally an evaluation of glyceryl tripalmitate matrices in the striatum was performed in this study in comparison to silicone rods, a biocompatible material commonly used in neurosurgery.

2. Materials and Methods

2.1. Materials

Chicken egg lysozyme (Grade I, M_w 14,000 Da), bicinchoninic acid disodium salt (BCA) and *Micrococcus lysodeikticus* were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany). R&D Systems (Wiesbaden-Nordenstadt, Germany) supplied carrier-free human recombinant brain-derived neurotrophic factor (hrBDNF, M_w 13,600 Da, frozen in 0.1 M sodium citrate, 0.3 M sodium chloride, pH 3) and human BDNF Quantikine® ELISA kit. Glyceryl tripalmitate (Dynasan® 116) and Witepsol® H37 were a gift from Sasol (Witten, Germany). Polyethylene glycol (PEG, M_w 6,000 Da), dichloromethane (DCM), tetrahydrofurane (THF) and Coomassie® Brilliant Blue G-250 were from Fluka (Buchs, Switzerland). Merck (Darmstadt, Germany) supplied copper-(II)-sulfate pentahydrate, sodium azide and all buffer salts in analytical grade. Fluorescein isothiocyanate (Isomer I) and all SDS-PAGE reagents were obtained from Serva (Heidelberg, Germany). Spectra/Por® Biotech membrane (MWCO 3,500 Da) was bought from Carl Roth (Karlsruhe, Germany). Disposable PD-10 desalting columns were from GE Healthcare (Munich, Germany). Tissue Tek® was purchased Sakura Finetek Europe (Zoeterwoude, Netherlands). 7 to 12 week-old (150–180 g) Fischer 344 female rats were obtained from Charles River (l'Arbresle, France). Antibody to glial fibrillary acidic protein (GFAP, astrocyte marker) was purchased from Dako (Trappes, France). Antibodies to cluster of differentiation 11b (CD11b, clone OX-42, microglial cell/macrophage marker), Natural Killer cell receptor (NKR-P1a, clone 10/78), and T cell receptor (TCR $\alpha\beta$, clone R73) were provided by BD Pharmingen (Le pont du Claix, France). Secondary antibodies were purchased from Valbiotech (Paris, France). Normal goat serum and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were supplied from Sigma–Aldrich (Saint Quentin Fallavier, France).

2.2. Preparation of protein-loaded lipid matrices

A schematic overview of the four different methods applied in this study to manufacture protein loaded lipid matrices is given in figure 1.

2.2.1. Different manufacturing strategies using lysozyme as model protein

(A) Powder mixture

960 mg glyceryl tripalmitate powder and 40 mg lyophilized protein (as obtained from manufacturer) were blended stepwise in an agate mortar. The mixture was directly used for compression.

(B) S/O dispersion

40 mg lyophilized protein were suspended in a solution of 960 mg glyceryl tripalmitate in 4 ml THF. Ultrasound was applied for 10 seconds (5 W, Digital Sonifier 250-D, 3 mm microtip, Branson, Frankfurt, Germany) before freezing the suspension in liquid nitrogen.

(C) W/O Emulsion

40 mg lyophilized protein (as obtained from manufacturer) were dissolved in 0.5 ml double-distilled water and subsequently emulsified into a solution of 960 mg glyceryl tripalmitate in 10 ml dichloromethane. The emulsion was vortexed, ultrasonicated for 10 seconds and immediately frozen in liquid nitrogen.

(D) PEG co-lyophilization

20 mg protein and 20 mg PEG were dissolved in 5 ml double-distilled water. After freezing in liquid nitrogen, the mixture was lyophilized at 6°C and 0.12 mbar for 30 hours in a benchtop freeze-dryer (Beta 2-16 with LMC-2 system control, Christ, Osterode, Germany). When vacuum was removed, 5 ml THF were added immediately to dissolve the PEG fraction of the lyophilisate, resulting in a suspension of solid protein particles. In case of formulations where the absence of PEG was desired, they were centrifuged down and washed twice with fresh THF after removal of the PEG-containing supernatant. Glyceryl tripalmitate (960 mg or 480 mg for formulations with and without PEG respectively) was dissolved in the suspension applying ultrasound for 5 seconds. Afterwards the mixture was frozen again in liquid nitrogen.

In the case of s/o dispersion, w/o emulsion and PEG co-lyophilization, organic solvent was removed from the frozen formulation under a vacuum of 6×10^{-3}mbar for 20 hours (Two stage High vacuum Pump E2M5, Edwards, Crawley, UK) and the resulting dry powder was ground in an agate mortar.

For all formulations (A-D), cylindrical matrices of 2 mm diameter, 2.2 mm height and 7 mg weight were prepared from the protein-loaded lipid powder by manual compression in a custom-designed compression tool made of hardened steel [20]. Compression force was controlled at 250 N for 10 seconds by a Perkin-Elmer hydraulic press (Perkin-Elmer, Ueberlingen, Germany). Matrices were weighed on an analytical balance to determine their exact weight prior to release studies.

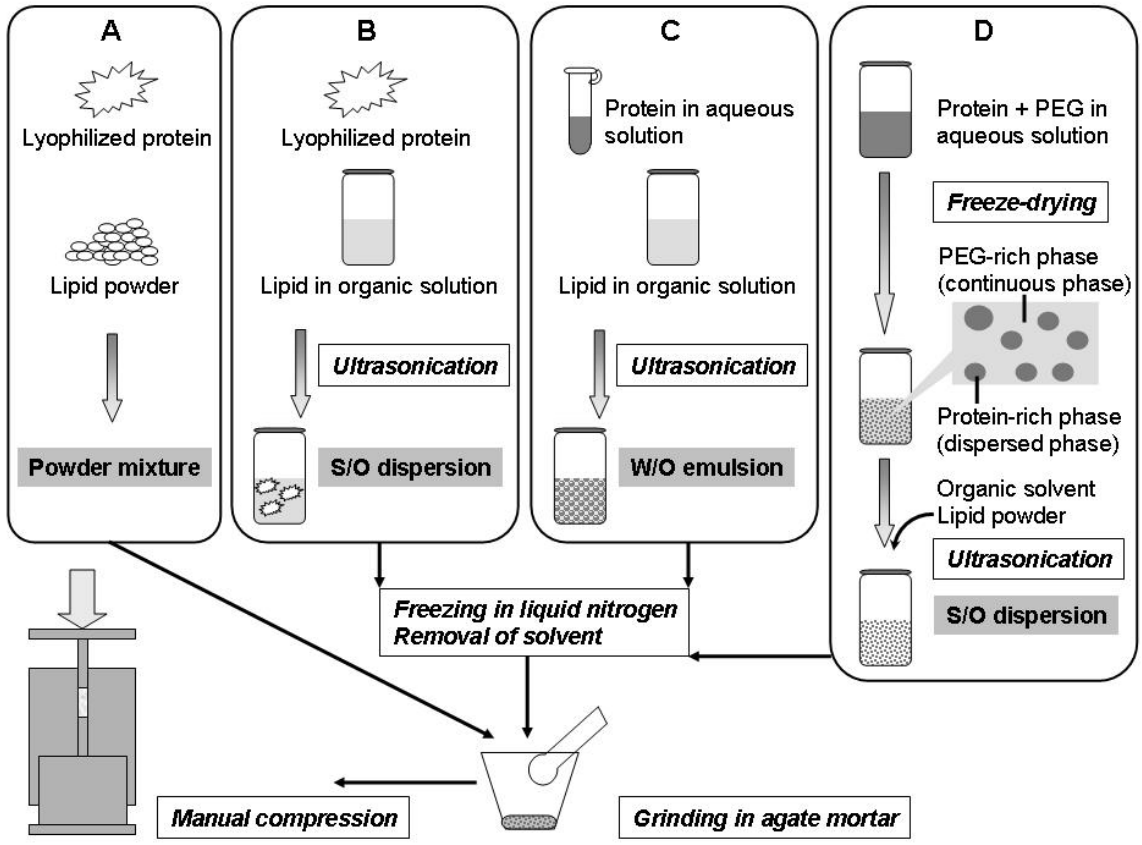

Fig. 1: Schematic overview of the different manufacturing strategies applied for protein-loading of lipid matrices: (A) powder mixture, (B) s/o dispersion, (C) w/o emulsion and (D) PEG co-lyophilization.

2.2.2. Preparation of BDNF-loaded matrices

BDNF was processed according to the PEG co-lyophilization method described under 2.2.1. (D). 1 mg of BDNF was formulated with 1 mg of PEG and 48 mg glyceryl tripalmitate. Prior to lyophilization with PEG, the protein was desalted by passage over disposable PD-10 desalting column to remove the buffer components it was supplied in.
The resulting powder mixture was compressed manually on a smaller compression tool yielding mini-cylinders of 1 mm diameter, 0.8 mm height and 1 mg weight. Compression force was held at 60 N for 10 seconds.

2.3. In vitro protein release

All vials were treated with Sigmacote® prior to use to prevent the adsorption of protein. Matrices were incubated in 1.5 ml phosphate buffer pH 7.4 supplemented with 0.01% sodium azide at 37°C. Buffer was replaced completely after each sampling interval and stored at -20°C until further analytics. To stabilize BDNF during release, 0.1% BSA was added to the phosphate buffer (0.02% sodium azide) and release was additionally performed at 4°C.

2.3.1. BCA micro-assay

100 µl sampled release buffer were incubated with 100 µl bicinchoninic acid disodium salt (BCA) working solution in a covered microtiter plate at 60°C for 1 hour as described by Smith et al. [21]. The intensity of color development, which is proportional to the protein content, was quantified by measurement of absorption at 562 nm after cooling to room temperature for 15 minutes, using a 96-well plate reader (CS-9301 PC, Shimadzu, Duisburg, Germany). A calibration curve was obtained from known concentrations of lysozyme and corrected for absorbance of buffer alone.

2.3.2. ELISA

The amount of BDNF released at 4 and 37°C was determined using a Quantikine® ELISA kit according to the manufacturer's instructions.

2.4. Protein integrity

2.4.1. Enzymatic activity

Lysozyme activity was tested using a modification of the method described by Shugar [22]. In brief, 100 µl of lysozyme sample or standard were placed in a cuvette and completed with 2.5 ml of a 0.015% *Micrococcus lysodeikticus* suspension in 66 mM potassium phosphate buffer pH 6.24. The decrease in absorption resulting from the enzyme's lytic activity was measured in 12 second-intervals for 2 minutes at 450 nm. The initial kinetic rate, proportional to enzyme activity, was obtained from the slope of the linear part of the curve. Lysozyme activity in samples was calculated from a calibration curve obtained with freshly prepared enzyme solution.

2.4.2. SDS - polyacrylamide gel electrophoresis (SDS-PAGE)

Protein was extracted from lipid matrices after formulation by dissolving the lipid cylinder in 2 ml THF and shortly applying ultrasound (5 sec, 5 W, Digital Sonifier 250-D connected to 3 mm microtip, Branson, Frankfurt, Germany). The protein was isolated by centrifugation (12,000 rpm, 10 min, Eppendorf centrifuge 5415R, Eppendorf, Wesseling-Berzdorf, Germany) and removal of the supernatant containing triglyceride. The procedure was repeated twice and solvent traces were removed afterwards under vacuum for two hours (Two Stage High Vacuum Pump E2M5, 6×10^{-3} mbar, Edwards, Crawley, UK) After solvent removal by vacuum, samples were submitted to reducing and non-reducing SDS-PAGE analysis using a Mighty Small II SE 250 unit (Hoefer Scientific Instruments, San Francisco, USA). Electrophoresis was run in a constant current mode at 30 mA on discontinuous stacking (5%) and separating gels (12.5%). Gels were stained by a colloidal Coomassie® Brilliant Blue G-250 dispersion as described in Westermeier [23].

2.5. Implant characterization

2.5.1. Distribution of FITC-labeled lysozyme in triglyceride matrices

Confocal microscopy was used to evaluate the distribution of protein within the matrix depending on different manufacturing strategies. To prepare FITC-labeled lysozyme, the method described by Bezemer et al. [24] was adopted. Shortly, 13.2 mg FITC in 0.66 ml DMSO were added stepwise to 100 mg lysozyme dissolved in 10 ml 0.1 M disodium carbonate buffer pH 8. The mixture was stirred in a dark room for 2 hours before dialysis

in a Spectra/Por® Biotech membrane (MWCO 3,500 Da) against phosphate buffer pH 7.1 for 24 hours. The resulting protein solution was freeze-dried and stored at -20°C until further use.

Fluorescently labeled lysozyme was incorporated into lipid matrices according to the four methods described above. For easier handling during cross sectioning and microscopic observation, lipid matrices were embedded by melt casting into a Witepsol® H37 cylinder of 1 cm inner diameter and 1 cm height and fixed on the sample holder of a cryomicrotome by freezing with Tissue Tek®. Cross sections were obtained by longitudinal cuts of the implants on a Microm HM 550 OMP cryotome (Microm, Walldorf, Germany). To protect the cutting area from moisture, Tissue Tek® was removed in the frozen state and cylinders were placed into a desiccator immediately.

Cross sections were visualized on a Zeiss Axiovert 200M confocal microscope coupled to a Zeiss LSM 510 scanning device (Carl Zeiss Co. Ltd., Germany). The inverted microscope was equipped with a Plan–Neofluar 5×/ 0.15 objective. FITC-lysozyme was excited at 488 nm and fluorescence was detected using a 505-530 nm band-pass filter. Images were processed using the LSM 5 software purchased from Carl Zeiss Co. Ltd., Germany.

2.5.2. In vivo brain biocompatibility [1]

Glyceryl tripalmitate matrices were produced under aseptic conditions by PEG co-lyophilization method with 1 mg BSA, 3.6 mg PEG 6,000 and 47.8 mg lipid. The resulting powder mixture was compressed manually on the small compression tool yielding mini-cylinders of 1 mm diameter, 2 mm height and 2 mg weight. Compression force was held at 60 N for 10 seconds. Platinum-cured, radiopaque, biocompatible silicone thread of the same diameter (Identi-Loop®, Degania Silicone Inc., Cumberland, RI, USA) was cut under aseptic conditions to pieces of 2 mm length and utilized as reference material.

Female Fischer 344 rats were anaesthetized with an intraperitoneal injection of a combination of xylazine (12 mg/kg, Rompun®, Bayer Pharma, Puteaux, France) and ketamine (40 mg/kg, Clorketam®, Vétoquinol SA, Lure, France). Afterwards, the animals were immobilized in a stereotactic head frame (Lab standard Stereotaxic, Stoelting, Chicago, IL, USA). Silicone or lipid implants were placed in the right striatum at the following coordinates: +3 mm lateral and +1 mm anterior to the Bregma and -5 mm deep

from the dura, with the tooth-bar set at 0 mm. The experiments were conducted according to the French Minister of Agriculture and the European Communities Council Directive of November 24th 1986 (86/609/EEC). The animals were kept in standard animal facilities with two rats per cage and given free access to food and water. They were housed in a temperature controlled and humidity-controlled room with 12-hour on-off light cycles.

Animals (n = 3) were sacrificed on post operative day 7 and months 1 and 2. The brains were removed and snap-frozen in isopentane cooled by liquid nitrogen and stored at -80°C. Fourteen-micron cryosections (CM 3050S, Leica, Nussloch, Germany) were fixed with ethanol (95%)/acetic acid (5%) or acetone and washed three times with phosphate-buffered saline (PBS). Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in PBS for 15 minutes. In order to block nonspecific binding, sections were incubated in PBS containing 10% normal goat serum. All incubations with primary antibodies [anti-(rat TCR αβ clone R73), anti-(rat CD11b/c clone OX-42) and anti-(rat NKR-P1a clone 10/78)] were performed overnight at 4°C at a 1:50 final dilution. Incubation with anti-(rat GFAP) antibody was performed at a 1:200 final dilution. Primary antibodies were detected using a rat-absorbed biotinylated anti-mouse or anti-rabbit IgG secondary antibody (1:200, Valbiotech, Paris, France), followed by the avidine–biotin complex method according to the supplier's directions (AbCys, Paris, France). Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride, permanently mounted (Eukitt®, Labonord, Villeneuve d'Ascq, France) and microscopically analyzed (Axioscope®2 optical microscope, Zeiss, Le Pecq, Germany).

2.6. Statistical analysis

All measurements were performed in triplicate and expressed as means ± standard deviation (SD). Statistical significance was assessed by one-way analysis of variance (ANOVA) in conjunction with a Dunnett's test (comparison versus control) at a level of $p<0.01$ (fig. 4).

[1] *In vivo* experiments performed by A. Sapin at INSERM U646, University of Angers, France

3. Results

3.1. Model protein loaded matrices

3.1.1. In vitro release

Release from the devices strongly depended on the preparation method of the lipid-protein mixture. A simple mixing of solid powders (fig. 2A) led to a fast release behavior that lasted no longer than 8 days, with 70% of the model drug loading liberated within the first 24 hours. Dispersing the protein as a solid within an organic solution of the matrix material (fig. 2B) proved to reduce the initial release to 15% on the first day, however, after 15 days, release seemed to be completed with more than 75% of the lysozyme loading still trapped in the matrix. Matrices prepared with the w/o emulsion method were capable of releasing protein for more than two months without the release profile leveling off (fig. 2C). A very similar profile was obtained for matrices produced by the PEG co-lyophilization approach, provided that PEG was not removed during the formulation procedure (fig. 2D). In that case, the hydrophilic load consisted of 2% PEG 6,000 and 2% lysozyme. When the loading was composed of 4% protein alone, i.e. in the absence of PEG, a much slower release was found, reaching only one third of the fraction set free by PEG-rich matrices in the same time period. The latter two strategies (C and D) were successful in preventing a burst release with an initial amount of only 14% (C) and 21 or 8% (D, formulation with/without PEG respectively) liberated in the first 24 hours. Both formulations of (D) seemed to have a potential for continued release longer than the time period monitored in this study, as was found for formulation (C) as well.

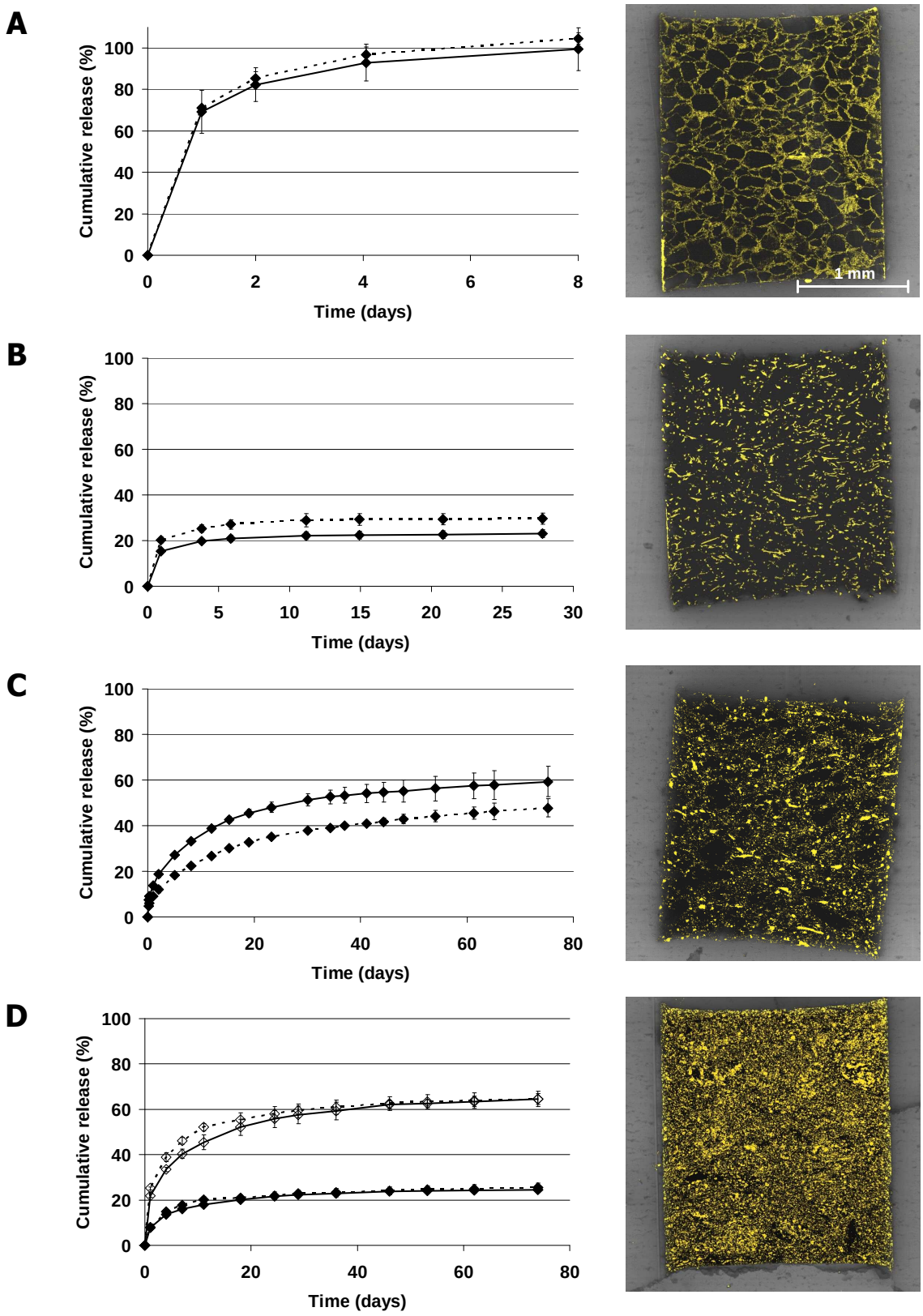

Fig. 2: In vitro release of lysozyme (♦) from matrices loaded with 4% protein by ***(A)*** *powder mixture,* ***(B)*** *s/o dispersion method,* ***(C)*** *w/o emulsion method or* ***(D)*** *PEG co-lyophilization. (◊), formulation containing 2% lysozyme, 2% PEG 6,000. Broken lines represent active protein. Images depict FITC-lysozyme distribution within matrices prepared by the corresponding method (determined by confocal microscopy).*

3.1.2. Protein integrity

Lysozyme activity in the release buffer was taken as a direct measure of protein integrity after incorporation into lipid matrices. Lysozyme seemed to have been encapsulated without suffering catalytic losses by powder mixture (fig. 2A), s/o dispersion (B) and PEG co-lyophilization (D), judged by the overlap in cumulative release of active and total protein as measured by *Micrococcus lysodeikticus* and BCA assay respectively. A deviation from that was found for the w/o emulsion method (C) where the release profile of lysozyme liberated in its active form remained below that of total amount of protein. That was mainly due to the first 30 days, where only 60 to 75% of released lysozyme were found in its enzymatically active form. After that time, between 3 and 5 µg were detected per sampling interval, both for active and total lysozyme, resulting in parallel slopes of the curves.

An extraction of the matrices after preparation and comparison of the recovered protein by SDS-PAGE revealed that lysozyme showed a tendency towards aggregation during the preparation by w/o emulsion as judged by the appearance of higher molecular weight bands in lane 4 (fig. 3). This was in contrast to all other manufacturing methods, for which no aggregation was observed.

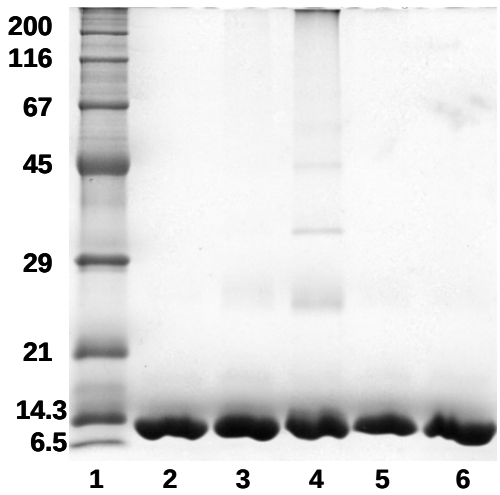

Fig. 3: SDS-PAGE analysis (non-reducing) of lysozyme extracted from matrices prepared by powder mixture (lane3), emulsion method (lane 4), s/o dispersion (lane 5) or PEG-co-lyophilization (lane 6). Lane 1 is the marker (M_w in kDa indicated on the left), lane 2 lysozyme bulk substance.

A simulation of the emulsification step by dispersing 50 µl lysozyme solution in 1 ml dichloromethane revealed the critical step during manufacturing (fig. 4): ultrasonication of the aqueous solution alone did not harm the enzyme, however, if it was carried out in the

presence of dichloromethane, no active lysozyme was recovered in the water phase after inducing phase separation of the emulsion by addition of 1 ml water and centrifugation. Instead, the presence of a white precipitate at the interface was observed, which showed the same pattern of higher molecular weight bands when analyzed by SDS-PAGE as observed for preparation method (C) in figure 3 (data not shown).

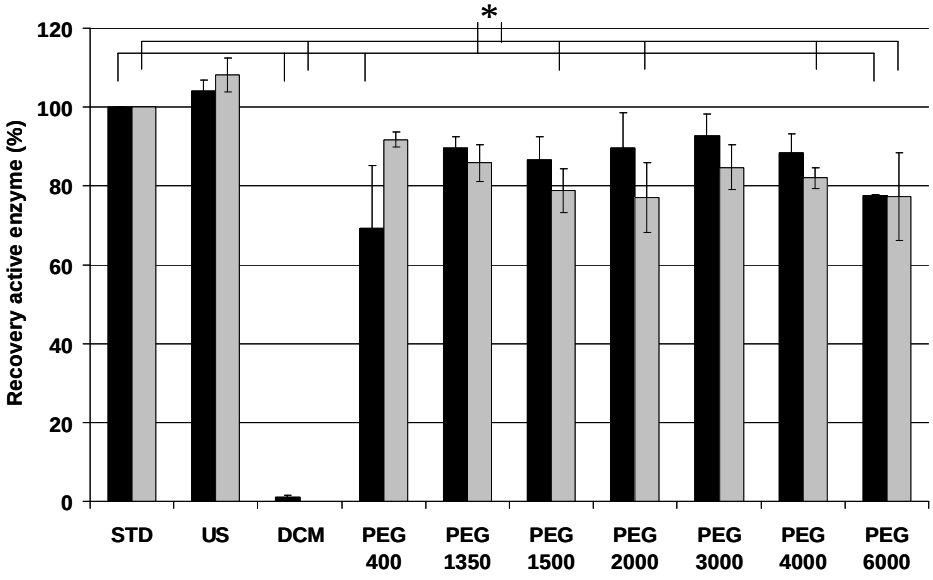

Fig. 4: Recovery (%) of active lysozyme in solution after emulsification in dichloromethane (black bars = 12 mg/ml, grey bars = 0.5 mg/ml lysozyme concentration in starting solution): (STD) lysozyme without treatment; (US) ultrasonication of lysozyme solution alone; (DCM) ultrasonication after emulsification in DCM without stabilizer; (PEG, M_w) ultrasonication after emulsification in DCM with PEG in aqueous solution. (*) $p<0.01$ in statistical comparison.

The evaluation of polyethylene glycols with different molecular weights added at 33% (w/v) to the aqueous phase before emulsification emerged as an effective approach to prevent loss of lysozyme activity to some extent. No clear tendency towards a correlation between PEG molecular weight and protective effect could be derived, nor was there a difference between experiments conducted at high (12 mg/ml) or low (0.5 mg/ml) concentration of lysozyme in the aqueous phase. When the PEG molecular weight exceeded 1,500 Da, a clouding of the aqueous phase was observed before emulsification for high concentrations and at 6,000 Da also for low concentrations of lysozyme. No white precipitate was detected at the interface between dichloromethane and water after emulsification in the presence of PEG, however, the enzyme could not be fully protected from harm as none of the recovered activities reached its original extent.

3.1.3. Implant characterization

3.1.3.1. Protein distribution within matrices

The distribution of lysozyme within lipid matrices was evaluated with the help of fluorescently labeled protein detected by confocal microscopy. Again, a striking difference between the four strategies was found. Protein incorporated by powder mixture (fig. 2A, bright areas correspond to fluorescence of the labeled protein, black regions result from localization of the lipid) appeared to be arranged in the form of a network surrounding areas of pure lipid in the matrices. The inverse situation was the case for cylinders resulting from s/o dispersion (fig. 2B) where zones of high protein content were embedded within the lipid. The emulsion strategy (fig. 2C) also resulted in these protein assemblies, however, with the surrounding lipid being interspersed with a significant portion of more finely distributed protein. A distinctly homogeneous and very fine distribution was the result of the PEG co-lyophilization approach leaving almost no areas void of protein (fig. 2D).

3.1.3.2. In vivo brain biocompatibility

The biocompatibility study of glyceryl tripalmitate matrices was performed in comparison to silicone rods, a biocompatible material commonly used in neurosurgery. None of the animals died precociously or exhibited any overt behavioral changes, obvious neurological deficits or weight loss, suggestive of systemic or localized toxicity from the surgical procedure or from the implants. At 7 days after the implantation, microglial cells and macrophages (anti-CD11b/c antibody) were observed along the injury due to implantation and around both lipid and silicone implant (fig. 5A). Additionally, only a few lymphocytes expressing αβ T-cell receptor and Natural killer cells were observed close to the implantation site, whereas very few of them were found around the implants. Finally, GFAP immunoreactivity (astrocytes visualization) was detected directly in contact with implants as well as farther away in striatum. At one month after the implantation, both implants, silicone and lipid, had conserved their shape. Isolated macrophages and microglial cells persisted as cicatricial tracks (fig. 5A). Furthermore, a very weak labeling of T cell receptors and no NK cells were observed. GFAP immunoreactivity was identical to 7 days: astrocytes were detected around both types of implants as well as in the surrounding tissue. (fig 5B). The same results were obtained after two months; no difference of labeling was found for silicone and glyceryl tripalmitate implants.

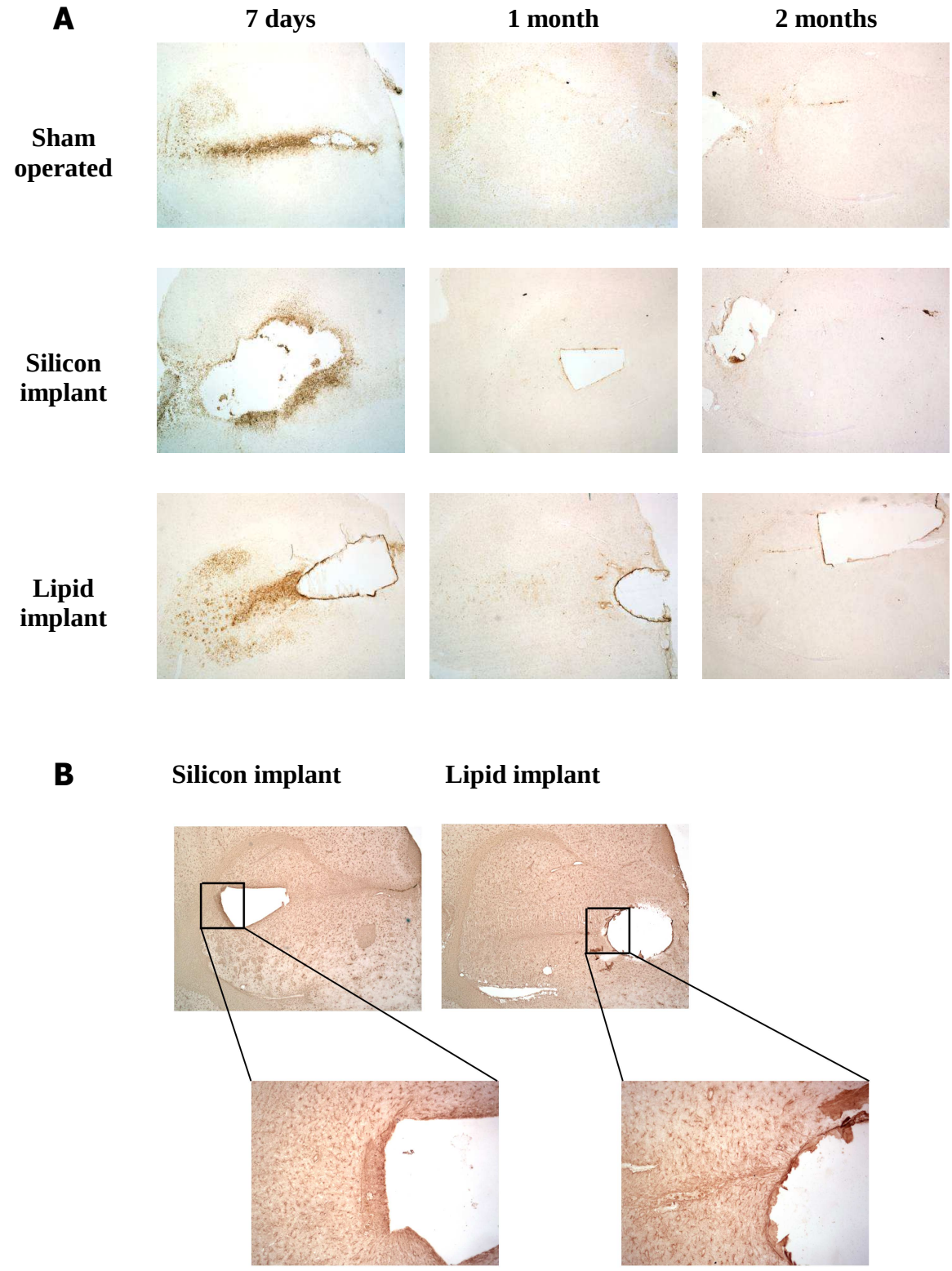

Fig. 5: Biocompatibility evaluation of glyceryl tripalmitate matrices in comparison to silicon rods. ***(A)*** *CD11b/c antigen visualized by OX-42 antibody at post-operative day 7 and month 1 and 2 observed at 2.5 fold magnification.* ***(B)*** *Astrocytes visualized by anti-GFAP antibody 1 month after implantation observed at 2.5 and 10 fold magnification.*

3.2. BDNF loaded matrices

As PEG co-lyophilization was superior to the other manufacturing techniques, BDNF was incorporated into lipid matrices by that strategy. Implants further reduced in size compared to lysozyme matrices were compressed and the release behavior was assessed at two different temperatures, 4 and 37°C. Release was faster at a higher temperature as can be seen in figure 6A summing up to about 60% after 30 days versus 20% at 4°C.

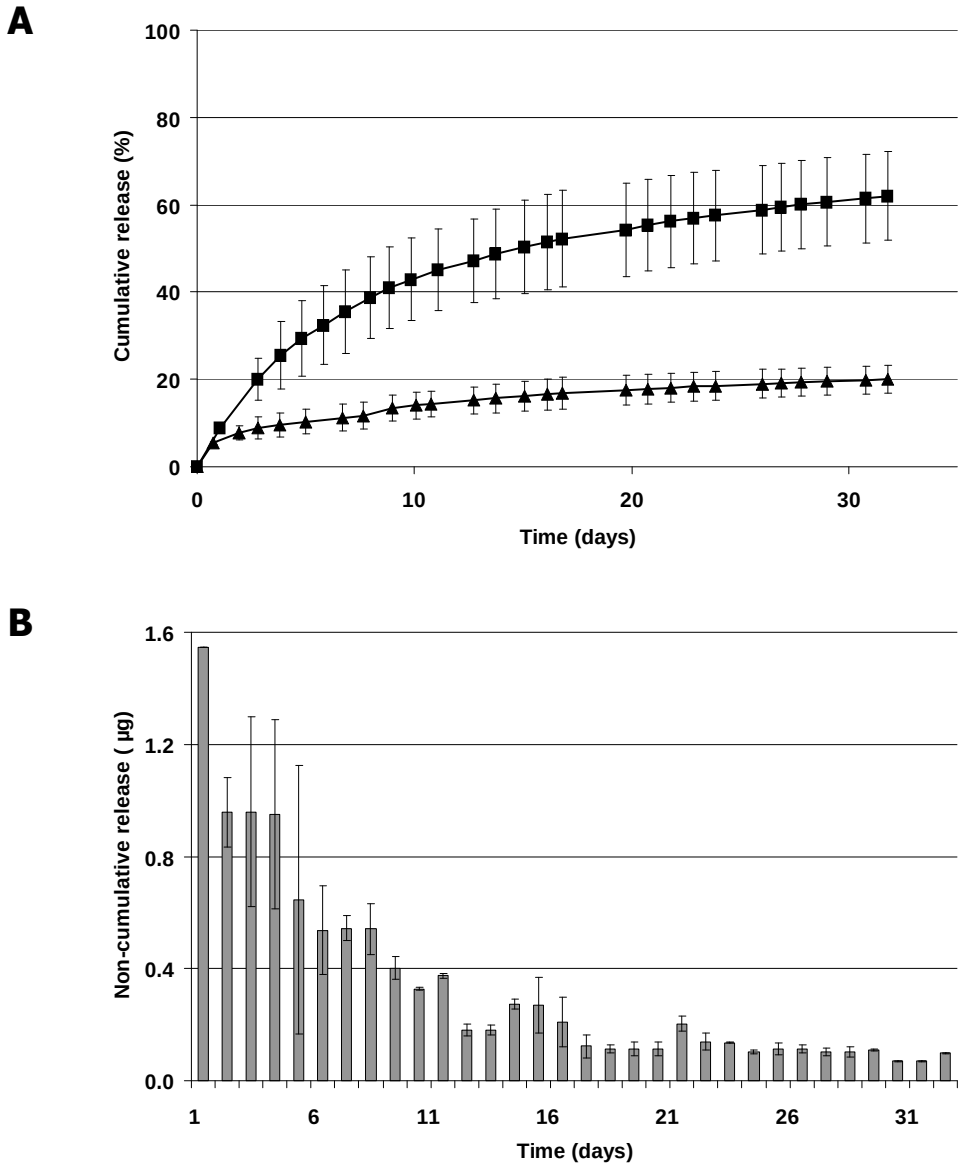

*Fig. 6: In vitro release of BDNF from glyceryl tripalmitate matrices incubated in phosphate buffer pH 7.4 (supplemented with 0.1% BSA) at 4°C (▲) and 37°C (■) depicted **(A)** as cumulative amount in % or **(B)** µg released per day at 37°C.*

An evaluation of protein incorporated into glyceryl tripalmitate by SDS-PAGE revealed no alteration from BDNF stock solution (fig. 7) directly after preparation. A second analysis after release for one month in BSA containing buffer held at two different temperatures resulted in the detection of faint bands at a molecular weight corresponding to BDNF dimer. No low molecular weight degradation products were detected. The bands at approximately 55 kDa in lanes 4 and 5 of the gel represent traces of BSA from the release buffer as can be concluded from a comparison with BSA alone (lane 6).

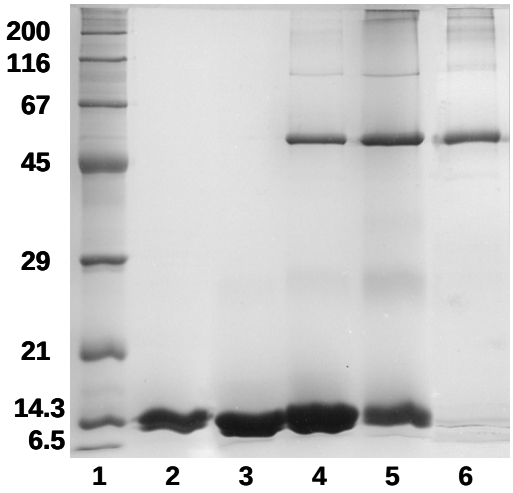

Fig. 7: SDS-PAGE analysis (non-reducing) of BDNF extracted from glyceryl tripalmitate matrices. Lane 1: marker (M_w in kDa indicated on the left); lane 2: BDNF bulk substance; lane 3, 4 and 5: BDNF extracted from matrices after preparation, release at 4°C, release at 37°C, lane 6: BSA.

4. Discussion

The first part of this study demonstrates the importance of understanding the influence of manufacturing strategies on the resulting protein distribution in lipid matrices. The lysozyme loaded lipid cylinders fabricated in this study did not differ as to their matrix material and also the amount and type of hydrophilic loading. However, regarding their *in vitro* release performance, a striking divergence in their capability to provide long-term protein release could be found.

Most manufacturing strategies for lipid cylinders have so far relied on the simplicity of the direct compression of a powder mixture of solid lipid and lyophilized protein [9-13], equivalent to method (A) investigated in this study. Thereby, satisfying release durations have only been achieved in the case of larger matrices with dimensions excluding the *in vivo* implantation into the rat brain. An acceleration in release is to be expected when the matrix size is reduced, both due to a higher surface area/volume ratio and shorter diffusion pathways for the drug to reach the matrix surface [25, 26]. This has been the case for formulation (A), not being able to withhold the protein for more than 8 days when cylinders of 2 mm diameter and 2.2 mm height were compressed from the powder mixture.

Confocal microscopy emerged as the ideal tool to provide background information about the inner structure of the matrices, suggesting a direct correlation between the homogeneity of the protein distribution within the matrix and the resulting release profile (fig. 2). To our knowledge, this is the first study giving an insight into the inner organization of protein-loaded lipid matrices. In the case of a powder mixture, figure 2 suggests the formation of a continuous network of protein, which can easily be penetrated by release buffer and leached out in a short time period. This network is probably pre-created when the finer protein powder spreads around the lipid powder particles during the grinding step in the agate mortar.

In order to inverse the arrangement, the concept of 'coating' the protein particles by lipid was pursued by precipitating the lipid onto solid particles finely suspended in its solution. There are several proofs of the fact that proteins display an increased stability when suspended as a solid in organic solvents [27, 28], as a reduction in conformational flexibility compared to aqueous/organic mixtures preserves secondary structure and thereby impairs denaturation [29]. Concept (B) evolved to be valuable with regard to a reduction of the initial release rate, however, the envisaged long-term release was not realized. Confocal microscopy illustrates that the protein starting material was not

sufficiently fine for a homogeneous distribution and therefore trapped within the matrix during release due to the lack of hydrophilic pores linking the inner regions of the matrix with the release buffer. The amount of 15% released lysozyme during the first day probably corresponds to the protein loading situated directly underneath the matrix surface after compression.

Several approaches have been discussed so far for the micronization of protein material, amongst them high pressure homogenization [6], jet milling [30], spray-drying or spray-freeze-drying [31, 32] or even grinding in a mortar under liquid nitrogen [7, 8]. However, these strategies have the disadvantage of requiring large amounts of starting material, as there often is a continuous loss of recovered protein after repeated micronization cycles. A concept often applied in finely and homogeneously distributing protein within polymeric microparticles, is the use of a w/o emulsion [33]. Vogelhuber et al. have also described this strategy for incorporating a small molecular weight fluorescent model substance, pyranine, into glyceryl tripalmitate matrices, which subsequently showed a sustained release for more than 120 days [20]. In method (C), this has been realized for lysozyme loaded lipid matrices, which exhibited a very promising release behavior for more than two months. Confocal microscopy confirms the presence of more finely distributed protein all over the cylinder with some larger protein assemblies. These are probably due to a phase separation caused by limited stability of the emulsion before freezing in liquid nitrogen, reflected in larger protein-containing areas in the final product. However, it had been anticipated that the use of an emulsion might bring about stability risks for the protein as it has been largely discussed in literature that the presence of a w/o interface can cause protein adsorption, unfolding and formation of soluble and insoluble aggregates [27, 34-36].

Indeed, the activity loss detected for released protein in this study can be traced back to the emulsion step in the preparation procedure (fig. 4). While there are reports about processing lysozyme by emulsion without harm to its structure [37, 38], others have found unfolding, covalent or non-covalent aggregation [34, 39-41] and precipitation at the interface [34, 36], which was assumed to originate in differences in experimental conditions [34] and sample preparation [36]. While van de Weert et al. [36] report full dissociation of non-covalent lysozyme aggregates in the presence of SDS by boiling, Park et al. [41] have found complete dissociation only in the presence of guanidine hydrochloride, not SDS. Results in this study obtained by PAGE analysis of samples boiled in the presence of SDS suggest a minor covalent aggregation by non-disulfide exchange reaction. Interestingly, Perez et al. [34] make reference to the solubility of the white

precipitate recovered from the interface in water – an observation paralleled in this study favoring a non-covalent nature of the aggregates. Only 70% (± 17) of the re-dissolved precipitate was tested biologically active, which is in good agreement with the fact that in this study only about 75% of released lysozyme from lipid matrices prepared by a w/o emulsion showed lytic activity on *Micrococcus lysodeikticus* cells. Presumably, lysozyme that was aggregated at the interface during incorporation into the lipid matrices, slowly re-dissolved during release without completely and correctly refolding.

In search for potential emulsion stabilizers, which comply with compactibility and release control, PEG emerged as a particularly interesting substance. Solid PEGs are mixable with lipids without decreasing their compressibility [9]. It is an excipient widely used in biomedical formulations for its non-toxicity and lack of immunogenicity [42] and has become known for its potential to protect protein drugs both during manufacturing and release [28, 43]. PEG is also supposed to inhibit protein aggregation by preventing its adsorption to the w/o interface [28]; a protective effect on NGF during emulsification has been reported for PEG 400 [44]. This study proves that also higher molecular weight PEGs show the potential to stabilize lysozyme during the emulsion step, however, no complete recovery of activity was achieved. A further increase in the PEG content of the aqueous phase would not be desirable, as it proved to compromise the triglyceride's potential for controlling release at high loadings in preliminary experiments. Apart from that, the clouding of the aqueous phase at high protein concentration and increasing molecular weight of PEG indicates PEG-induced protein precipitation, suggesting a further increase in PEG content would remain without additional protective effect. Mild precipitation of proteins by PEG is a commonly used purification method in biotechnology, and it is assumed that most proteins precipitate in a reversible state allowing correct refolding [45].

Morita et al. [46] were the first to describe a use of PEG in formulation strategies by applying a freezing-induced phase separation for protein micronization. A modification of that strategy was developed for lipid implants as described in method (D). The great advantage of this procedure is the possibility to perform all manufacturing steps in one vial, minimizing the loss of material. Very promising release profiles have been obtained from the formulation with and without PEG, suggesting an ongoing release even after the investigated period of 75 days in this study. The possibility to adjust the amount of PEG in the formulation enables its use in tailoring of the release profile. According to confocal microscopy, this method showed a superior performance in homogeneously distributing the protein in a very fine state within the whole matrix. As lysozyme was found to be fully

active without the formation of any aggregates, this was the strategy chosen for formulating BDNF matrices.

The homogeneous protein distribution obtained by PEG co-lyophilization was the crucial prerequisite allowing for further size reduction in comparison to lysozyme model matrices. With 1 mm diameter and 0.8 mm height, BDNF implants to our knowledge represent the smallest lipid matrices produced by compression so far, and fully comply with the aim of *in vivo* brain application via a needle. The feasibility of glyceryl tripalmitate matrix application to brain tissue was proved by the biocompatibility evaluation performed with model implants in comparison to silicone rods and complements data obtained from subcutaneous testing [47]. The study was conducted with BSA implants loaded by PEG co-lyophilization, as BSA might serve as a potential future carrier protein for low-dose application of highly potent protein drugs. No difference of recruitment of labeled cells (astrocytes, T lymphocytes, NK cells, macrophages and microglial cells) was observed in brain exposed to lipid or silicone implants at post-operative day 7 and month 1 and 2. Lipid implants thus were tolerated as well as silicone rods, an acknowledged biocompatible material.

As figure 6 illustrates, BDNF loaded matrices are capable of continuously releasing protein for more than one month. The amount of drug liberated per day decreased from 1.5 to about 0.1 µg/day, when matrices were incubated at 37°C, while drug release was lower at a lower temperature probably due to a diffusion controlled release mechanism. According to [48, 49], a desirable release profile would be in the range of 1 to 1.5 µg/day for at least 2 to 3 weeks. To this end, the release rate would have to be enhanced, e.g. by increasing the PEG content in the formulation. Results obtained via SDS-PAGE analysis suggest that protein was not aggregated or degraded in the formulation step, as compared to the stock solution. However, incubation conditions seem to have affected encapsulated protein in a way, that faint aggregate bands could be visualized on the gels by Coomassie staining. The effect was more pronounced at higher temperature, suggesting stability problems due to the incubation. This has been identified as a critical issue before [28, 43, 50, 51], however, strategies for preserving protein activity within the implant require a more detailed knowledge about mass transport phenomena within the matrices. In this context, a closer insight into the release mechanism would be desirable in order to be able to predict the influence of excipients on release behaviour. A preliminary examination of diffusion patterns of fluorescently labelled protein within lipid matrices gave very promising results for the PEG co-lyophilization method and will be the subject of further investigations [52].

Altogether, lipid matrices show great promise for a controlled targeted delivery of protein drugs such as BDNF to the brain tissue, where only few approaches have been attempted so far [5].

5. Conclusion

This study has revealed the importance of understanding the inner organization of controlled release matrices, both with regard to the choice of a suitable manufacturing method and intended release performance. Variations in the preparation strategy can not only strongly affect protein stability, but also alter the arrangement of the drug within its carrier, compromising its potential for long-term controlled release. PEG co-lyophilization proved as a valuable strategy to produce matrices with finely and homogeneously distributed protein. Cylinders of 1×0.8 mm can potentially be introduced into the rat brain, where they are well tolerated, potentially enabling an evaluation of the performance of protein drugs such as BDNF for the treatment of brain diseases. In order to be able to improve conditions within the matrix during protein release, the question of mass transport phenomena occurring during incubation should be addressed in more detail.

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Chapter 4

In vitro Investigation of Lipid Implants as a Controlled Release System for Interleukin-18

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Abstract

Operating on the inductive and effective phases of an anti-tumor immune response and uncovering pivotal functions that may reduce cancer cell growth, interleukin-18 (IL-18) appears to be an attractive candidate for the sustained local adjuvant immunotherapeutic treatment of brain gliomas. The objective of this work was to develop IL-18 loaded lipid implants as a controlled delivery system. For the preparation of protein loaded triglyceride matrix material, a solid-in-oil (s/o) dispersion technique was chosen, for which protein particles in the micrometer range were first prepared by co-lyophilization with polyethylene glycol (PEG). Implants of 1 mm diameter, 1.8 mm height and 1.8 mg weight were manufactured by compression of the powder mixture in a specially designed powder compacting tool. The *in vitro* release behavior of ^{125}I-Bolton-Hunter-radiolabeled IL-18 was assessed in a continuous-flow system. A cell culture assay was established for the determination of bioactivity of released IL-18. Implants showed a continuous release of 10 to 100 ng IL-18 per day for 12 days. A progressive integrity loss was observed with ongoing release, which would be related to protein degradation during incubation. The initially released fraction proved complete retention of bioactivity, indicating that the manufacturing procedure had no detrimental effects on protein stability.

1. Introduction

Malignant tumors of the CNS represent an illness, where therapeutic approaches so far have only been able to achieve a short extension in life span of the patient [1]. Conventional treatment includes surgical removal of the tumor, radiation therapy and chemotherapy. Despite efforts and advances in neuroradiology and neurosurgical techniques, median survival time of glioblastoma patients is less than one year. Those tumors tend to recur within centimeters of their original location and novel approaches are urgently needed to treat the disease.

In this context, local immunotherapy has become an area of growing interest. Tumor cells express proteins foreign to the host, which render them vulnerable to an immune response. The immune reaction can be activated by local application of cytokines; potential bioactive agents include interleukins (IL), interferons (IFN) and colony-stimulating factors [2, 3]. IL-18 is a cytokine, which was chosen by our group for its prospects of being used as a therapeutic agent in this regard.

First described as interferon-γ (IFN-γ) inducing factor (IGIF) in 1989 [4], IL-18, also called IL-1F4, is a molecule made up of a single peptide chain of 18.3 - 18.4 kDa produced primarily by macrophages and mononuclear blood cells from an inactive precursor. IL-18 belongs to the IL-1β cytokine superfamily and interacts with a heterodimeric receptor complex (IL-18 receptor-α and IL-18 receptor-β) to mediate a wide range of immune and non-immune biological effects [5]. In addition to stimulating IFN-γ production from macrophages, T cells and NK cells, IL-18 stimulates the production of IL-2, GM-CSF and chemokines (IL-8, MIP-1β and MCP-1) from peripheral blood mononuclear cells. IL-18 also promotes the development of Th1 cells from Th0 cells. IL-18 is able to enhance T and NK cell cytotoxicity towards tumor cells and exerts chemoattractive effects that have so far been demonstrated on Th1 T-lymphocytes [6], dendritic cells [7] and NK cells [8]. Additionally, IL-18 is known to reduce angiogenesis *in vivo*, an effect, which results in hypovascularization of treated tumors [9]. Collectively, IL-18 activities suggest the possibility of applying this cytokine to local tumor immunotherapy.

In parallel to gene therapy studies, currently the most common technological method to deliver therapeutic factors directly to a specific brain region involves surgically implanting pump or cannula systems [10]. Apart from functional difficulties, such as the clogging of the pump system, the bioactive molecule itself sets a limitation to this approach. Interleukins are protein drugs and have short half-lives and limited stability in the aqueous

reservoir of a pump system. Recent efforts thus have focused on designing controlled release devices that enable the delivery of intact biomolecules directly to the tumor site [11].

To our knowledge, no delivery system for IL-18 has been described so far, limiting the opportunities to investigate its potential as a therapeutic agent in an animal model *in vivo*. With the intent to overcome this deficit, lipid implants were devised as a controlled release system for IL-18. Glyceryl tripalmitate, a physiological triglyceride with interesting properties suggesting its use as matrix material for long-term release of bioactive compounds [12-14], served as an alternative to polymeric release systems. Triglycerides are biocompatible and biodegradable molecules [15] that can avoid many disadvantages of their counterparts [16]. Indeed, no swelling occurs [17] and the formation of acidic degradation products, which can result in a pH shift or even covalent attachment to the protein drug [18], are not of concern.

To plan for potential testing in an animal tumor model, the implants must be small enough to be inserted into the rat brain by stereotaxy and capable of releasing biologically active IL-18 in a controlled fashion. According to our estimations, for the rat brain a dose of 10-100 ng of IL-18 (ED_{50} 3–6 ng/ml) per day and implant would likely be a valuable objective. This is based on analogous reports about growth factors with a similar ED_{50} exhibiting significant effects when released from PLGA microspheres [16, 19]. A method for incorporating protein into lipid matrix material was established. Particular interest was given to investigating the *in vitro* release performance and an assessment of the bioactivity of released protein in a cell culture assay.

2. Materials and methods

2.1. Materials

Rat recombinant IL-18 (rrIL-18), IL-2, IL-12, interferon-γ (IFN-γ), mouse recombinant IL-18 (mrIL-18), ELISA DuoSet antibodies directed against rat IFN-γ and human anti-CD28 were obtained from R&D Systems (Abingdon, UK). Rat anti-CD3 was purchased from BD Biosciences (Le Pont de Claix, France). Dynasan®116 (glyceryl tripalmitate) was obtained from Sasol (Witten, Germany). Polyethylene glycol (PEG, M_w 6,000 Da) and tetrahydrofurane (THF) were supplied by Fluka (Buchs, Switzerland), bovine serum albumin (fraction V, M_w 66 kDa), gelatin, dithiothreitol (DTT), Sigmacote®, MOPS (3-[N-morpholino]propanesulfonic acid), EDTA and sodium azide were from Sigma–Aldrich (Saint Quentin Fallavier, France). Bolton-Hunter reagent (N-succinimidyl-3-(4-hydroxy-3-[^{125}I]-iodophenyl)propionate, monoiodinated, 250 µCi) was purchased from Perkin-Elmer Life Sciences (Courtaboeuf, France), sodium sulfate from VWR International (Fontenay sous Bois, France), PD-10 columns and Sephadex® G 25M from Amersham Bioscience (Orsay, France).

2.2. Methods

2.2.1. ^{125}I-labeled interleukin-18

IL-18 was radiolabeled using Bolton-Hunter reagent. Ten microgram of mrIL-18 were dissolved in 105 µl 0.1 M borate buffer pH 8.5 and added to the vial containing dried 250 µCi (9.25 MBq) Bolton-Hunter's reagent. The reaction was performed at 4°C with gentle vortexing for 30 minutes. To stop the reaction, 0.5 ml of a 0.2 M glycine solution in 0.1 M borate buffer pH 8.5 were added. The mixture was incubated for another 10 minutes at 4°C. Radiolabeled protein was purified by gel filtration using a Sephadex PD-10 column, which had been pre-equilibrated prior to use with 0.5% gelatin solution for 1 hour at 4°C. The reaction mixture was added to the column, and the reaction vial was washed four times with 200 µl of 0.1 M borate buffer pH 8.5. Radiolabeled protein was eluted with 4 ml double distilled water. Fractions of 200 µl were collected in silanized glass tubes and counted with a gamma counter Cobra™ II Auto-Gamma (Packard Bioscience, Rungis, France). The peak fractions were pooled and used for preparation of protein loaded lipid powder. The ^{125}I-mrIL-18 amount in the final formulation was negligible when compared

to total IL-18 amounts used for implant preparation (150 µl peak fraction contained about 1 µg of ^{125}I-mrIL-18 representing less then 5% of total IL-18 including 25 µg free rrIL-18) and, therefore, not considered when performing a bioassay on released protein.

2.2.2. Implant manufacture

IL-18 loaded lipid implants were prepared in a two-step process, which was optimized for handling small batch sizes in one single vial. In a first step, protein was co-lyophilized with PEG to obtain microparticles according to a modified method described by Morita et al. [20]. Figure 1 shows a schematic outline of the different steps. Two different amounts of PEG were studied (2 and 7% with regard to total implant weight) in order to investigate the possibility to tailor the release profile by varying the amount of hydrophilic excipient; the theoretical protein content of the matrices was 2%.

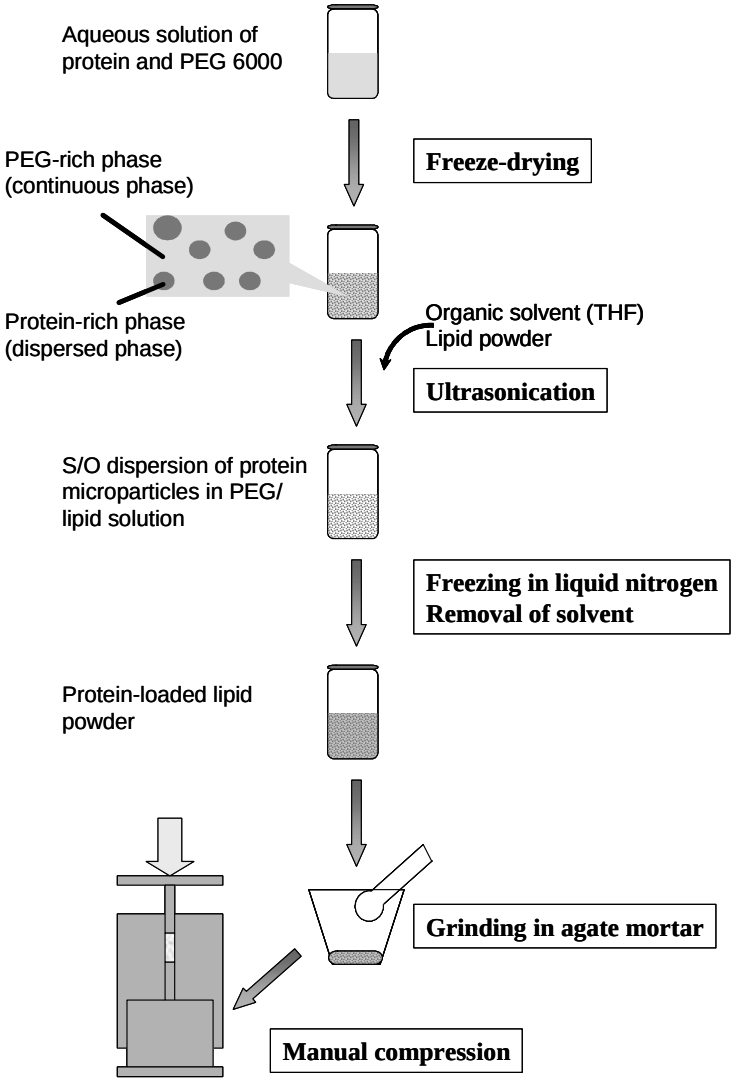

Fig.1: Schematic representation of the manufacturing procedure for protein loaded lipid implants.

Briefly, 25 µg rrIL-18 dissolved in 48 µl MOPS buffer pH 7.2 (20 mM MOPS, 50 mM Na_2SO_4, 0.5 mM EDTA and 0.5 mM DTT) were added to a solution of 500 µg BSA and either 525 µg or 1838 µg PEG 6,000 in 100 µl water in a 2 ml safe-lock micro test tube (Eppendorf, Wesseling-Berzdorf, Germany). 150 µl of elution fraction from Bolton Hunter labeling containing radiolabeled ^{125}I-mrIL-18 were added, and the solution was rapidly frozen in liquid nitrogen before freeze-drying for 20 hours. After removal of vacuum from the freeze-dryer, 250 µl of THF were added immediately to the lyophilizate to dissolve PEG. 25.2 mg (or 23.9 mg for the formulation with the higher PEG content) of powdered glyceryl tripalmitate were placed in the vial. By applying ultrasound (3 sec, 5 W, Microson XL2007, with a 3.2 mm microprobe, Microsonix, Servilab, Le Mans, France), the protein particles were disaggregated and dispersed in organic solvent, and simultaneously triglyceride was dissolved. The mixture was placed under liquid nitrogen until the THF was completely frozen. By applying a vacuum, the organic solvent was removed, leaving behind a powder formulation. The powder was removed from the micro test tube and ground in an agate mortar.

A specially designed compression tool made of hardened steel [17] was used to compress this powder to form cylindrical implants of 1 mm diameter, 1.8 mm length and 1.8 mg weight. Compression force was controlled at 60 N for 10 seconds by a Perkin-Elmer hydraulic press (Perkin-Elmer, Ueberlingen, Germany). Prior to any further investigations, the lipid matrices were weighed on an analytical balance to exactly calculate the content of incorporated protein. Each implant was measured for incorporated radioactivity and the homogeneity of protein distribution was judged from radioactivity/mg ratio for all implants.

2.2.3. In vitro release

Conditions for the *in vitro* release study were chosen such as to minimize stability problems after release from lipid matrices. A continuous flow system, which had been previously developed for the release of proteins from microparticles by Aubert-Pouessel et al. [21] was used for this study. The system consists of an assembly of adsorption resistant material (polyetheretherketone, PEEK®), composed of an unpacked Omega® HPLC tube (4.6 mm inner diameter × 5 mm, Upchurch Scientific, Oak Harbor, WA) fitted with 0.5 mm frits and connected with HPLC tubing at each end. The HPLC tube itself comprises an approximate volume of 1 ml and is big enough to accommodate several implants. The system allows great flexibility with regard to incubation conditions. While the tube itself

can be kept at a temperature of choice (in this study 4, 20 and 37°C), denaturation hazards for released protein are minimized by collecting eluted buffer at 4°C in a refrigerated chamber. A continuous flow is held upright by a syringe pump (PhD 2000, Harvard Apparatus, France) supplying MOPS buffer pH 7.2 (containing 0.1% BSA, 0.02% sodium azide) to the column inlet at 1 µl min^{-1}. Eluent was collected for 24 hours in silanized tubes. The total amount of released protein was determined by counting radioactivity (cpm) with a gamma counter Cobra™ II Auto-Gamma (Packard Bioscience, Rungis, France). Radioactive decay was considered and corrected. Samples were frozen at −20°C until further analysis by bioassay.

2.2.4. Interleukin-18 stability [1]

To assess the biological activity of IL-18, a bioassay allowing the quantification of γ-IFN produced by IL-18 stimulated primary rat splenocytes enriched in T-lymphocytes was developed. Briefly, spleens obtained from 9-weeks old Sprague-Dawley female rats were mechanically dissociated. Splenocytes were then isolated on a Ficoll gradient in order to eliminate debris, platelets, granulocytes and red blood cells. After differential adhesion for 1 hour at 37°C/ 5% CO_2 on plastic culture dishes to remove other unwanted adherent cells (e.g. macrophages), primary rat splenocytes enriched in T-lymphocytes were cultured in RPMI medium containing 10% fetal bovine serum (both from Biowhittake Europe, Verviers, Belgium). They were subsequently primed for activation during 24 hours in the presence of 0.3 µg/ml anti-CD28 in supernatant and in a dish previously coated for 1 hour at 37°C/ 5% CO_2 with 0.5 µg/ml anti-CD3 antibodies and further washed twice with Hank's balanced salt solution. Effects of IL-18 on the production of γ-IFN by isolated splenocytes, after 48 hours stimulation in the presence of low amounts of IL-12 and IL-2 (0.1 and 0.4 ng/ml, respectively), were finally determined in supernatants by using a DuoSet ELISA system, according to the manufacturer's instructions (R&D Systems, Abingdon, UK).

[1] Experiments performed by E. Garcion at INSERM U646, University of Angers, France

3. Results

3.1. Manufacturing procedure

The described manufacturing procedure allowed an easy handling of small batch sizes for the preparation of lipid implants loaded with highly potent proteins. All preparation steps could be performed in the same vial, thus avoiding a loss of material. Figure 1 outlines the main steps of the process. The first step aims at the formation of protein microparticles by freeze-drying an aqueous PEG–protein solution. Varying the amount of PEG present in this solution allows for control of the total PEG content of the formulation. Solid protein particles were obtained by selectively dissolving PEG with the addition of THF. Homogeneous dispersion of these particles and the dissolution of glyceryl tripalmitate were achieved by shortly applying ultrasound. The formulation was frozen a second time in liquid nitrogen to induce the precipitation of dissolved lipid on dispersed protein microparticles, resulting in a very fine and homogeneous distribution of the bioactive agent within the lipid powder mixture after removal of the solvent. Radioactivity/mg was found to vary less than 1% between different implants, affirming the homogeneity of the powder formulation.

3.2. In vitro release characteristics

Figures 2A and B show release profiles of ^{125}I-IL-18 from glyceryl tripalmitate implants. Release of cytokine was investigated under the influence of different concentrations of PEG as a hydrophilic excipient. Relative amounts were 2 and 7% with regard to total implant weight (fig. 2A and B, respectively). As many proteins show increased stability problems at elevated temperatures, the conditions in the reconstitution medium after lyophilization were previously found to play a significant role [22]. It could be seen in preliminary stability studies that IL-18 activity decreased as a function of incubation temperature (data not shown). In order to be able to assess the ratio of bioactive/total released protein, temperatures lower than 37°C were included in the experimental design to limit possible degradation during release.

The implants show a continuous release behavior without a burst effect (fig. 2A and B) for the time period investigated, i.e. 3 weeks. Depending on the temperature at which the column containing the matrices was kept, different velocities could be distinguished. A higher temperature correlated with an increase in release rate. Comparing the formulations

with different amounts of PEG (fig. 2A versus 2B), an interesting effect could be seen: a slight increase in release with increased PEG content was only achieved when implants were incubated at 37°C. In contrast, at 4 and 20°C a higher PEG content seemed to influence protein release in a way that less IL-18 was released compared to implants with 2% of the excipient incubated at the same temperature.

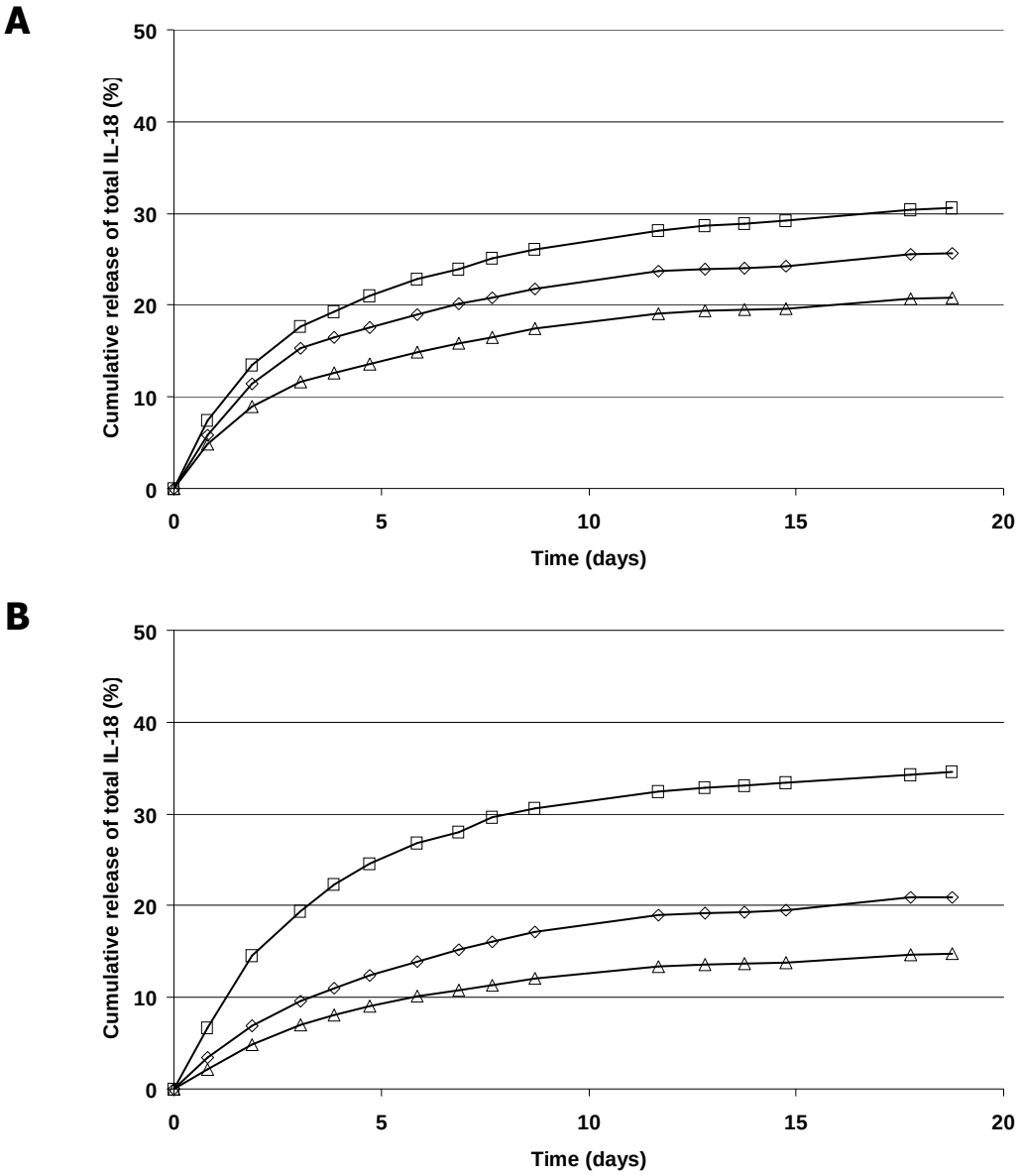

*Fig.2: Cumulative in vitro release of ^{125}I-IL18 from lipid implants containing **(A)** 2% and **(B)** 7% PEG 6,000 incubated at different temperatures: 4°C (Δ), 20°C (◊) and 37°C (□).*

As can be seen in figure 3, a very good approach to the envisaged dosage regime was achieved with the preparation containing 7% PEG.. Release of total IL-18 was found to be in the desired range for the first 12 days of this study: 10 to 100 ng of cytokine were

liberated into the release medium per day. IL-18 release from the formulation containing 2% PEG fulfilled the requirements as well, however with a lower daily release rate (results not shown).

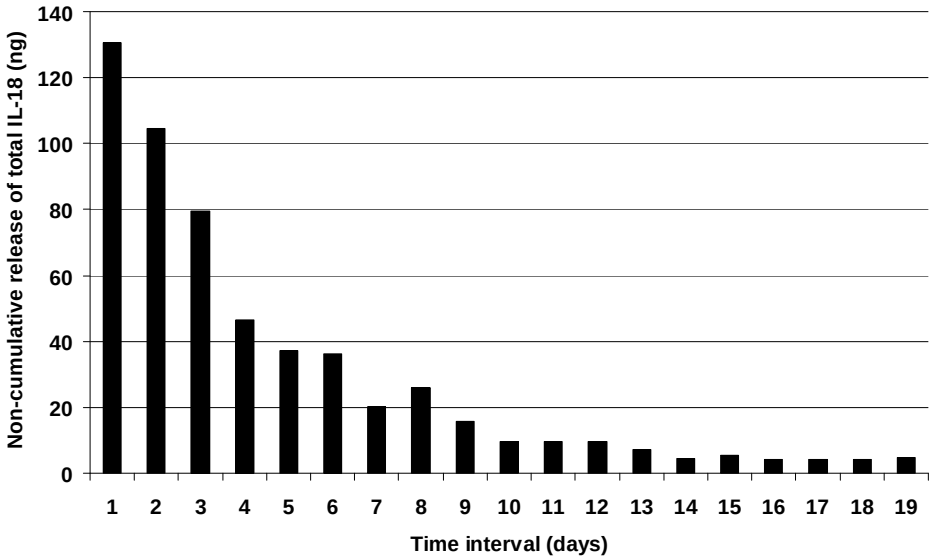

Fig. 3: In vitro release of ^{125}I-IL-18 from implants containing 7% PEG, incubated at 37°C in ng/day.

3.3. Biological activity of released protein

Knowing the release profile of IL-18, it was possible to assess bioactivity of the interleukin by taking an appropriate amount of sample and comparing it to a standard substance. As the best release profile was obtained with implants containing 7% PEG 6,000, further analysis was made considering this condition.

Figure 4A shows the amounts of IFN-γ that are produced by splenocytes after stimulation by IL-18. On the left hand side, the response to different concentrations of standard IL-18 is depicted. On the right hand side, IFN-γ production after stimulation of cells with IL-18 released from lipid implants can be found (day 1–8 and 11 of the release study). The theoretical concentration of these samples was 5 ng/ml bioactive rrIL-18 as calculated from the release of radioactively labeled ^{125}I-mrIL-18. It can be seen that on day 1 samples seem to contain fully bioactive protein, whereas samples from later time points in the study do not induce the same amount of IFN-γ production, as compared to 5 ng/ml standard IL-18. This result is depicted in figure 4B as calculated percentages of released protein found in its bioactive form by referring to the standard value for 5 ng/ml. It can be clearly seen that on day 1 (fig. 4B), the protein was released in its bioactive form, as a response to the

elution buffer was approximately 100% compared to standard. In the progression of the study, the fraction of protein that was released without a change in integrity diminished.

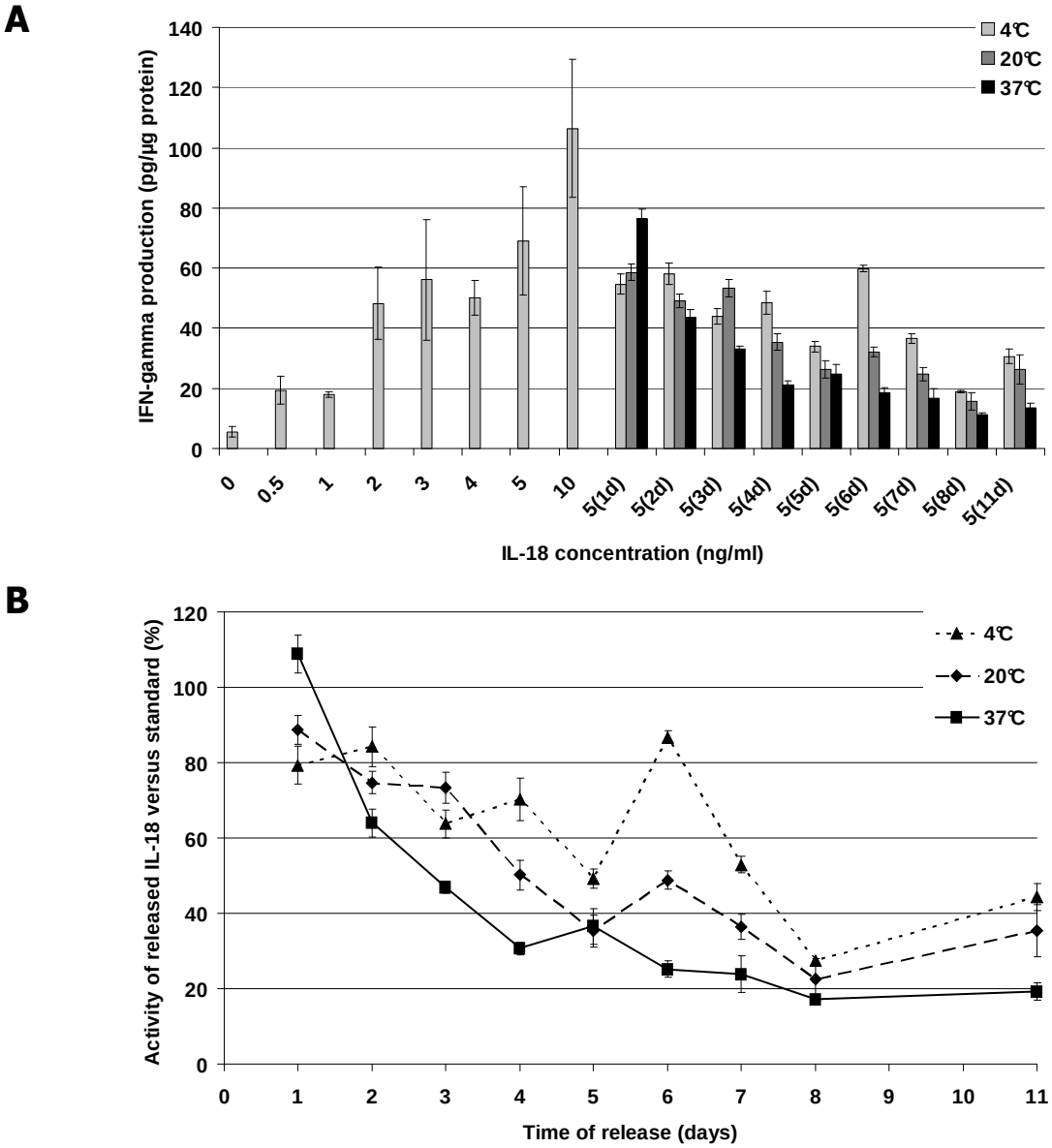

Fig. 4: Biological activity of IL-18 ***(A)*** *measured as IFN-γ production of splenocytes stimulated with known concentrations of IL-18 standard (0-10 ng/ml) compared with IL-18 released from implants containing 7% PEG (theoretical concentration 5 ng/ml, day of release in brackets), and* ***(B)*** *expressed in % as relative amount of IFN-γ production compared with standard IL-18.*

4. Discussion

4.1. Manufacturing procedure

To our knowledge, this is the first report of triglyceride implants being small enough for an implantation into the brain of mice or rats. A diameter of 1 mm allows for the stereotactical insertion of the matrices by pushing them through a needle, thus allowing the *in vivo* investigation of incorporated bioactive compounds intended for cerebral application. This miniature size, however, brings about a significant challenge in the manufacturing procedure, as a fine and homogeneous distribution of the active compound within the system has to be ensured. First experiments with lysozyme – a protein model compound with similar molecular weight and isoelectric point as IL-18 – demonstrated that a simple physical mixture of lyophilized protein and lipid powder by grinding in a mortar could not guarantee a release profile in the desired range, as protein was completely released from matrices within 8 days [22, 23]. However, if protein microparticles were incorporated by the PEG co-lyophilization approach, the continuous release for over 80 days was observed [12], suggesting that the fineness of protein distribution within the lipid matrix influences the release profile. Therefore, this strategy was chosen for the development of IL-18 loaded implants.

Morita et al., who first described the use of co-lyophilization of proteins with polyethylene glycol for formulation purposes [20], explain the formation of spherical protein particles by the aqueous phase separation phenomenon. When an aqueous mixture of protein and PEG is freeze-dried, two distinctive aqueous phases form, resulting in a separation of polymers in the freeze-dried product as well. The method can be applied to a wide variety of proteins.

A similar strategy for protein encapsulation has been suggested for polymer microparticles [24-27]. However, until now, investigations to determine the actual PEG content in the final formulation are missing although Morita et al. discuss a contribution of residual PEG amount to release profiles for microparticles prepared by s/o/w method [26]. In the case of implant formulation, the incorporation of PEG amount can be controlled, as all steps are performed in the same vial and no loss of excipient by diffusion to an outer aqueous phase [28] can occur. Different strategies are possible with regard to the fate of polyethylene glycol. Preliminary studies showed that it would be easy to remove by centrifugation of the protein particles and re-suspension in new solvent before the addition of lipid matrix material [12]. Here, it was allowed to remain inside the formulation as a release modifier.

4.2. In vitro release characteristics

The *in vitro* release testing of a formulation is expected to deliver results regarding performance as a controlled release carrier and retention of biological activity of the incorporated substance. When dealing with fragile compounds like protein drugs, special care has to be taken that *in vitro* release conditions do not exert deleterious effects upon the drug during release and storage of the samples [12, 28]. With a continuous-flow release system as described by Aubert-Pouessel et al., the risk of protein degradation after release is minimized as the buffer flow transports released protein away from the elevated temperature in the incubation chamber and eluent can be stored in a refrigerated chamber [21]. We chose to perform release studies in MOPS pH 7.2 buffer system, as this was recommended to be the most stable medium by the supplier (R&D Systems, Abingdon, UK). This also coincides with our own observations as IL-18 does not preserve its biological activity after incubation from 1 to 3 hours at room temperature in phosphate, borate, bicarbonate and citrate buffers pH 7.4 used on their own (less than 25% recovery, data not shown). In parallel to using the best IL-18 stabilizer medium and in order to further reduce risk of unspecific adsorption, 0.1% BSA was added as a stabilizer to the release medium [29].

The clear dependence of IL-18 release rate on temperature suggests a diffusion-controlled migration of hydrophilic substance within water filled pores in the lipid matrix. Apart from temperature, further impact factors on release rate of IL-18 from lipid matrices can be identified. Two excipients known for acting as release modifiers were added to the formulation: BSA and PEG. BSA or HSA are commonly used as diluent proteins [21, 30, 31]. Jiang and Schwendeman intentionally added BSA to their microparticle formulation in order to increase the release rate of their model drug by amplifying the porosity of the matrix [32]. A ratio of 20:1 for BSA:IL-18 was chosen from experience with release of BSA alone, taking into account that a potential *in vivo* application requires a sufficient daily release to reach a concentration range, in which IL-18 would be active at the tumor site. PEG 6,000 was left inside the formulation for similar reasons and used in two different relative amounts per implant, as presented in figures 2A and B (2 and 7%, respectively). It is well known for its ability to increase release rate from slowly degrading delivery systems [32-34] and lipid matrices [35, 36] by acting as a porogen. In this study, an increase of PEG content from 2 to 7%, however, evoked only a slight increase in total amount of released protein − 30 versus 35%, respectively – an effect which could only be

observed at an incubation temperature of 37°C. At lower temperatures the paradox observation of lower release rates at a higher PEG content was made. This might be explained by a slower dissolution rate of the excipient at lower temperatures and a rise in viscosity in the water filled pores of the implant and thus a hindrance of diffusion of protein – an effect which presumably is enforced in the presence of higher PEG amounts. At 37°C, dissolution of PEG may not be the limiting step, thus allowing the excipient to act its part as a porogen.

Although release enhancers were present in the formulation, an incomplete release was observed. Protein aggregation following initial rehydration of particles during the release study might be a mechanism inside lipid implants leading to the formation of aggregates, which are mainly held responsible for non-release from microparticles and matrix systems due to adsorption and diffusional problems [24, 35, 37].

Considering, however, that the fineness of lysozyme distribution had a significant effect on its release behavior, as mentioned above, a possible explanation might as well be found in percolation theory [38]: The lipid material itself is inert and protein can only be released if aqueous buffer is able to penetrate into the matrix to dissolve the protein. During this process, pores are created where protein is transferred from the solid state into solution. If the ratio of lipid material/protein is too high, an interconnecting network of hydrophilic substance is not possible and areas persist where protein is completely excluded from accession by buffer. This can result in an incomplete release of incorporated drug.

Taking this into account, it seems likely that non-release from lipid implants is caused by the lack of a continuous pore system when protein is finely distributed inside the matrix. This could be addressed by further optimizing the content of porogens in the formulation.

4.3. Biological activity of released protein

Being able to perform a bioassay on released protein allows for the gathering of valuable information about the quality of the manufacturing procedure as well as for the prediction of *in vivo* performance of the drug carrier. The finding of complete preservation of IL-18 integrity at day 1 indicates that protein encapsulation was successfully performed without damage to the protein with the above-described manufacturing procedure. PEG is generally acknowledged as a cryoprotectant [22], although there are proteins, for which integrity losses during co-lyophilization have been reported [39]. Suspending proteins in various organic solvents is considered to be possible without causing solvent-induced structural

perturbations, provided they are suspended in an anhydrous state as they display less conformational flexibility than in the aqueous/organic mixtures used for microparticle preparation [40].

Although release conditions were chosen such as to minimize stability hazards for the released protein, a progressive decrease in the biological activity of IL-18 was detected over 11 days. Interestingly, the decrease in bioactivity of IL-18 is more pronounced for incubation at 37°C followed by 20 and 4°C. As protein degradation pathways can be enhanced at elevated temperatures, these results suggest that the mechanism leading to a loss of activity of released protein takes place during incubation rather than manufacturing. In the literature, reports about the similar behavior of bioactive compounds during release can be found: NGF, which was released from PLGA microspheres was only entirely recognized by ELISA on day 1 [41]; γ-chymotrypsin specific activity was found to decrease over release from PLGA microparticles [24, 25]. A progressive recognition loss was also measured for GDNF formulated in PLGA microspheres, although the release medium had previously been optimized for preservation of GDNF integrity [42].

A moisture induced aggregation related to the slow hydration of encapsulated proteins [22, 28] could be responsible, indicating the need for further stabilization of the protein during release. Approaches discussed in the literature for constricting moisture-induced aggregation with regard to matrix material include an increase in polymer hydrophobicity in order to limit the amount of moisture sorbed in the matrix [28], which however also increases the risk of adsorption phenomena. A second possibility is to ensure rapid hydration of the particles, which can be achieved by the incorporation of hydrophilic additives, such as PEG [24, 25, 32] or BSA [31]. A bell shaped aggregation versus water content dependency has been observed for several proteins, indicating that protein stability may increase again above certain water content, probably due to a dilution effect. This was especially observed in the presence of PEG [37]. Future investigations will therefore be directed towards identifying molecular events occurring during rehydration and release more closely inside the lipid matrices. A correlation between the amount of incorporated PEG and release seems desirable with regard to both the problems of incomplete release and protein stability.

5. Conclusions

IL-18 loaded lipid matrices have been developed, which seem fit for intracerebral implantation into rat or mouse brains by stereotaxy. A manufacturing procedure has been developed, which permits small batch sizes and prevents losses of substance due to a single-pot arrangement, therefore allowing the processing of highly potent, expensive protein drugs. The protein could be homogeneously distributed within the lipid matrix material and bioactivity upon initial liberation from the carrier was fully preserved. Further investigations are necessary to improve protein stability during release as a decrease in IL-18 bioactivity over release time suggests degradation of protein during incubation. A controlled release of protein in the desired therapeutic range of 10 to 100 ng/day was measured during the first 12 days of the study. An *in vivo* evaluation of these matrices is currently being undertaken in order to evaluate their performance in glioblastoma treatment on the basis of an animal model.

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Chapter 5

Confocal Microscopy for the Elucidation of Mass Transport Mechanisms Involved in Protein Release from Lipid-based Matrices

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Abstract

It was the aim of this study to identify the governing mechanisms during protein release from cylindrical lipid matrices by simultaneously visualizing mass transport and correlating the data with *in vitro* dissolution testing. Glyceryl trimyristate cylinders of 2 mm diameter, 2.2 mm height and 7 mg weight were manufactured by compression of a protein-lipid powder mixture prepared by a polyethylene glycol (PEG) co-lyophilization technique. Protein, PEG and the release buffer were fluorescence-labeled and their distribution visualized and quantified at different stages of the release process by confocal microscopy in parallel to the quantification in the release buffer. The different quantification strategies were found to yield identical results. Buffer penetration and protein release occurred simultaneously from the outer regions of the cylinder progressing towards the center. Release from the top and bottom of the matrix was not negligible but much slower than penetration from the side, probably due to an oriented arrangement of lipid flakes during compression. At 6% protein loading, buffer penetration was complete after 4 days, while only 60% of the protein was liberated in that time and release continued up to day 63. Protein release kinetics from the lipid were purely diffusion controlled and could be described using the power law equation $M_t/M_\infty = kt^n$ with an average time exponent n of 0.45 (± 0.04) for loadings varying between 1 and 8%. A percolation threshold at 5% pure protein loading and 3-4% mixed loading (PEG and protein at a 1:1 mass ratio) could be identified.

1. Introduction

In controlled release science, substantial research efforts are devoted to the evaluation of suitable matrix materials for the delivery of protein drugs [1]. Triglycerides have gained growing attention in this context due to their favorable properties: as physiological substances they have shown good biocompatibility tested subcutaneously and in the brain [2, 3]; they are easily compactable and display high long-term stability upon incubation without swelling [4], which qualifies them as a good alternative to commonly used polymeric matrix materials. A successful incorporation into microparticles or cylinders and release thereof has been reported for several peptide and protein drugs and model substances, amongst them insulin [5-7], somatostatin [8], IFN α-2a [9], BSA, hyaluronidase [10], lysozyme, BDNF [3] and interleukin-18 [11].

Although stability risks related to the incorporation into polymers, such as the development of an acidic microclimate during incubation [12-14] and the formation of detrimental polymer degradation products [15] can be circumvented by the use of triglyceride matrices, in parallel studies, we have encountered problems during release testing, manifesting in a progressive activity loss [11] or the occurrence of aggregates [3]. These effects were more pronounced at higher incubation temperatures suggesting an instability mechanism related to the conditions during *in vitro* dissolution testing. Although a recent mathematical evaluation of data from triglyceride systems has implied a diffusion controlled release mechanism [16], it would be extremely valuable to have a more detailed insight into the mass transport phenomena inside the matrices and obtain quantitative data about the expansion of the aqueous buffer environment, probably being the key towards a better understanding of protein stability. Confocal laser scanning microscopy (CLSM) combines all required features for this task - fluorescently labeled model substances can be visualized within a matrix in a semiquantitative manner [17], allowing the co-localization of several substances in a multitracking mode.

It was therefore the aim of this study to correlate the data obtained from *in vitro* dissolution testing with the information assessed by CLSM to propose a release mechanism and get sensitized for potential stability hazards for proteins incorporated into the matrices. To this end, fluorescent tags were attached to the model substances: protein was labeled with fluorescein isothiocyanate (FITC), PEG with carboxytetramethylrhodamine succinimidyl ester (TAMRA-SE) and buffer was visualized by adding sulforhodamine 101 hydrate

(SRH). Protein was loaded onto lipid matrix material by a PEG co-lypohilization technique, previously reported by our group for the processing of IL-18 [11].

2. Materials and methods

2.1. Materials

Chicken egg lysozyme (Grade I, M_w 14,000 Da), bovine serum albumin (BSA, Cohn fraction V, M_w 66,000 Da), bicinchoninic acid disodium salt (BCA), sulforhodamine 101 hydrate (SRH) and Sephadex G10 were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany). Fluorescein isothiocyanate (Isomer I) and all SDS-PAGE reagents were obtained from Serva (Heidelberg, Germany). Molecular Probes (Leiden, Netherlands) supplied 6-carboxytetramethylrhodamine, succinimidyl ester (6-TAMRA-SE, M_w 528 Da) and Fluka (Buchs, Switzerland) polyethylene glycol (PEG, M_w 6,000 Da), dichloromethane (DCM), tetrahydrofurane (THF), Coomassie® Brilliant Blue G-250, N-ethyldiisopropylamine (DIPEA) and dimethylsulfoxide (DMSO). Methoxy-poly(ethylene glycol)-amine (mPEG-NH_2, M_w 5,000 Da) was from Squarix (Marl, Germany). Copper-(II)-sulfate pentahydrate and sodium azide were purchased from Merck (Darmstadt, Germany). GE Healthcare (Munich, Germany) supplied a HiPrep™ 26/10 desalting column and Carl Roth (Karlsruhe, Germany) Spectra/Por® Biotech membrane (MWCO 3,500 Da). Tissue Tek® was obtained from Sakura Finetek Europe (Zoeterwoude, Netherlands). Witepsol® H37 and glyceryl trimyristate (Dynasan®114) were a gift from Sasol (Witten, Germany).

2.2. Fluorescence labeling of model substances

2.2.1. Labeling of PEG

150 mg mPEG-NH_2 and 1 mg 6-TAMRA-SE were dissolved in 10 ml of dichloromethane. DIPEA was added at a molar concentration equal to 6-TAMRA-SE. The solution was stirred under exclusion of light at room temperature for 20 hours before the solvent was evaporated under vacuum. Then, 2.5 ml double-distilled water were added to dissolve the product and the mixture was separated on a Sephadex G10 column (50 ml, 2.5×10 cm) Fractions of 0.5 ml were collected and analyzed by thin-layer chromatography for TAMRA-mPEG content and purity (eluent chloroform/ methanol 3:1 (v/v), TLC aluminium sheets silica gel 60 F_{254}, Merck, Darmstadt, Germany). Solutions containing labeled TAMRA-mPEG were freeze-dried and stored at -20°C until further use.

2.2.2. Labeling of BSA

5 mg FITC were dissolved in 0.4 ml DMSO and slowly added to 50 mg BSA in 5 ml 0.1 M disodium carbonate buffer pH 9.0. The mixture was gently stirred under exclusion of light at room temperature for 2 hours. Low molecular weight compounds were removed by passage over a HiPrep^{TM} 26/10 desalting column. Fractions containing labeled protein were freeze-dried and stored at -20°C until further use. SDS-PAGE analysis was performed to compare the integrity of labeled to native BSA.

2.3. Preparation of protein-loaded lipid matrices

Protein loaded lipid matrices were obtained by co-lyophilizing a solution of protein in the presence of PEG 6,000 as described recently [11]. Briefly, equal amounts of protein and PEG were dissolved in double-distilled water and freeze-dried at 6°C and 0.12 mbar for 30 hours in a benchtop freeze-dryer (Beta 2-16 with LMC-2 system control, Christ, Osterode, Germany). The PEG fraction of the lyophilisate was dissolved after removal of vacuum by subsequent addition of THF resulting in a suspension of solid protein particles in organic solvent. To test for the influence of PEG in the formulation, different strategies were pursued. Table 1 gives an overview of the formulations investigated in this study. PEG was either left in solution and glyceryl trimyristate added at this stage or PEG was removed before the addition of triglyceride. This could be achieved by repeatedly centrifuging the solid protein particles down (12,000 rpm, 10 min, Eppendorf centrifuge 5415R, Eppendorf, Wesseling-Berzdorf, Germany), removing the PEG-containing supernatant and adding fresh THF. After dissolving triglyceride by shortly applying ultrasound (5 sec, 5 W), the dispersion was frozen in liquid nitrogen and the organic solvent was removed under a vacuum of 6×10^{-3} mbar for 20 hours (Two Stage High Vacuum Pump E2M5, Edwards, Crawley, UK). The resulting dry powder was ground in an agate mortar and compressed in a custom-designed compression tool made of hardened steel [4] to form cylindrical matrices of 2 mm diameter, 2.2 mm height and 7 mg weight. A compression force of 250 N was maintained for 10 seconds using a Perkin-Elmer hydraulic press (Perkin-Elmer, Ueberlingen, Germany). Matrices were weighed on an analytical balance to determine their exact weight prior to the following release studies.

Protein (mg)	PEG 6,000 (mg)	Water (ml)	Triglyceride (mg)	THF (ml)	Total hydrophilic loading (%)	Protein loading (%)
3	3	1	595	2	1	**0.5**
3	3	1	295	1.5	2	**1**
5	5	1	325	1.5	3	**1.5**
5	5	1	240	1.5	4	**2**
8	8	2	305	1.5	5	**2.5**
10	10	2	315	1.5	6	**3**
10	10	2	230	1.5	8	**4**
3	-	1	295	1.5	1	**1**
5	-	1	245	1.5	2	**2**
5	-	1	160	1.5	3	**3**
8	-	2	190	1.5	4	**4**
8	-	2	150	1.5	5	**5**
10	-	2	155	1.5	6	**6**
15	-	2	175	1.5	8	**8**
30*	15*/15	5	940	6	6	**3**
30*	-	5	470	6	6	**6**

Tab. 1:Composition of formulations investigated in this study. Where (-) is marked in the PEG column, the excipient was initially contained as equal amount to protein and removed after co-lyophilization. (*) stands for fluorescently labeled compounds. Lysozyme served as a model protein in the experiments with different hydrophilic loadings, whereas FITC-BSA was employed for the other experiments. The total hydrophilic loading is the sum of the PEG and the protein content.

2.4. Release studies

2.4.1. Quantification of protein release

Matrices were incubated in 1.5 ml phosphate buffer pH 7.4 supplemented with 0.01% sodium azide at 37°C. In regular time intervals, buffer was exchanged completely and analyzed for protein content using a micro BCA assay as described by Smith et al. [18]. Briefly, 100 µl sample and 100 µl bicinchoninic acid disodium salt (BCA) working solution were mixed in a 96-well plate, covered and incubated at 60°C for 1 hour. Absorbance was read at 562 nm on a 96-well plate reader (CS-9301 PC, Shimadzu, Duisburg, Germany) after cooling to room temperature. Calibration curves were prepared for FITC-BSA and lysozyme separately and corrected for blank readings.
When fluorescent model substances were contained within the matrix, at each sampling time, one set of matrices (n = 6) was removed from the incubator and frozen at -20°C. Except for cylinders from buffer penetration studies, matrices were freeze-dried prior to further processing.

2.4.2. Quantitative determination of PEG

The PEG quantity in the release buffer was assayed by adopting a method described in [19]. In brief, a 31% (w/v) solution was prepared from ammonium sulfate dissolved in 0.5 M sodium acetate buffer pH 5 and fluorescein disodium salt hydrate was added to a final concentration of 77 µM. 300 µl of this solution were added to 40 µl sample and allowed to settle in the dark for 30 minutes at room temperature before absorption was measured at 475 nm using a 96-well plate reader. PEG solutions of known concentrations were assayed to obtain a calibration curve.

2.5. Confocal microscopy

2.5.1. Analysis of protein content by cylinder cross-sections

For easier handling during cross sectioning and microscopic observation, lipid matrices were embedded horizontally (n = 3) or vertically (n = 3) into a cylinder by melt casting Witepsol® H37 into a ring of 1 cm inner diameter and 1 cm height placed in a flat bottom plastic mold. After solidification in the dark for 2 hours, the cylinder was fixed on a sample holder by freezing with Tissue Tek® and cross sections through the matrix center were obtained by cutting on a Microm HM 550 OMP cryotome (Microm, Walldorf, Germany). To protect the cutting area from moisture, Tissue Tek® was removed in the frozen state and cylinders were placed immediately into a desiccator.

2.5.2. Confocal images

Cross sections were visualized using a Zeiss Axiovert 200M confocal microscope coupled to a Zeiss LSM 510 scanning device (Carl ZeissCo. Ltd., Germany). The inverted microscope was equipped with a Plan–Neofluar 5×/ 0.15 objective. FITC-BSA was excited at 488 nm and fluorescence was detected using a 505-530 nm band-pass filter whereas SRH was excited at 543 nm and recorded with a 560 nm long-pass filter. Images were taken in the multitracking mode. TAMRA-PEG was visualized by excitation at 543 nm and detection above 585 nm using a long-pass filter. Images were processed using the LSM 5 software purchased from Carl Zeiss Co. Ltd., Germany. To ensure that pixel intensities in the images were proportional to drug content, pixel intensity levels were adjusted when the image for time 0 was taken, so that maximum and minimum values were between 0 and 255 grey value in each photomultiplier channel [17]. Then, all images determined for quantitative evaluation were taken with the same instrumental settings.

2.5.3. Image analysis

Cross sections of matrices incubated for different time periods were compared regarding fluorescence profiles for FITC-BSA and SRH. Therefore images from transversal cuts were divided into sections resulting in a grid of 20 concentrical circles with 0.05 mm interspace (see fig. 1) using LSM 5 software.

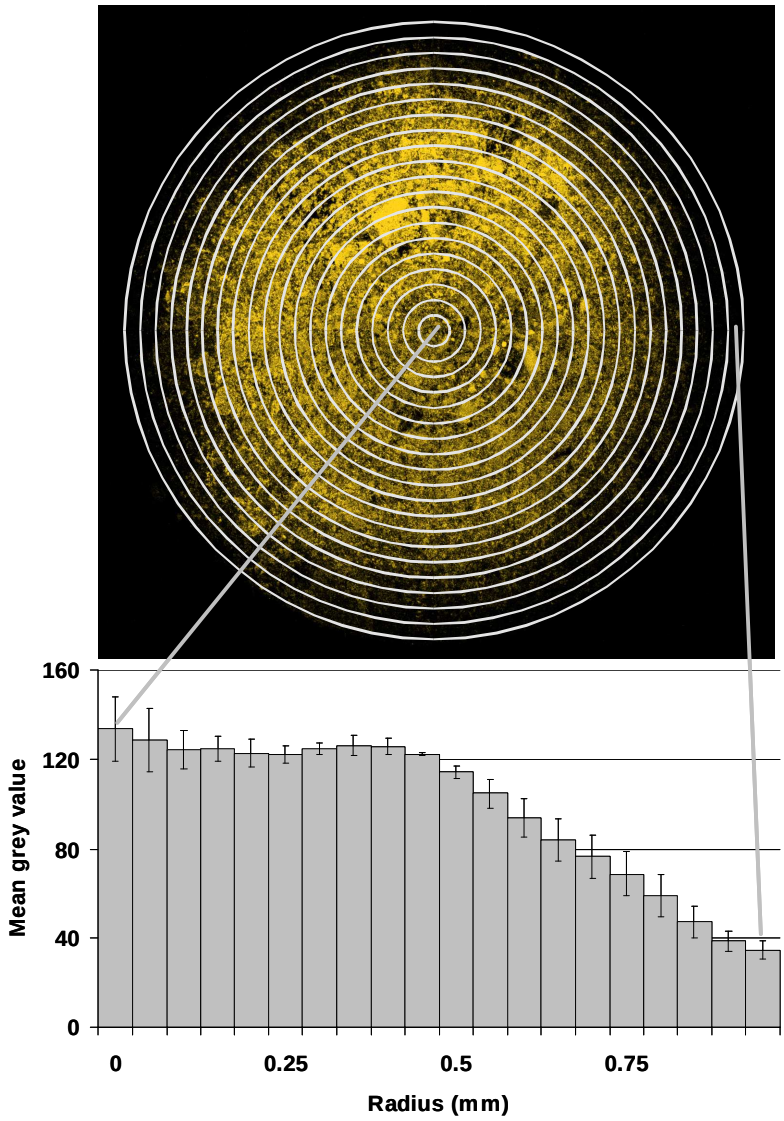


Fig. 1: Schematic illustration of confocal image analysis. Grey bars display mean grey value assessed in concentrical rings superposed as depicted above. Error bars represent standard deviation from evaluation of three different cylinders (cross section) imaged after identical incubation periods. (Example image was taken after 2 days of incubation, matrix loaded with 6% FITC-BSA).

For each ring n, the area A_n and the mean grey value as a measure for fluorescence intensity (MGV_n, given by the LSM 5 software as the sum of the grey values of all pixels in the ring divided by the number of pixels) were noted. Total fluorescence I of the cylinder cross section was determined applying equation (1) taking into account ring area. For each time point, the transversal cuts from 3 individual matrices were analyzed.

$$I = \sum_{n=1}^{20} A_n \times MGV_n \quad (1)$$

2.5.4. Investigation of buffer penetration velocity

In parallel to release studies, buffer penetration into the cylinders was monitored on a separate set of matrices by adding 0.1 mg/ml sulforhodamine 101 hydrate to phosphate buffer pH 7.4 that served as release medium. The buffer was exchanged completely at the same time points as for the release study and cylinders were frozen at -20°C before analyzed as described above.

2.5.5. Buffer penetration depth

In addition to image analysis according to 2.5.3., for SRH penetration studies, longitudinal cuts (n = 3) were compared regarding penetration depth in the radial or axial direction by measuring the distance of the buffer penetration front from the surface of the matrix at the half height and the half diameter using the LSM 5 software.

2.6. Statistical analysis

All measurements were made in triplicate and expressed as means ± standard deviation (SD).

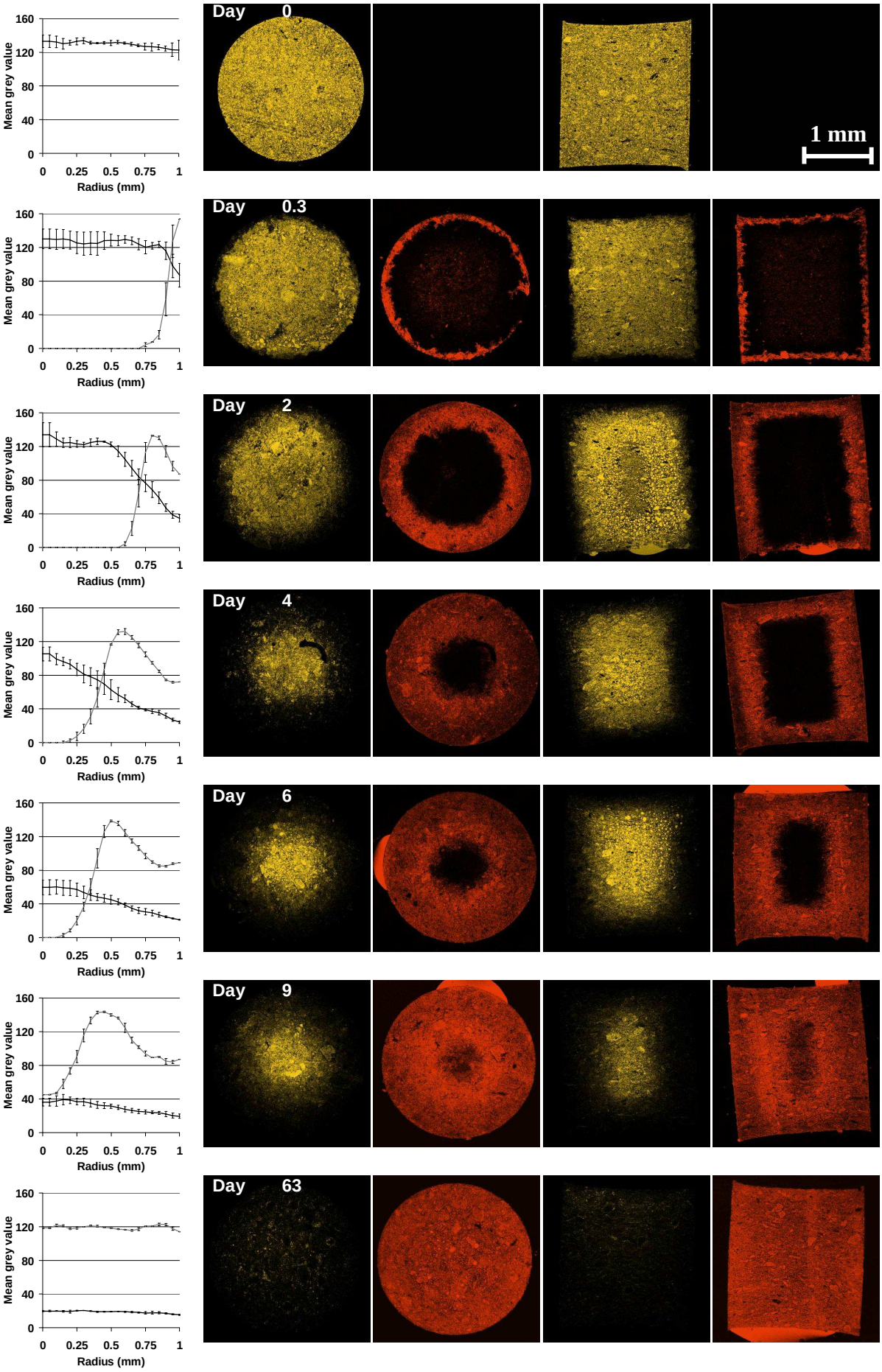
3. Results

3.1. Distribution of fluorescently labeled protein and buffer

In figure 2, the progress of release buffer penetration and protein release as determined by confocal microscopy can be followed in parallel. While buffer started to penetrate the matrices from the surface progressing towards the center (radius = 0 mm) of the cylinder, the protein in the matrix was depleted simultaneously. In the end, the matrix was void of protein and completely penetrated by buffer. To the left of each set of images, a quantitative evaluation of the situation within the cross section can be found. Before day 4, the protein content within the matrix seemed to be only affected in the outer regions with the loading in the center corresponding to the initial value. A linear slope could be detected across the protein diffusion front. From day 4 on, protein was also depleted from the center of the matrix, where the content gradually decreased until the end of release. Although the main peak of the buffer penetration front had not yet reached the matrix center at that time, it was preceded by a diffusion front, which stretched towards the center of the cylinder from day 6 to 9.

Next page:

Fig. 2: Changes in fluorescence profiles of cylinder cross sections. Results from image analysis are shown in the first column (black line: FITC-BSA, grey line: buffer with SRH). Images depict FITC-BSA fluorescence (left) together with the corresponding SRH penetration status (right) in pairs. The first two images were taken of a transversal cross section, the last two from a longitudinal cut. Samples were taken after 0, 0.3, 2, 4, 6, 9 and 63 days of incubation (from top to bottom) of matrices loaded with 6% FITC-BSA.


Day 0
Day 0.3
Day 2
Day 4
Day 6
Day 9
Day 63
1 mm
Mean grey value
Radius (mm)
0
40
80
120
160
0.25
0.5
0.75
1

When measuring the buffer penetration depth in FITC-BSA loaded matrices, the interesting observation was made, that penetration was more extensive from the radial direction of the cylinder compared to the axial direction (fig. 3). When the matrix was penetrated completely by buffer (fig. 4), the black areas, indicative of lipid localization, create an impression of a preferential orientation in horizontal layers within the matrix structure.

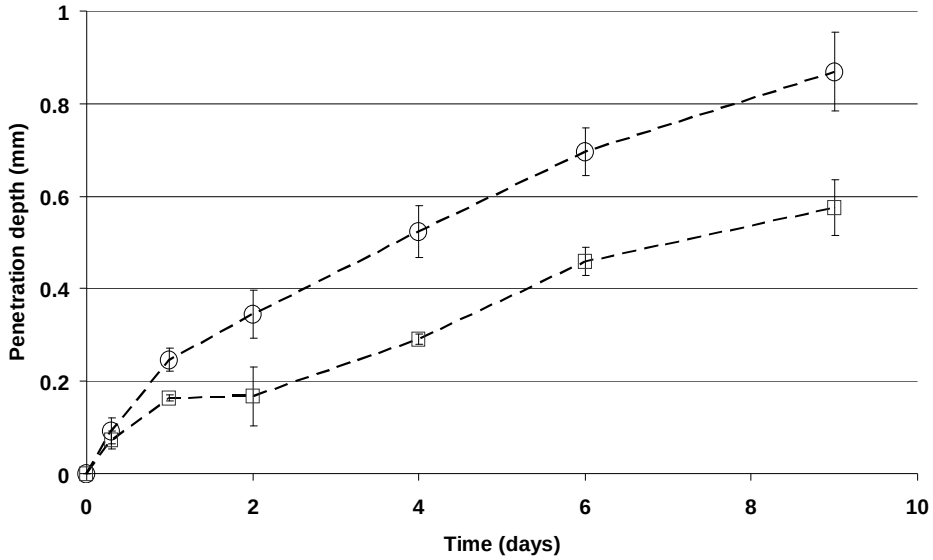


Fig. 3: Comparison of radial (○) and axial (□) penetration depth of phosphate buffer pH 7.4 containing 0.1 mg/ml sulforhodamin 101 hydrate into glyceryl trimyristate matrices loaded with 6% FITC-BSA.

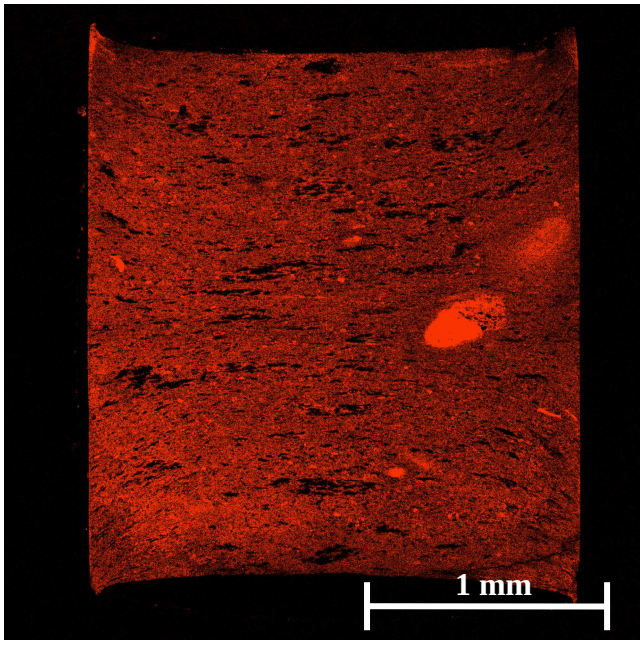


Fig. 4: Longitudinal cross section through a glyceryl trimyristate cylinder after complete release of its protein loading (6% FITC-BSA) and 100% penetration of release buffer with 0.1 mg/ml SRH.

Figure 5 depicts results for TAMRA-PEG depletion, which were not evaluated quantitatively. It can be seen that labeled PEG is also distributed finely and homogeneously all over the matrix and changes in fluorescence distribution over time closely resemble the ones found for FITC-BSA.

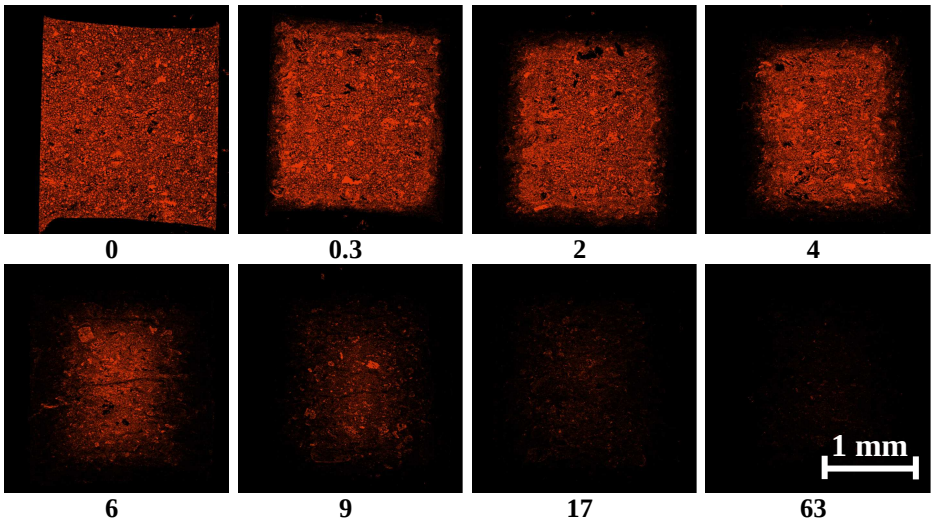


Fig. 5: Changes of TAMRA-PEG fluorescence profiles in longitudinal cylinder cross sections of matrices loaded with 3% protein and 3% PEG (equal amounts of TAMRA-PEG and PEG 6,000). Samples were taken after incubation for the number of days indicated underneath.

3.2. In vitro release of fluorescently labeled BSA

The release of FITC-BSA from glyceryl trimyristate matrices was determined both by measuring the amount of protein liberated into the release buffer by micro BCA assay and quantification of the non-released fraction with the help of confocal microscopy. Figure 6A gives an overview of the results with the solid line representing the release profile obtained by protein assay. More than 85% of the matrix loading were released within the first 20 days with only a little increase up to 92% until day 63.

The data points obtained by analysis of non-released FITC-BSA by confocal microscopy (black squares) are located close to the release profile in figure 6A, indicating that both methods of quantification provide identical information.

In the image, also values from confocal analysis of penetration of buffer containing SRH were included (triangles): Closed triangles represent the amount of buffer determined in the cross sections after the different incubation periods related to the amount of buffer measured at day 63 in %. While these values were obtained applying equation (1) as described under image analysis (2.5.3.), open triangles result from radial penetration depth measurements on longitudinal cross sections (2.5.5.) and are expressed in % of the radius already dyed by SRH. It can be seen, that buffer penetration can also be taken as indicative of the amount of protein released, however, it was completed earlier than protein release.

Figure 6B represents a plot of the release data according to the power law equation $M_t/M_\infty = kt^n$ introduced by Ritger and Peppas [20] with the exponent $n = 0.45$ for cylindrical, non-swellable matrices. In the equation, M_t is the cumulative absolute amount of drug released at time t and M_∞ the absolute cumulative amount released at infinitive time, which should be equal to the amount incorporated. k is a constant incorporating structural and geometric characteristics. A good linear correlation ($R^2 = 0.9989$) was obtained for values up to 60% of cumulative release with a lag period occurring during the first 8 hours of release.

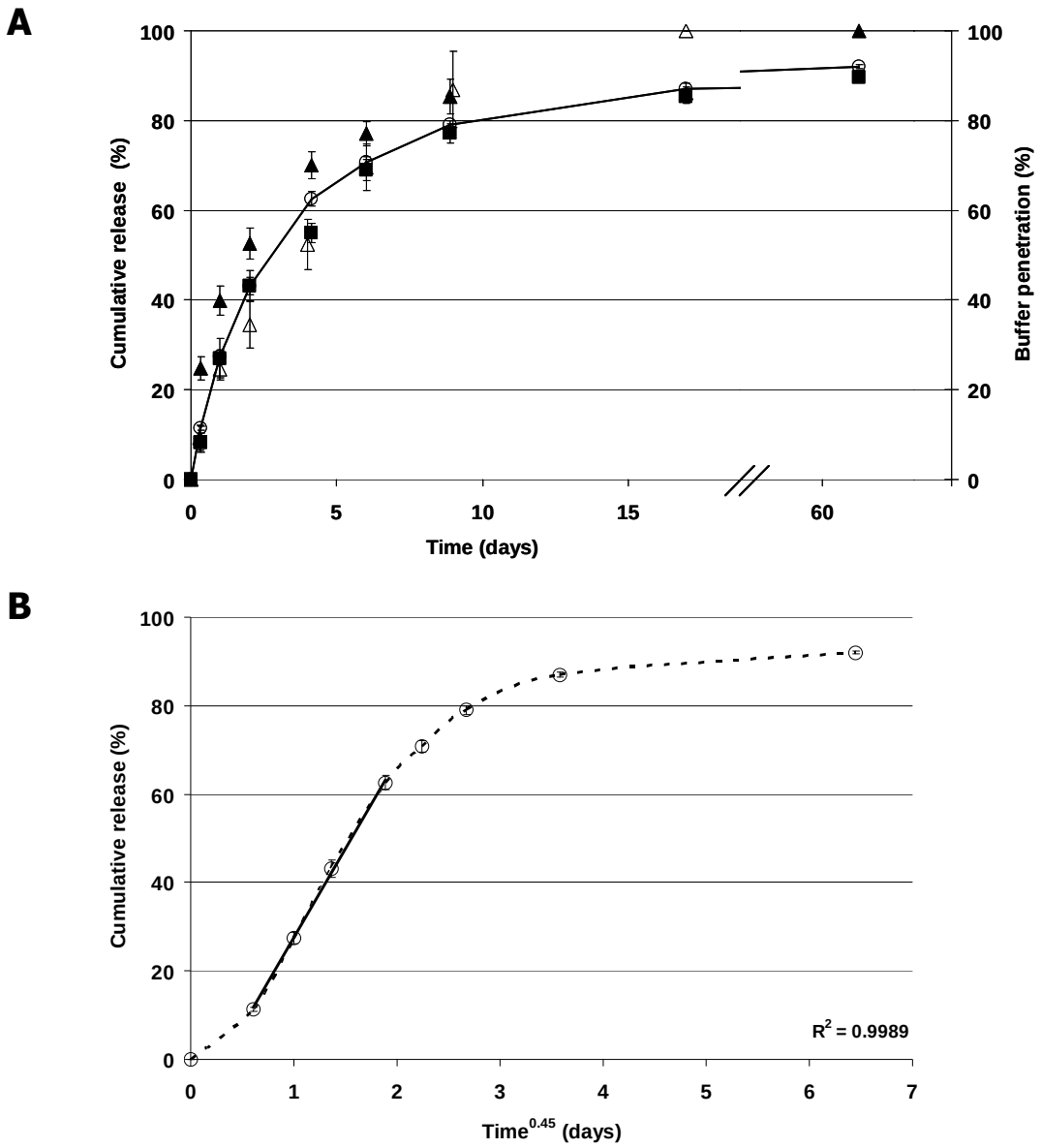


Fig. 6: ***(A)*** *In vitro release of FITC-BSA from glyceryl trimyristate matrices loaded with 6% protein (○) and correlation with release quantified by confocal image analysis of non-released FITC-BSA (■). Comparison to quantification of penetrated buffer containing SRH by confocal image analysis (▲) and radial penetration depth measurements (Δ).* ***(B)*** *Cumulative amount released plotted versus time$^{0.45}$. The black line corresponds to the linear approximation with R^2 = 0.9989.*

3.3. In vitro release of lysozyme from matrices with different loading

The hydrophilic loading of the lipid matrices varied between 1 and 8%. One set of matrices was loaded exclusively with protein (fig. 7A), whereas a second set was produced, where the loading was composed of equal amounts of protein and PEG 6000 (fig. 7B and C). All experiments showed a strong dependence of the release rate on the loading with faster release occurring at higher loadings.

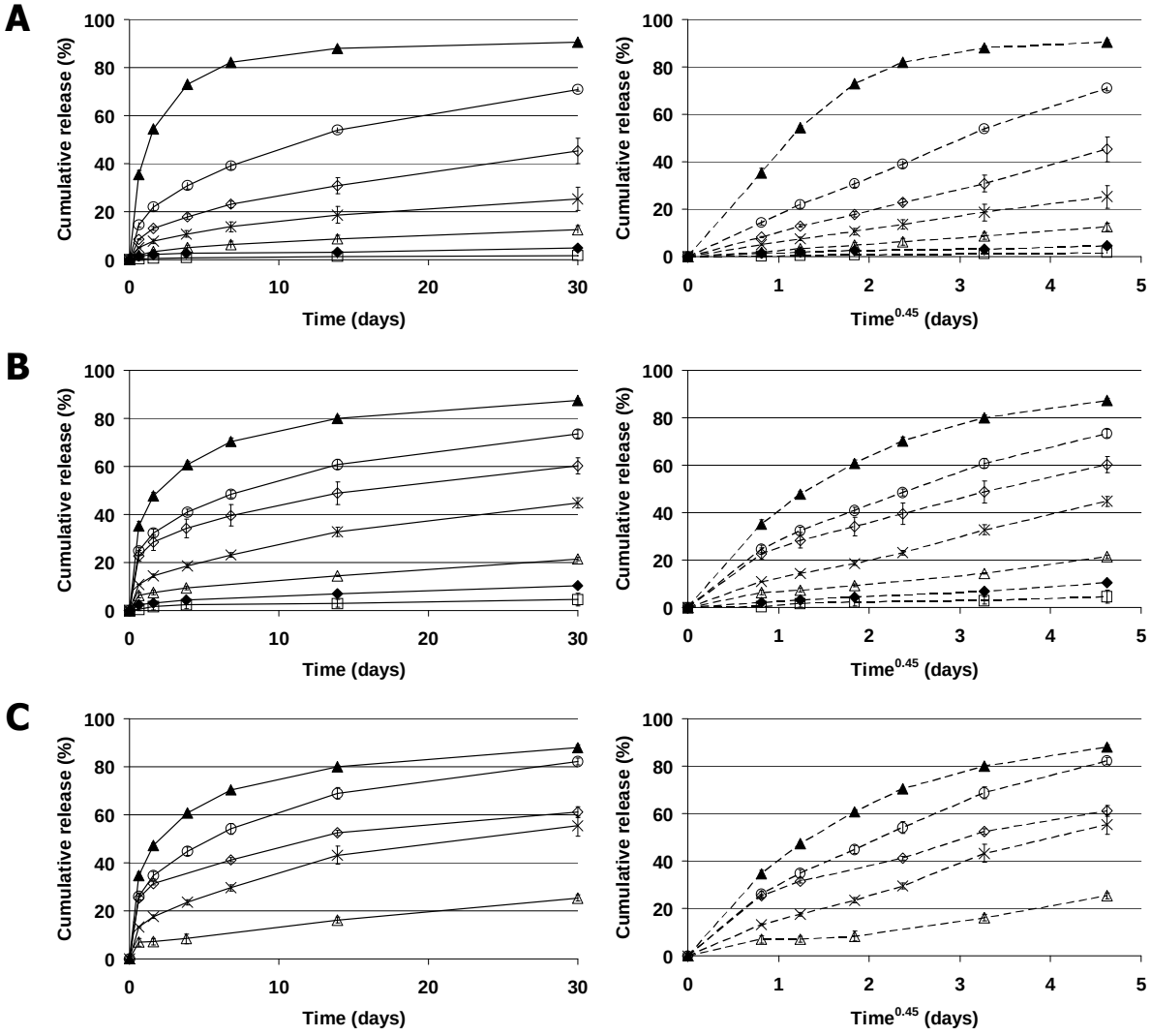


Fig. 7: ***(A)*** *In vitro release of lysozyme from glyceryl trimyristate matrices (loading consisted exclusively of protein).* ***(B, C)*** *Matrices loaded with equal amounts of protein and PEG 6,000:* ***(B)*** *protein release,* ***(C)*** *PEG release. Total hydrophilic loading was (□) 1%, (♦) 2%, (Δ) 3%, (×) 4%, (◊) 5%, (○) 6% and (▲) 8% according to table 1.*

In the range of 1 to 5% hydrophilic matrix components, protein release was faster from matrices containing PEG (fig. 7A and B) whereas at 6%, no difference was observed and at 8% matrices without PEG showed an initially faster release. Figure 7C depicts the amount of PEG detected in the release buffer for matrices with 3 to 8% hydrophilic loading (PEG release for 1 and 2% loading was below the detection limit), which corresponds closely to the amount of lysozyme released, supporting the idea of a parallel release.

A plot of the logarithm of time versus the logarithm of the cumulative lysozyme amount released in % as in figure 8 enables the determination of the time exponent *n* (equal to the slope) for the linear part of the curve according to the power law equation. A linear relationship with good correlation coefficients was observed with all graphs having the same slope but different y-axis intercepts. The mean of the slopes (excluding the value for 2% loading) equaled 0.45 (± 0.04).

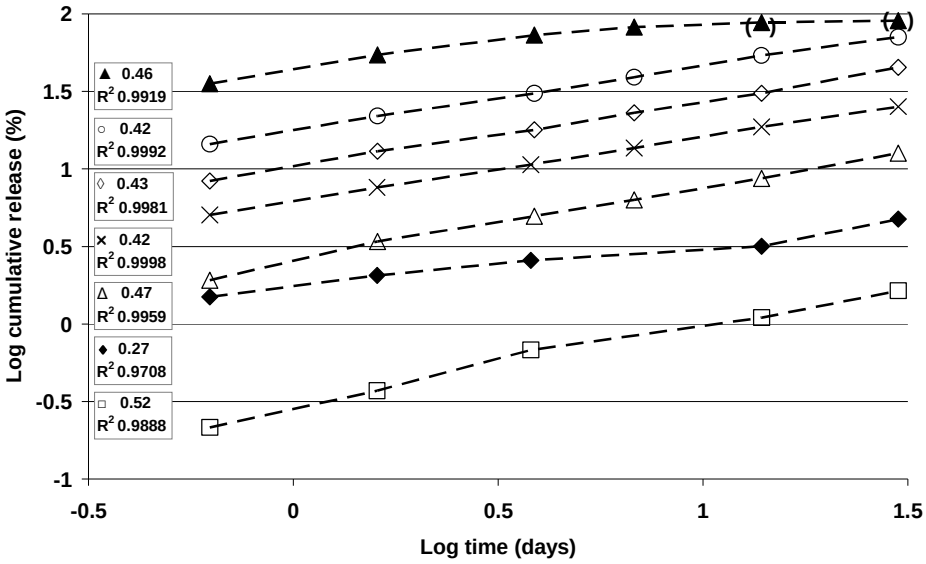


Fig. 8: Logarithm of cumulative release (%) of lysozyme versus logarithm of time for glyceryl trimyristate matrices loaded with (□) 1%, (♦) 2%, (Δ) 3%, (×) 4%, (◊) 5%, (○) 6% and (▲) 8% protein according to table 1. Boxes on the left hand side indicate the value for the linear slope of the curve and its correlation coefficient R^2.

To linearize release data, time$^{0.45}$ was then plotted versus cumulative release (%) (fig. 7, images on the right). In the case of lysozyme, a very good correlation was found; for the profiles of PEG containing matrices, a bend was observed after the first values (corresponding to 15 hours) before the profile displayed a linear slope.

Due to the different release rates, the amount of model substance released within 30 days was also found to be a function of the matrix loading. This is illustrated in figure 9, with the results shaping into a sigmoidal curve having a inflection point at approximately 5% for matrices loaded with lysozyme alone and between 3 and 4% for matrices with equal content of PEG and lysozyme.

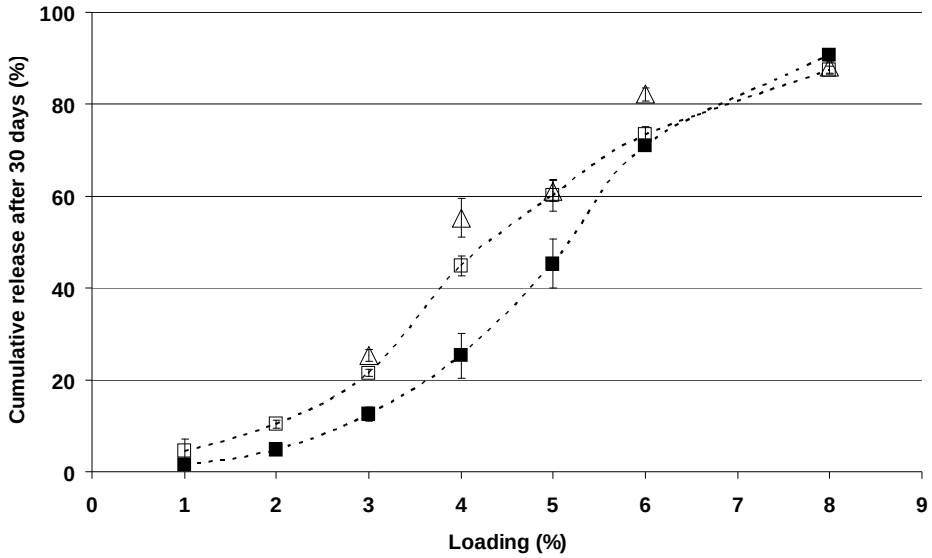


Fig. 9: Amount of protein released after 30 days (%) versus matrix loading for matrices containing only protein (■) or both protein and PEG 6000 (□) according to table 1. (Δ) represents released amounts of PEG 6000.

4. Discussion

Incorporation of protein into lipid matrix material creates an environment for long-term release (figs. 6, 7). Confocal images taken of the matrix cross sections prior to release document a very fine and homogeneous distribution of protein all over the cylinder (fig. 2, day 0). The porosity of such a matrix, i.e. the space theoretically accessible for release buffer by capillarity and drug dissolution, is composed of void space and drug filled space [21]. A compression force of 250 N in this study assured, that air porosity, i.e. void space, was reduced to a minimum, rendering the incorporated amount of drug and excipient the main impact factors for release.

The images in figure 2 indicate that buffer penetration and protein release are closely related processes. Images were all taken with the same set of parameters in this study with prior adjustment of the instrument settings ensuring that the pixel grey values were proportional to the model substance content allowing for a quantitative analysis. The good agreement between the data from methods quantifying FITC-BSA release (BCA assay of released protein and confocal image analysis of non-released protein) with data from analyzing protein penetration into the matrices (fig. 6A) supports the hypothesis that buffer only penetrates into the matrices, where protein is dissolved and diffuses out. In contrast to SRH penetration depth measurement, SRH profile image analysis of horizontal cross sections indicated a slightly higher buffer content in the matrix than protein released. As can be seen in figure 2, SRH dye distribution was not homogeneous in the region already penetrated by release buffer with a peak occurring at the buffer penetration front and a decrease in fluorescence towards the matrix surface. Possibly, the height of that peak might be amplified by a faint bleed-through of FITC-fluorescence at high concentrations of freshly dissolved FITC-BSA at the buffer penetration boundary. On day 63, when protein has been completely released, SRH distribution was homogenous all over the cylinder.

Buffer penetration depth results in figure 6A stemmed from radial measurements of longitudinal cross sections. Interestingly, axial penetration through the cylinders' flat top and bottom surface was less extensive (fig. 3). As the matrix was manufactured by compression of a powder consisting of anisometric triglycerides flakes, an orientation of the flakes under compression force seems plausible, leading to a higher tortuosity in axial than in radial direction and a better accessibility from the sides of the cylinder. The image of a cylinder completely penetrated by buffer (fig. 4) supports this hypothesis, as the black

areas in the image, where lipid is located, seem to display a preferential orientation of layers in horizontal direction.

60% of protein was released during the first 4 days (fig. 6A) of the study, during which the buffer slowly progressed towards the center. The profile of protein distribution within the matrix (fig. 2, diagrams) showed a linear slope between the buffer penetration front and the cylinder's surface, a typical characteristic of purely diffusion controlled release processes [22, 23]. When protein started to get depleted also from the center of the cylinder, the protein concentration gradient – being the driving force for its diffusion – gradually decreased, resulting in a slowing of the release rate.

For better visualization, release data was linearized (fig. 6B) by applying the power law equation. Lipid matrices represent the special case of cylindrical, non-swellable matrices, where this exponent is postulated to be 0.45 [20] for pure Fickian release behavior. The model allows a prediction up to 60% cumulative release, which in the case of lipid matrices coincides with the slowing of release due to the decrease in diffusion gradient after complete penetration of the matrices by release buffer. In figure 6B, a lag phase can be observed during the first 8 hours of release, which is probably due to FITC-BSA being slightly more hydrophobic than the unmodified protein [24], thus showing a reduced capacity to induce matrix wetting by the release buffer and therefore causing a lag in buffer penetration [25].

The time period between the buffer penetration front reaching the center of the matrix and complete protein release might be critical for protein stability as the remaining loading has to face an aqueous environment while still being inside the matrix. Thus one approach to limit stability hazards during incubation might be a decrease of buffer penetration speed, e.g. by using a more hydrophobic triglyceride as matrix material. Lower loadings would also represent an alternative, however with the risk of incomplete release.

To test the impact of different loadings, matrices were fabricated with lysozyme as a model substance, which potentially enables the determination of enzymatic activity. Depending on the loading, the fraction of protein having access to the matrix surface via pores created by the release buffer upon its dissolution may vary explaining the shift of the release profiles in figure 7. A preliminary evaluation of lysozyme enzymatic activity using the *Micrococcus lysodeikticus* assay [26], however, allowed no conclusions as to protein stability, as lysozyme retained its activity regardless of the loading (data not shown), probably due to its general high stability [27].

In order to assess the time exponent *n* according to the power law equation at different loadings, release data was plotted as the logarithm of cumulative release versus logarithm of time (fig. 8), where the slope is representative of the exponent *n*. The values determined from figure 8 by linear correlation showed an excellent fit and were independent of the matrix loading (the profile at 2% loading was considered as outlier due to the inconsistent high release at the beginning). The mean value of 0.45 (± 0.04) for *n* corresponds to literature data, where *n* was postulated to equal 0.45 for pure Fickian diffusion in non-swelling matrices as mentioned above [20, 22]. When plotting the release profiles versus $t^{0.45}$ (fig. 7), a linearization of the release data could be achieved for all loadings. Release curves of matrices containing hydrophilic PEG showed a slight bend at the beginning, which was maybe due to a better initial wetting. Additionally, PEG – by being able to intersperse the lipid phase upon compression – may increase the amount of protein having access to the surface upon first contact with release buffer. PEG release seems to occur in parallel to lysozyme liberation, only slightly exceeding it up to loadings of 6%, presumably due to the lower molecular weight of the PEG 6,000 resulting in a faster diffusion. In general, the replacement of half the hydrophilic loading by PEG led to an increase in release (fig. 9) and a higher amount of lysozyme liberated at day 30 for 1 to 6% loading. A sigmoidal shape of the profile in figure 9 was interpreted according to Siegel and Langer [28] as indicative for the hydrophilic loading, above which 100% of the drug can be released as an interconnected network of pores is formed. This critical porosity is reached at the inflection point in the curve, which seems to be at a higher loading for matrices containing only protein (5%) than for PEG-containing matrices (3-4%). The homogeneous distribution of PEG detected by confocal microscopy (fig. 5) suggests a function as a porogen, preparing a diffusion pathway for the protein. Lower molecular weight and its ability to intermingle with the lipid upon compression might be responsible for slightly lowering that value. Generally, percolation theory as described by Bonny and Leuenberger [29, 30] has identified porosities of 20 to 30% as typical threshold values of inert matrices. However, a dependence of this value on the ratio of drug/matrix particle size was observed shifting it towards lower porosities as the drug particle size decreases [31, 32]. As the PEG co-lyophilization method leads to a micronization of the protein in the first preparation steps resulting in protein particle sizes below 10 µm (data not shown), the low values obtained in this study are not surprising.

5. Conclusion

A quantitative correlation of *in vitro* dissolution tests and changes of internal matrix composition evaluation by confocal microscopy allowed for the determination of mass transport mechanisms within lipid matrices. Protein release from triglycerides was governed by simultaneous buffer penetration, protein dissolution and its diffusion out of the matrices. Experiments at different loadings all point towards the diffusion being the rate determining step. Potential protein stabilization approaches should address the time span between complete buffer penetration of the matrix and 100% release of the remaining loading, which would be exposed to an aqueous environment before leaving the matrix. However, more sensitive model proteins than lysozyme should be employed to address this topic.

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Chapter 6

Influence of Wettability and Surface Activity on Release Behavior of Hydrophilic Substances from Lipid Matrices

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Abstract

The aim of this study was to investigate the role of matrix and drug properties on controlled release from triglyceride matrices. Mini-cylinders of 2 mm diameter, 2.2 mm height and 7 mg weight were produced by compression of lipid powder obtained by using a polyethylene glycol (PEG) co-lyophilization method for the model substances lysozyme and dextran (M_w 4,000 Da, labeled with fluorescein isothiocyanate (FITC-dextran)). Lysozyme was released with decreasing velocity from glyceryl trilaurate, -myristate, -palmitate and -stearate for more than 14 months. Release correlated well with triglyceride lipophilicity defined by the chain length of the fatty acids. Contact angle measurements and the analysis of buffer penetration visualized by confocal microscopy emphasized the role of matrix wettability as a prerequisite for release. A comparison with FITC-dextran revealed that the protein itself enhances matrix wettability and hence its release due to its surface active properties. FITC-dextran remained trapped within the matrix only to be released at lower compression force or after the addition of surfactant. Employing PEG as a porogen proved to be an effective release enhancer only in the presence of protein, where an almost complete release of lysozyme within 426 days was observed irrespective of the type of triglyceride the matrices were made of. Protein added externally to the release buffer at 0.1% (w/v) was not efficient in lowering the contact angle and increasing the release rate of FITC-dextran. Tween®20 and 81 could be used in different concentrations (0.1, 0.01 and 0.001% (w/v)) to alter lysozyme and FITC-dextran release profiles: the presence of the polysorbates was the dominant impact factor for both protein and FITC-dextran; resulting release rates showed a close dependence on the contact angle of the respective release medium and triglyceride matrix material. However, results have to be interpreted with care, as both Tweens® seem to act not only by reducing the release medium contact angle but also by moderately affecting interparticulate adhesion of the matrix material.

1. Introduction

Protein delivery is an area of growing interest in the field of controlled release technology due to an increasing number of potential therapeutic biomacromolecules made available by the progress in biotechnology and genetic engineering [1, 2]. Particular attention is given to materials capable of embedding and releasing proteins over extended periods of time in a predictable fashion [3], improving the patients' quality of life during parenteral therapy. Triglycerides have been established as a suitable matrix material in this context by successful incorporation and release of peptides and proteins from microparticles and cylindrical matrices [4-8]. In parallel studies to this one, we have investigated the use of confocal microscopy to analyze protein distribution [9] and drug release mechanisms [10] for triglyceride cylinders. Release was found to be a diffusion controlled mechanism, initiated by controlled buffer penetration into the matrix. This process can be strongly dependent on wettability [11], i.e. the tendency of a liquid to spread on a given surface and consequently penetrate into pores. Several reports emphasize the influence of surface wettability on model substance release from matrix materials such as polymers [12-15], waxes [16-19], fatty acids [20] and polyglycerol esters of fatty acids [21, 22], however only low molecular weight chemical compounds have been examined as model substances so far.

It was the aim of this study to investigate, whether wettability effects play a role in release of high molecular weight compounds from triglycerides as well, and how this could be used to tailor release profiles.

Most studies that have been conducted with triglycerides as matrix materials for protein encapsulation so far have only focused on one lipid. However, the triglycerides were not identical in the respective studies: glyceryl trimyristate was used for the delivery of TAMRA-BSA, hyaluronidase [8], lysozyme and FITC-BSA [10], glyceryl tripalmitate was applied to incorporate insulin [23], interleukin-18 [4], lysozyme and BDNF [9] and glyceryl tristearate for the encapsulation of interferon α-2a [6]. Thus, a comparison of the effect of triglyceride properties is not possible due to the different manufacturing strategies and model substances. Therefore, we chose four triglycerides, which differed in fatty acid chain length from 12 to 14, 16 and 18 C-atoms, causing distinct changes of properties such as solubility in organic solvents, melting points and compactibility.

To clarify, whether the properties of the model substance itself contribute to matrix wettability, two different high molecular weight model compounds were selected:

lysozyme (M_w 14,000 Da) and FITC-dextran (M_w 4,000 Da), representing a substance with inherent surface activity and one without. They possess similar hydrodynamic radii (1.6 nm and 1.4 nm respectively) [24] and can both be processed by the PEG co-lyophilization method, which is based upon an aqueous phase separation between PEG and a macromolecule upon freezing [25].

In parallel to release studies, wettability of the matrices was assessed by the ability of release buffer containing the fluorescent dye sulforhodamine 101 hydrate to penetrate into the matrix structure, investigated by confocal microscopy, and compared to data obtained by contact angle measurements of the release medium on the respective triglyceride.

2. Materials and Methods

2.1. Materials

Chicken egg lysozyme (Grade I, M_w 14,000 Da), FITC-dextran (M_w 4,000 Da), sulforhodamin 101 hydrate (SRH), bicinchoninic acid disodium salt (BCA) and bovine serum albumin (BSA, Cohn fraction V, M_w 66,000 Da) were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany). ICI Specialty Chemicals (Essen, Germany) supplied Tween®20 and 81. Tissue Tek® was from Sakura Finetek Europe (Zoeterwoude, Netherlands). Polyethylene glycol (PEG, M_w 6,000 Da), dichloromethane (DCM) and tetrahydrofurane (THF) were bought from Fluka (Buchs, Switzerland). Glyceryl trilaurate, -trimyristate, -tripalmitate, -tristearate (Dynasan®112, 114, 116 and 118) and Witepsol® H37 were a gift from Sasol (Witten, Germany). Copper-(II)-sulfate pentahydrate, ammonium sulfate, orthophosphoric acid 85%, sodium azide and all buffer salts in analytical grade were purchased from Merck (Darmstadt, Germany). Fluorescein disodium salt hydrate was obtained from Lancaster Synthesis (Morecambe, England).

2.2. Lipid matrix preparation

In order to obtain a model substance loaded lipid powder suitable for compression, a PEG co-lyophilization method described previously by our group was applied [4]. Briefly, 10 mg of the model substance (either lysozyme or FITC-dextran) were dissolved in 2 ml of an aqueous solution of 10 mg PEG 6000. After freezing the mixture in liquid nitrogen, it was lyophilized at 6°C and 0.12 mbar for 30 hours in a benchtop freeze-dryer (Beta 2-16 with LMC-2 system control, Christ, Osterode). To dissolve the PEG fraction of the lyophilisate, 1.5 ml of THF were added immediately after vacuum removal resulting in a s/o dispersion of solid protein or dextran microparticles. PEG was either separated by centrifugation of the solid protein particles, removal of the supernatant and repeated washing with new THF, or left within the formulation. Triglyceride was added as a powder and dissolved by applying ultrasound for 5 seconds (5 W, Digital Sonifier 250-D, 3 mm microtip, Branson, Frankfurt, Germany). After freezing in liquid nitrogen, organic solvent was removed from the mixture under a vacuum of 6×10^{-3} mbar for 20 hours (Two stage High vacuum Pump E2M5, Edwards, Crawley, UK), and the resulting dry powder was ground in an agate mortar. Cylindrical matrices of 2 mm diameter, 2.2 mm height and 7 mg

weight were prepared from the model substance-loaded lipid powder by manual compression in a custom-designed compression tool made of hardened steel [26]. Compression force was controlled at 250 N for 10 seconds by a Perkin-Elmer hydraulic press (Perkin-Elmer, Ueberlingen, Germany). Matrices were weighed on an analytical balance to determine their exact weight prior to release studies.

2.3. Quantification of model substance release

2.3.1. BCA micro assay

The amount of lysozyme in the release buffer was determined by incubating 100 µl sample and 100 µl bicinchoninic acid disodium salt (BCA) Micro-Working Reagent at 60°C for 1 hour in a covered 96-well plate as described by Smith et al. [27]. After cooling to room temperature, absorption was determined at 562 nm by using a 96-well plate reader (CS-9301 PC, Shimadzu, Duisburg, Germany). Protein amount was calculated from standard curves obtained from known concentrations of lysozyme in either buffer alone or with added surfactants of the appropriate concentrations (0.1, 0.01 or 0.001%). Measurements were corrected against blank using the respective buffer.

2.3.2. Quantitative determination of PEG

The PEG quantity in the release buffer was determined by measuring the absorbance of fluorescein after its partitioning into an aqueous two-phase system as described in [28]. Briefly, a working solution was prepared from ammonium sulfate dissolved in 0.5 M sodium acetate buffer pH 5 to a final concentration of 31% (w/v) and fluorescein disodium salt hydrate at a final concentration of 77 µM. 300 µl working solution were added to 40 µl sample and allowed to stand in the dark for 30 minutes at room temperature before absorption was measured at 475 nm by using a 96-well plate reader. PEG solutions of known concentration were assayed to obtain a calibration curve.

2.3.3. Fluorescence measurement

The amount of FITC-dextran released into buffer was determined by measuring the fluorescence of diluted sample (excitation 490 nm, emission 520 nm, spectrofluorophotometer RF-1501, Shimadzu, Duisburg, Germany) and calculating the concentration from a standard curve.

2.4. Contact angle measurement

Microscope cover slips (18×18mm, Menzel-Glaeser, Braunschweig, Germany) were spin-casted with glyceryl trilaurate, -myristate, -palmitate and -stearate from a solution of the lipid in dichloromethane. The contact angle of phosphate buffer alone or containing different concentrations of Tween®20 and 81 (0.1, 0.01, 0.001%) and BSA (0.1%) was measured at an OCA 15 Contact Angle System (Dataphysics, Filderstadt, Germany). Results were obtained by calculating the average of 10 individual measurements.

2.5. Buffer penetration study

In order to be able to visualize and quantify buffer penetration speed into lipid matrices under different conditions, an identical setup as in the release studies was used with the addition of sulforhodamin 101 hydrate (SRH) to release buffer at 0.1 mg/ml. The buffer was exchanged completely in regular time intervals identical to sampling release. Each time one set of matrices (n = 3) was removed from the incubator and frozen at -20°C.

A comparison was intended for different triglycerides, presence/absence of PEG from matrices, presence of different surfactants added to the release buffer in different concentrations and the pure triglyceride versus model substance loaded matrix (for an overview of the experimental setup see table 1).

Confocal microscopy was used to evaluate the distribution of fluorescent dye within the matrices after certain periods of time. For easier handling during cross sectioning and microscopic observation, lipid matrices were embedded by melt casting into a Witepsol® H37 cylinder of 1 cm inner diameter and 1 cm height and fixed on a sample holder by freezing with Tissue Tek®. Cross sections were obtained by longitudinal cuts of the matrices on a Microm HM 550 OMP cryotome (Microm, Walldorf, Germany). Fluorescence of buffer penetrated into the matrices was visualized on a Zeiss Axiovert 200M confocal microscope coupled to a Zeiss LSM 510 scanning device (Carl ZeissCo. Ltd., Germany). The inverted microscope was equipped with a Plan–Neofluar 5×/ 0.15 objective. SRH was excited at 543 nm and fluorescence was recorded with a 560 nm long-pass filter. Radial penetration depth was measured as described in [10] using the LSM 5 software purchased from Carl Zeiss Co. Ltd., Germany and expressed in % of the cylinder radius.

2.6. Scanning electron microscopy

The morphology of the matrices was investigated by scanning electron microscopy (SEM). The cylinders were mounted on aluminum stubs covered with adhesive conductive carbon tape (LeitTabs; Plannet GmbH; Wetzlar, Germany) and coated with a layer of 1.4 nm gold/palladium (SEM auto-coating unit E2500; Polaron Equipment Ltd.; Watford, UK). The micrographs were obtained at 5.0 kV on a scanning electron microscope (JSM 840, Jeol; Eching, Germany).

2.7. Porosity estimation

Triglycerides loaded with 6% lysozyme were compressed under a range of different forces to achieve a controlled series of porosities, and cylinder height was measured (n = 3) using a digital sliding caliper (Absolute Digimatic CD-15CP, Mitutoyo, Andover, U.K). Porosity ε was calculated by the equation (1) adapted from Siegel et al. [29].

$$\varepsilon = \frac{(1-L)W}{\rho V} \quad (1)$$

L is the loading, and *W* the mass of a cylinder, *V* its volume calculated from the caliper measurements, and ρ the density of the matrix material (as given by the supplier).

2.8. Statistical analysis

All measurements were collected (n = 3) and expressed as means ± standard deviation (SD). Statistical significance was assessed by one-way analysis of variance (ANOVA) in conjunction with a multiple comparison test (Tukey test) at a level of $p<0.01$ (fig. 3) and with Dunnett's test (comparison versus control) at a level of $p<0.01$ (fig. 8).

3. Results and discussion

3.1. Influence of the wettability of the matrix material

The choice of triglyceride had a tremendous impact on the resulting release of the model protein lysozyme as can be seen in figure 1. The amount of protein released with time decreased in the same order as the fatty acid chain length in the matrix increased. Release lasted more than 14 months with the slope of the curves suggesting an ongoing lysozyme liberation even beyond the time range of the experiment.

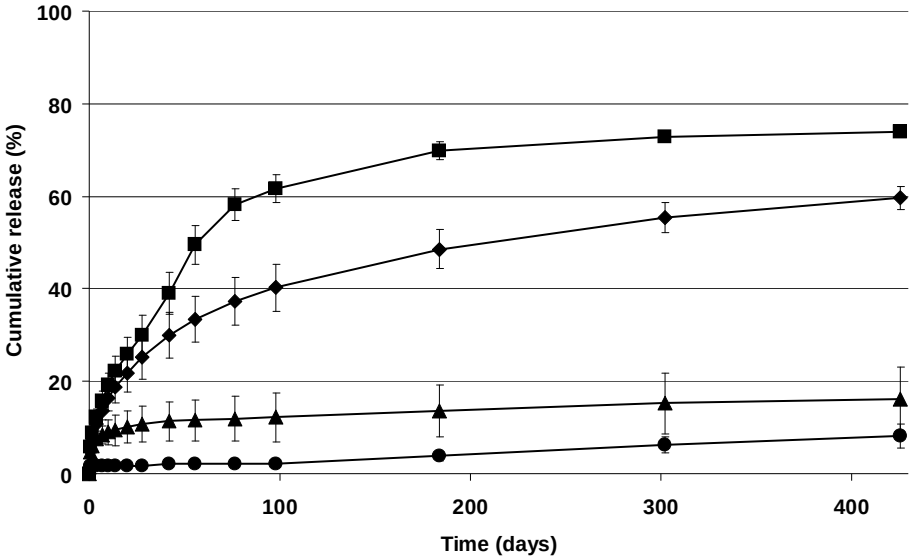

Fig. 1: In vitro release of lysozyme from triglyceride matrices loaded with 4% protein. Matrix material was either (■) glyceryl trilaurate, (♦) -trimyristate, (▲) -tripalmitate or (●) –tristearate.

To exclude possible effects of different porosities within the triglyceride matrices due to different compaction behavior, powder was compressed under a range of different pressures to achieve a controlled series of porosities. Release was indeed found to depend upon compaction force within the range of 50 to 500 N as can be seen in figure 2A. In general, a faster protein liberation was observed at lower compaction force for all investigated matrices. However, triglycerides behaved similarly on increased compression force by reduction in porosity (fig. 2B), and no tendency for an impact on the results of figure 1 could be deduced.

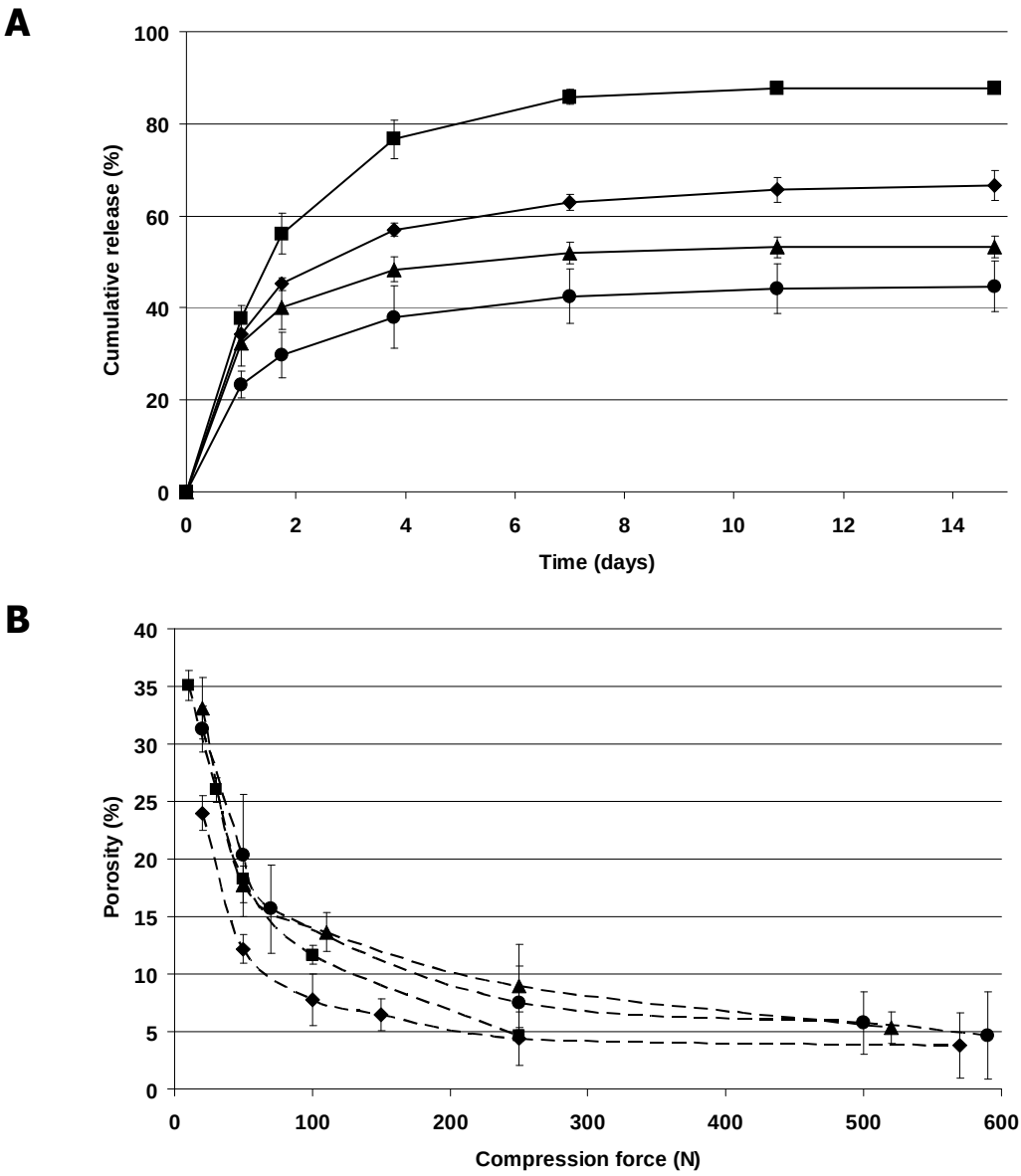

Fig. 2: ***(A)*** *Release profiles obtained for glyceryl trimyristate matrices loaded with 6% lysozyme compressed at (■) 50 N, (♦) 150 N, (▲) 250 N and (●) 500 N.* ***(B)*** *Influence of compression force on porosity of different triglycerides (6% lysozyme loading): (■) glyceryl trilaurate, (♦) –myristate, (▲) –palmitate and (●) –stearate.*

Therefore, the reason for the deviations in release behavior was sought in the different lipophilicity of the triglycerides, suggesting that wettability could be the key factor for explaining the observations described above.

The contact angle of a liquid on a given surface can be taken as a direct measure for wettability [30]. However, the determination of contact angles on compacts is impeded, because surface energy can change during compaction [30]; moreover, surface roughness and penetration of the wetting liquid into the matrix can distort the results. Therefore, lipids were cast as films from an organic solution [14, 31] in this study. In addition, results

from contact angle measurements were backed up by data from confocal imaging of the buffer penetration velocity into selected formulations (tab. 1).

Figure 3 shows the resulting contact angles of release buffer (black bars) on different triglycerides, which increase with longer fatty acid chain length of the lipid, indicating a reduction in wettability.

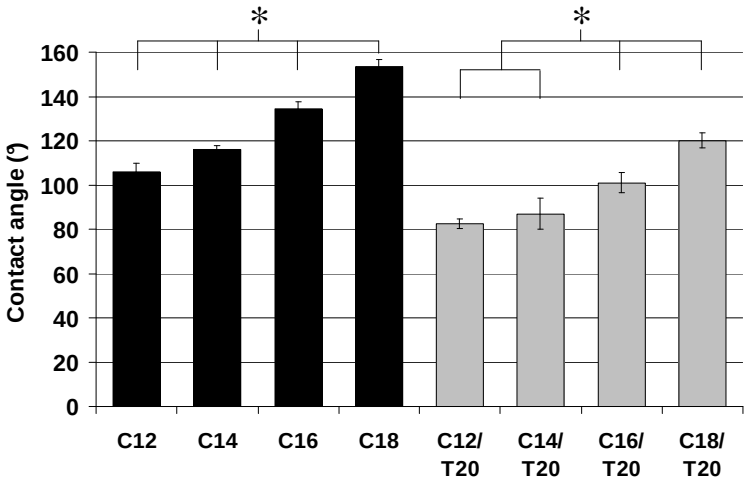

Fig. 3: Contact angle of phosphate buffer pH 7.4 (black bars) and buffer containing 0.1% Tween®20 (T20, grey bars) on glyceryl trilaurate (C12), -trimyristate (C14), -tripalmitate (C16) and –tristearate (C18), (∗) p<0.01 in statistical evaluation.

Generally, a contact angle above 90° can be regarded as poor wettability [30], where the use of surfactants in the release medium is an option to improve wetting of the pores in a release system [13, 15, 32-34]. In this context, 0.1% Tween®20 was added to the phosphate buffer and release was again determined from matrices loaded with 4% lysozyme resulting in the profiles depicted in figure 4. The time scale for the first matrices to reach more than 80% release was significantly reduced from 14 months to 14 days. However, the presence of surfactant could not compromise the differences between the triglycerides and again a sequential order according to fatty acid chain length was found both for release profiles (fig. 4) and contact angle measurements (fig. 3, grey bars), indicating that wetting of the matrices was still dependent on the triglyceride type even in the presence of surfactant.

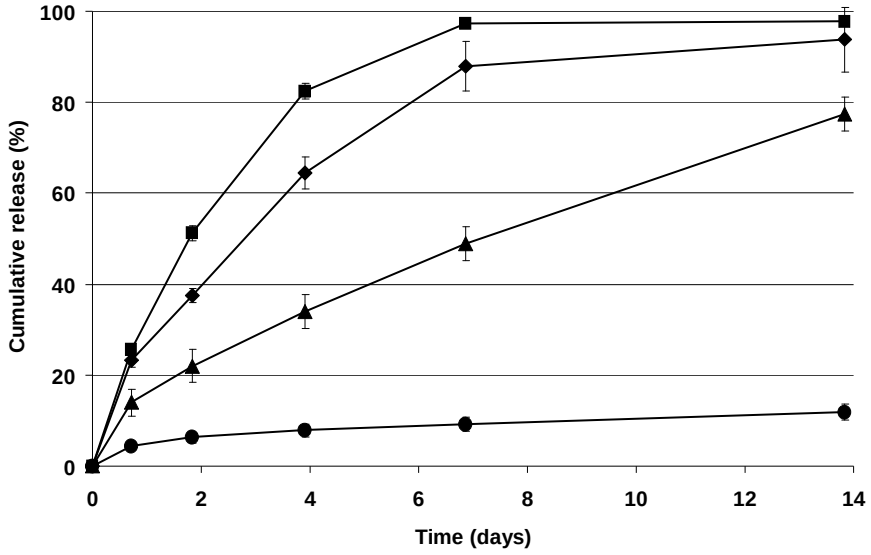

Fig. 4: In vitro release of lysozyme from triglyceride matrices consisting of (■) glyceryl trilaurate, (♦) -trimyristate, (▲) –tripalmitate, (●) –tristearate and 4% protein in phosphate buffer pH 7.4 containing 0.1% Tween®20.

To improve wetting by addition of a hydrophilic excipient to the matrix itself [21] instead of supplementing the release buffer, PEG was left in the formulation by omitting the centrifugation step after lyophilization. Thus, matrix behavior could be modified in a way, that all triglycerides reached close to 90% cumulative release in parallel after 14 months (fig. 5A). However, the role of PEG seemed to be more complicated than functioning as a mere wetting agent. Having a closer look at PEG and protein release during the first 6 months, surprisingly revealed an inverted order of the PEG release profiles (figs. 5A and B). In this time period, PEG release was slowest from glyceryl trilaurate, followed by -trimyristate, -palmitate and –stearate. Possibly, PEG is capable of interacting with the matrix material during matrix preparation and release, the better the more hydrophilic the triglyceride is. The slower PEG release may affect the liberation of lysozyme contrarily to the different matrix wettabilities in a way, that the resulting lysozyme release profiles from different triglycerides all turn out to be very similar.

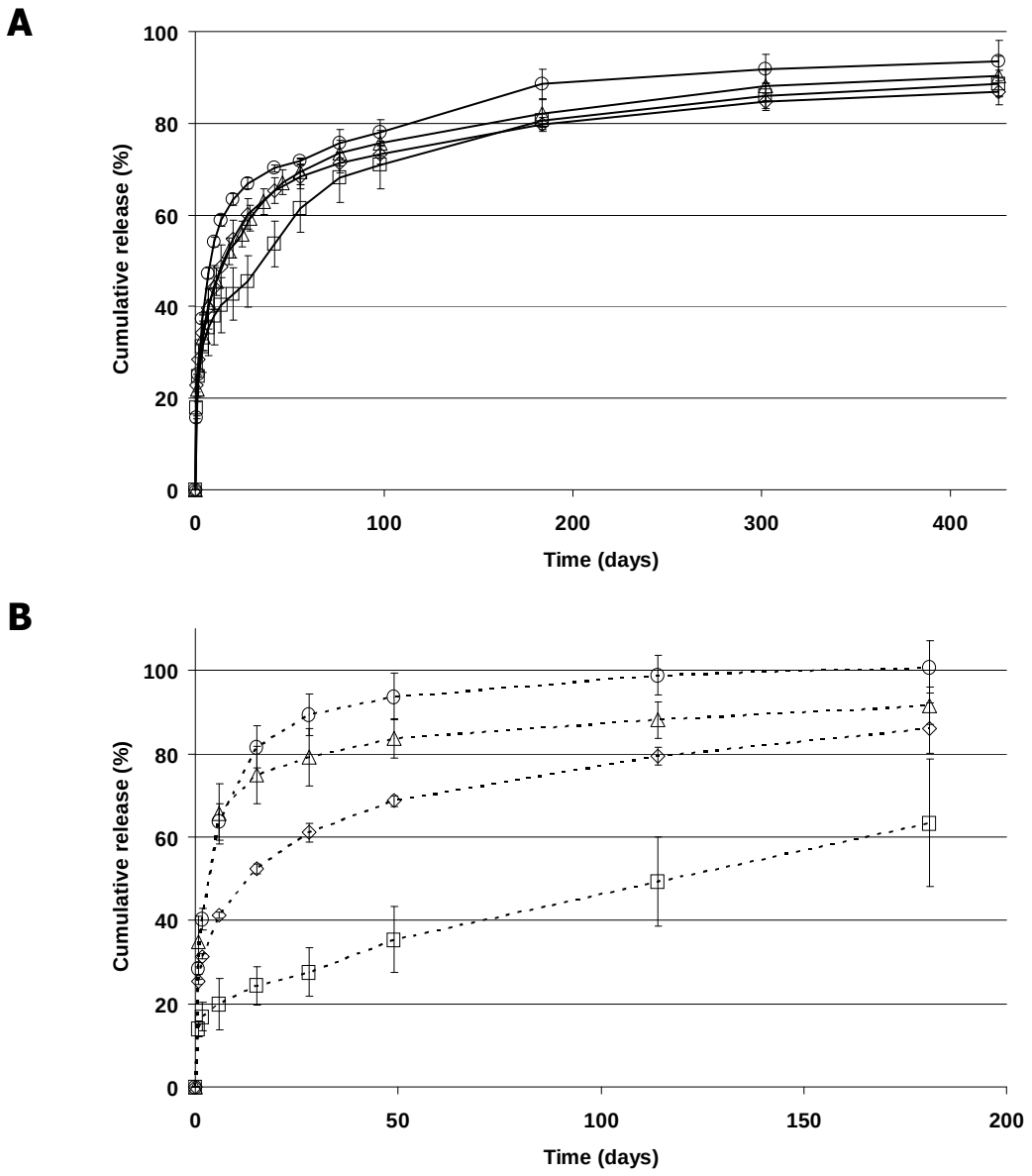

*Fig. 5: In vitro release of lysozyme **(A)** and PEG 6,000 **(B)** from triglyceride matrices loaded with 2% protein and 2% PEG 6000. Matrix material was either (□) glyceryl trilaurate, (◊) -trimyristate, (Δ) –tripalmitate or (○) -tristearate.*

Matrix penetration of release buffer can follow both dissolution of drug particles and capillary forces acting along the pores within the matrix, which draw the release liquid into the matrix [35]. The latter was described by Rosilio et al. [14] with the Washburn equation (2), which relates the distance L moved by the solvent front in the time t to the surface tension γ and the viscosity η of the liquid, the contact angle θ and the capillary radius R.

$$\frac{L^2}{t} = R\gamma\frac{(\cos\theta)}{2\eta} \quad (2)$$

For contact angles below 90°, cos θ is positive and drug release would occur due to buffer penetration by capillary forces. Contact angles above 90° result in negative values for cos θ, representing a negative capillary pressure, which would prevent liquid from entering the matrix. Contact angles determined for triglycerides (fig. 3) mainly ranged above 90°; despite this, buffer was able to penetrate the matrices as detected by confocal microscopy (tab. 1) and induce release.

Matrix composition		**Penetrating medium**	**Penetration depth (%) ± SD**	**Cumulative release (%) ± SD**
Glyceryl trilaurate	(250 N), 4% lysozyme	Release buffer	39.5 ± 2.1	19.3 ± 2.5
Glyceryl trimyristate	(250 N), 4% lysozyme	Release buffer	25.4 ± 2.9	16.3 ± 2.8
Glyceryl tripalmitate	(250 N), 4% lysozyme	Release buffer	20.1 ± 2.7	9.0 ± 2.6
Glyceryl tristearate	(250 N), 4% lysozyme	Release buffer	10.1 ± 1.7	1.7 ± 0.3
Glyceryl tripalmitate	(250 N), 2% lysozyme, 2% PEG	Release buffer	60.7 ± 4.5	45.5 ± 2.9
Glyceryl trimyristate	(250 N)	Release buffer	<1	-
		Buffer + 0.1% BSA	<1	-
		Buffer + 0.1% Tween®20	51.0 ± 1.9	-
		Buffer + 0.01% Tween®20	19.0 ± 2.4	-
Glyceryl trimyristate	(50 N)	Release buffer	5.5 ± 2.0	-
Glyceryl trimyristate	(250 N), 3% FITC-dextran, 3% PEG	Release buffer	4.2 ± 1.3	4.4 ± 0.5
		Buffer + 0.01% Tween®20	60.1 ± 2.7	60.4 ± 4.3
		Buffer + 0.01% Tween®81	34.6 ± 4.7	23.3 ± 0.8

Tab. 1: Buffer penetration depth (radial) measurements after 10 days (7 days for matrices with FITC-dextran) depending on different release media and matrix composition.

Cassie and Baxter have suggested that the cosine of the contact angle of a given surface would be the sum of the cosines of the different components [31]. Thus, proteins as surface active substances [36] might contribute actively to the reduction of buffer-lipid contact angle enabling further progression of the release buffer within the hydrophobic matrix.
In this context, it became highly interesting, how a model substance without inherent surface activity would behave within the system, when processed using the same manufacturing method.

3.2. Influence of the surface activity of the model substance

In a first step, lysozyme and FITC-dextran were incorporated into glyceryl trimyristate matrices and release compared for matrices compressed at 50 and 250 N. Figure 6 shows that both model substances were released quickly at low compression forces, indicating that model substance solubility and possible interactions with the matrix material were not hindering their release. A low compression force mainly increases pore size and air porosity, which accounts for the air entrapped within the matrix during compression and is independent of amount of drug. However, if compression force was increased to 250 N where porosity is mainly due to the space filled with drug (see fig. 2B), only lysozyme was released in a controlled fashion, while FITC-dextran remained trapped within the matrix; the small amount of substance detected in the buffer probably being due to the fraction dissolved at and directly underneath the cylinder surface. Both formulations contained 3% model substance and 3% PEG, which seemed to fail in acting as a porogen in the absence of surface active molecules.

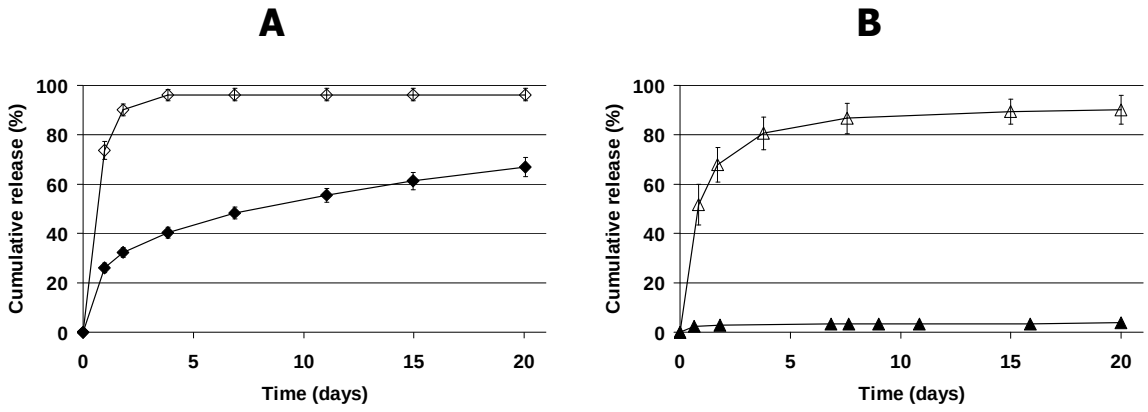

*Fig. 6: In vitro release of lysozyme **(A)** and FITC-dextran **(B)** from matrices compressed at 50 N (open symbols) and 250 N (closed symbols). Loading was 3% model substance, 3% PEG 6,000 in glyceryl trimyristate.*

Confocal microscopy revealed that buffer was not able to penetrate into matrices in the absence of any hydrophilic loading, neither at 50 nor at 250 N (tab. 1). In the presence of model substance and the excipient PEG, buffer was only found to penetrate into lysozyme loaded matrices compressed at 250 N whereas FITC-dextran matrices could not be intruded. This suggests that at low compression forces, buffer proceeds within the matrix by dissolving water-soluble drug, whereas at high compression forces, pore size is largely restricted and capillary effects play a significant role. Here, only the surface active protein is able to induce a wetting of the pores in a lipophilic matrix.

3.3. Role of surfactants

Solvent impenetrability of certain pores, which are below a critical minimum diameter has been related to the Washburn equation before [15] and can be overcome as described above by the addition of surfactant to reduce the contact angle and improve wetting. Figure 7A indicates, that FITC-dextran release could be triggered by the addition of surfactant to the release buffer at different time points resulting in fast and complete liberation of the matrix loading. A dependence of the slope of the release profile on surfactant concentration was observed during this experiment. To further investigate this effect, two polysorbate surfactants with different hydrophilic-lipophilic balance (HLB), Tween®20 (HLB 16.7) and Tween®81 (HLB 10) [37] were employed in different concentrations ranging from 0.001 to 0.1% in order to vary the contact angle of the release medium. When added to the release buffer from the beginning of the study (fig. 7B), the slope of the FITC-dextran release profile corresponded closely to the adjusted contact angle (fig. 8), a faster liberation being observed at better wetting of the matrices.

In parallel to surfactants, one experiment was conducted with 0.1% (w/v) BSA added to the release buffer. However, neither an increase in FITC-dextran liberation nor a lowering of the contact angle on glyceryl trimyristate was induced (figs. 7B, 8). It can be presumed, that conditions at the solvent front boundary within a lipid matrix loaded with protein would include higher concentrations than 0.1%, therefore, an attempt to assess contact angles at higher BSA concentrations (1 and 10%) was made, however lipid films were detached from the glass slides at contact with BSA solution, thus no measurement was possible. Presumably, protein can act its role in lowering the contact angle better when incorporated within the matrix and being present locally in high concentrations upon

contact with release medium, a phenomenon, which has also been observed for some surfactants [13, 38].

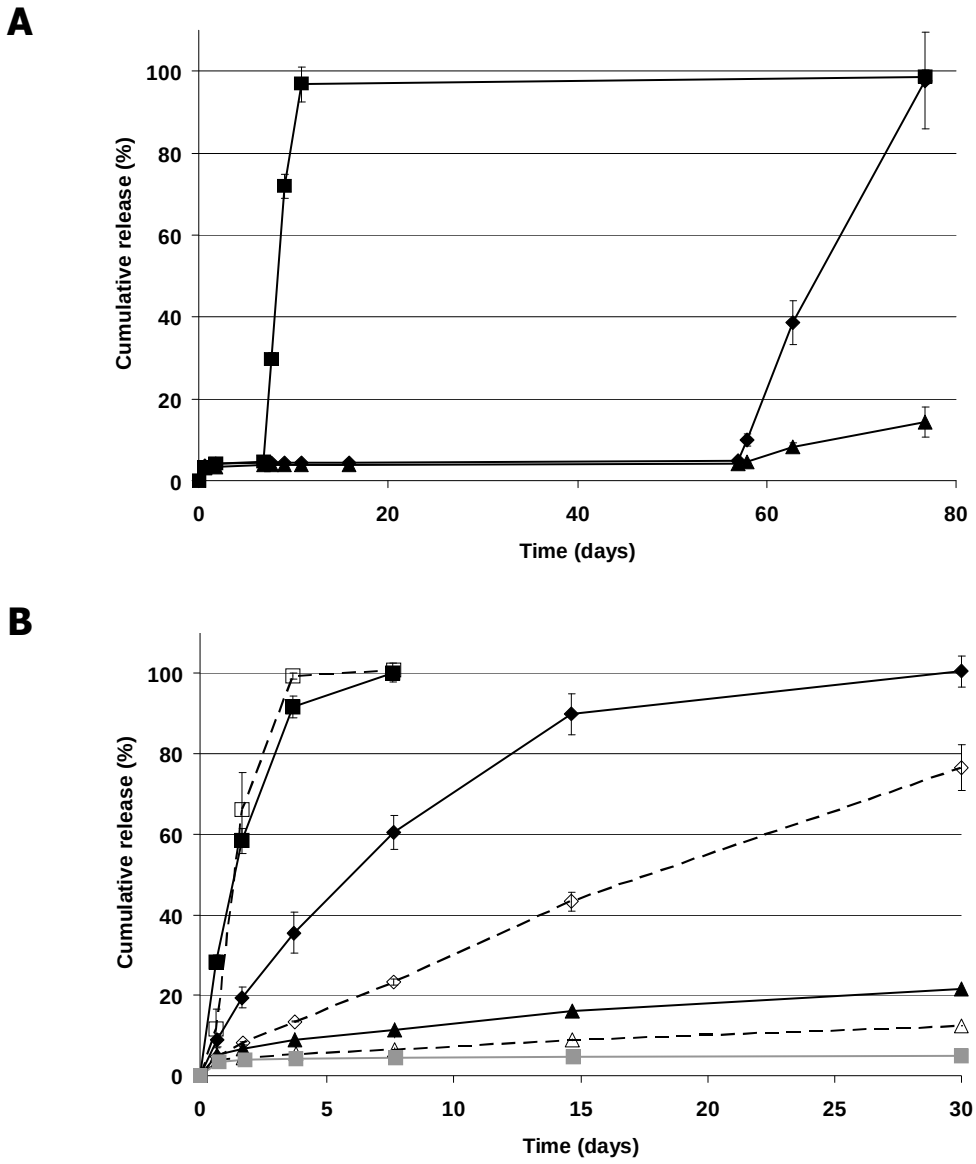

Fig. 7: In vitro release of FITC-dextran (M_w 4,000 Da) from glyceryl trimyristate matrices (loaded with 3% FITC-dextran, 3% PEG) incubated in phosphate buffer pH 7.4 containing Tween®20 (closed symbols) or 81 (open symbols) in concentrations of (■, □) 0.1 %, (♦, ◊) 0.01% or (▲, Δ) 0.001%. The grey curve results from incubation of matrices in buffer with 0.1% BSA. ***(A)*** *Surfactant was added to release buffer after 7 or 58 days.* ***(B)*** *Surfactant was contained in the release buffer from the beginning of the experiment.*

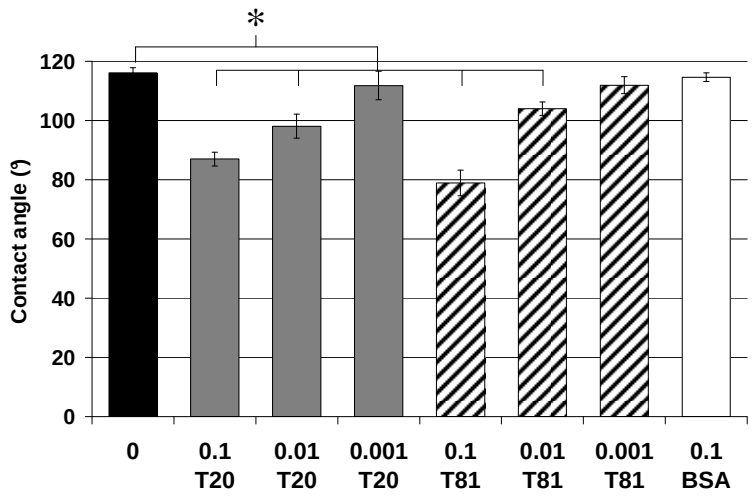

Fig. 8: Contact angle of phosphate buffer pH 7.4 on glyceryl trimyristate without additives (black bar), with 0.1, 0.01 or 0.001% Tween®20 (T20, grey bars), with 0.1, 0.01 or 0.001% Tween®81 (T81, hatched bars) and with 0.1% BSA (open bar); (∗) p<0.01 in statistical evaluation.

Results of lysozyme release from different triglycerides in buffer without and with 0.1% Tween®20 and FITC-dextran release in buffer with different concentrations of Tween®20, 81 and BSA were plotted against the corresponding contact angles in figure 9.

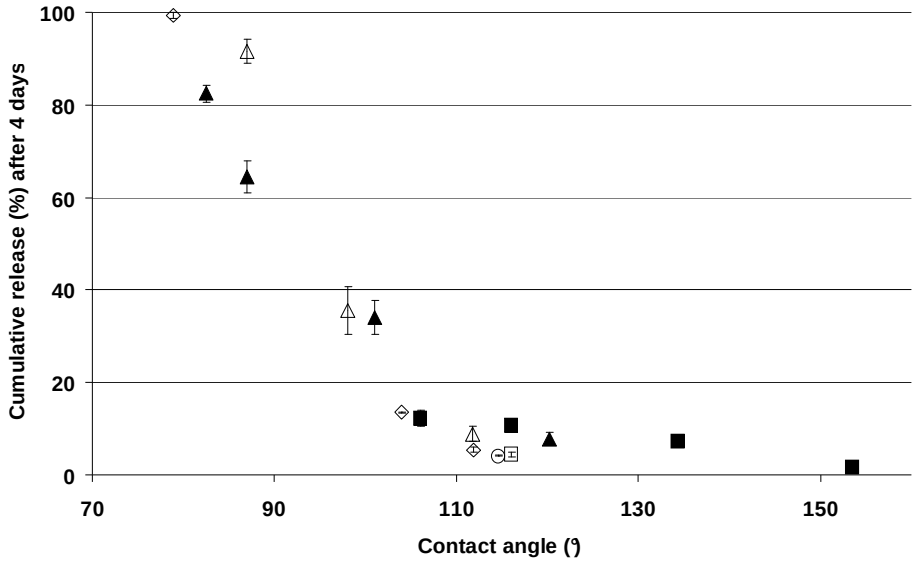

Fig. 9: Correlation between contact angle of phosphate buffer pH 7.4 on triglyceride films and the amount of released model substance after 4 days. Closed symbols represent lysozyme, open symbols FITC-dextran. Phosphate buffer contained no additives (■, □), Tween®20 (▲, Δ), Tween®81 (◊) or BSA (○).

Surface active effects of the protein appear to be negligible in the presence of surfactant and both lysozyme and FITC-dextran release show a close relationship to the contact angle

adjusted by surfactant. Confocal microscopy confirmed the dominant impact of surfactant, as buffer penetration in the presence of surfactant was found to be dependent only upon surfactant concentration (fig. 10) and did not rely on the presence of model substance in the matrices. The correlation for lysozyme release and contact angles in the absence of Tween® (fig. 9, black squares) deviates from the general trend observed in the presence of surfactants, showing release at surprisingly high contact angles (>130°). However, these contact angles were measured with buffer on pure triglyceride films. As discussed above, the surface activity of the protein probably lowers the contact angle locally when additionally incorporated into the triglyceride matrix, thus inducing release.

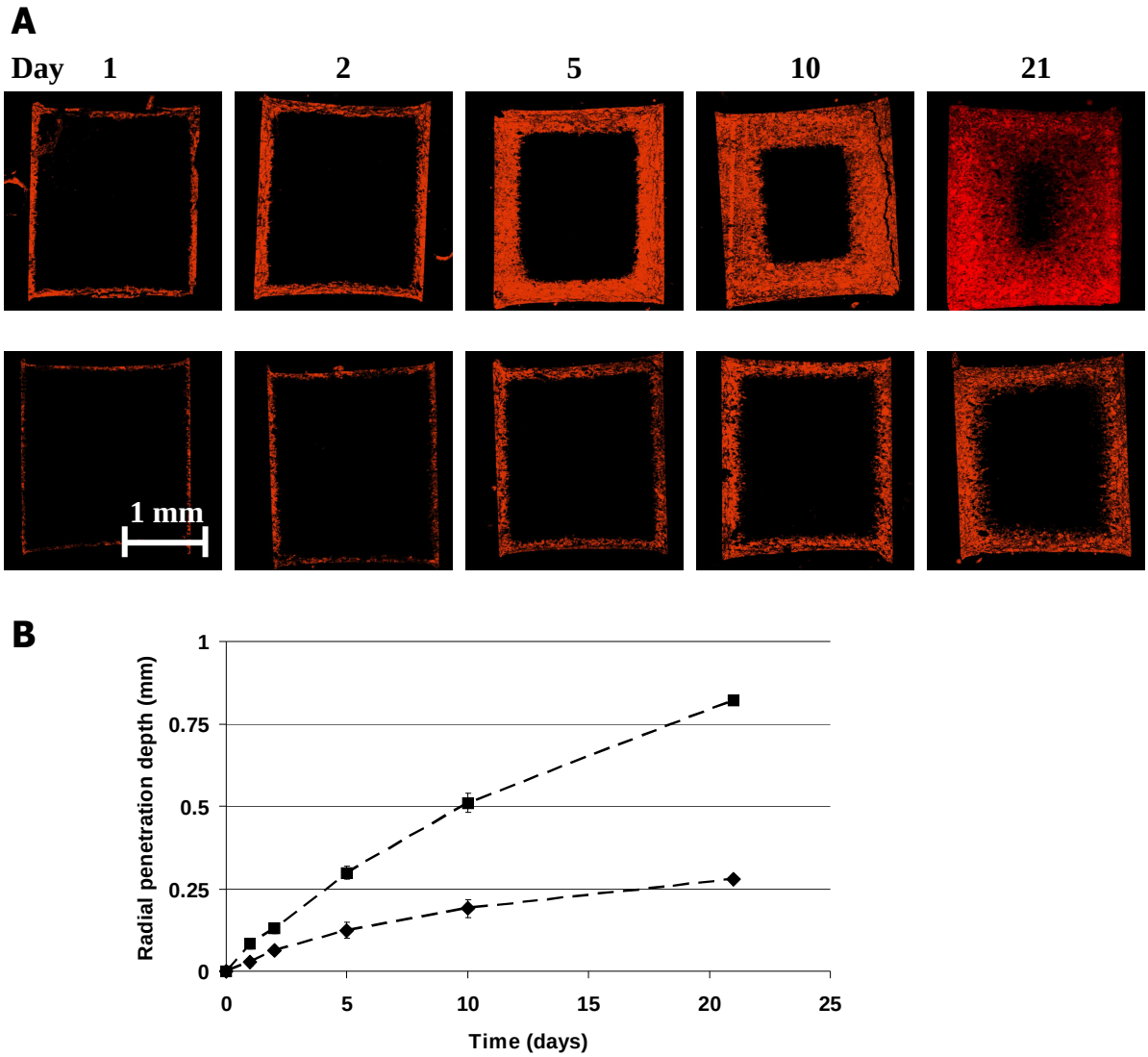

Fig.10: ***(A)*** *Penetration of phosphate buffer pH 7.4 containing 0.1% Tween®20 (upper row) or 0.01% Tween®20 (lower row) into pure glyceryl trimyristate matrices. Cross sections were taken after incubation for the number of the days indicated above.* ***(B)*** *Quantification of radial penetration depth from confocal images for buffer containing (■) 0.1% Tween®20 or (♦) 0.01% Tween®20.*

It is generally accepted that it is not essential for a surfactant to be present above its critical micelle concentration (CMC) to perform its function as a wetting agent [30]. In this study, the choice of concentrations included the use of Tween®20 at 0.1% (w/v), which is above its CMC as determined by ring tensiometry (data not shown). For reasons of better comparison of the two surfactants, Tween®81 was employed in the same concentrations, while its CMC was found to be at a higher concentration (data not shown). Solubilization effects on release behavior cannot be completely excluded at the higher surfactant concentrations, possibly also affecting water solubility of the lipid with Tween®20 being an oil in water surfactant. Thus, matrices were characterized by weight analysis and SEM before and after incubation.

At concentrations below its CMC, Tween®20, the surfactant with the higher HLB value induced a better wetting and buffer penetration. At a surfactant concentration of 0.1%, surprisingly Tween®81 led to faster release of FITC-dextran, which could be interpreted by the help of SEM images showing that Tween®81 obviously led to a disruption of the matrix at this concentration (fig. 11D). Both Tweens® seem to have slightly affected the surface structure of the lipid matrices (fig. 11 A-F, I) in comparison to cylinders incubated without surfactant (fig. 11H), where the surface appeared to be smooth. However, solubilization of the matrix material could be excluded as the weight loss during release corresponded exactly to the amount of model substance liberated in that time period for all experiments except for buffer containing 0.1% Tween®81 (data not shown). Having a HLB of 10, this surfactant is more lipophilic in comparison to Tween®20 and may interact with the lipid matrix material, which would explain the weight gain of approximately 7.7% (± 1.4) compared to the theoretical value after 100% model substance release. Interestingly, the cracks in the matrix mainly occur in the horizontal direction, which could indicate an effect of Tween®81 by local disruption of the interparticulate adhesion after compression, a mechanism, which was also discussed for different surfactants incorporated into Eudragits® [13, 38]. A loosening of the lipid flakes on the surface (fig. 11I) was also observed in general cylinder appearance after incubation in surfactant containing buffer for the other concentrations of both Tweens®. Thus, the effect of surfactants cannot only be exclusively explained by a better wetting, however, contact angle measurements can be a valuable indication for the expected release behavior.

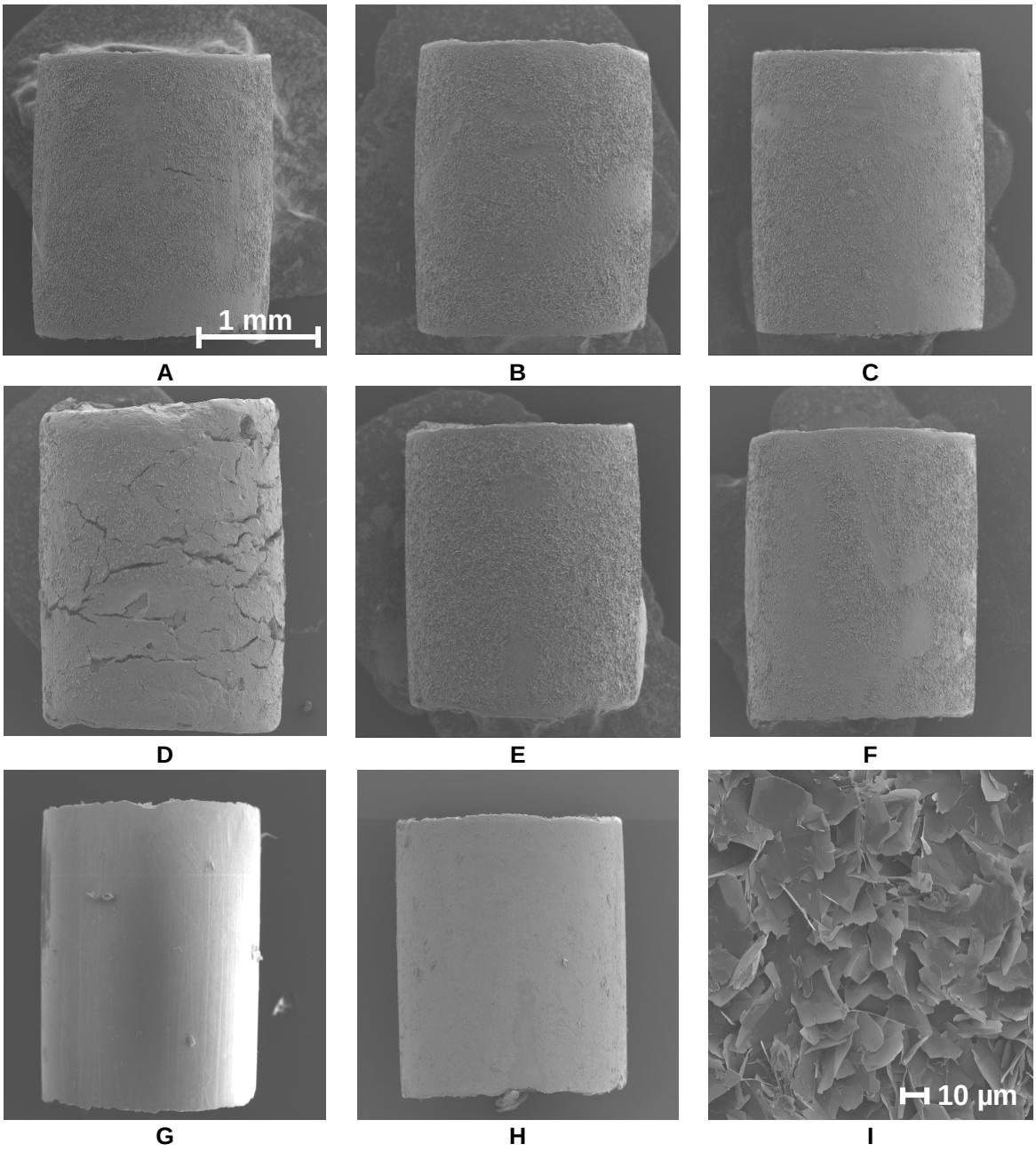

*Fig.11:Scanning electron microscopy images taken at 27× magnification of cylinders after incubation in release buffer containing Tween®20 (top row, **A-C**) or Tween®81 (middle row, **D-F**). Concentrations were 0.1, 0.01 and 0.001% (from left to right). The bottom row shows a cylinder before **(G)** and after **(H)** incubation in release buffer without additives. The last image **(I)** depicts a 500× magnification of the surface of matrices incubated with surfactant. Matrices were taken from the release experiment described under figure 7B, where time of incubation was 8 days (0.1% Tween®) and 30 days (all others).*

4. Conclusion

Wettability effects play a decisive role in release of high molecular weight model compounds from triglyceride matrices. Thereby, both matrix and drug properties are crucial for the resulting release behavior. For a protein, the release can be tailored by varying the lipophilicity of the matrix material via choosing different fatty acid chain lengths of the triglyceride. Contact angle measurements were valuable for predicting release behavior, however, incorporated proteins seem to induce buffer penetration even at contact angles above 90° by their inherent surface activity. FITC-dextrans remained trapped and depend upon the external addition of surfactant to the release buffer to induce wetting of the matrix pores. Surfactants can be added to the release buffer to tailor release profiles for both proteins and non-surface active drugs and can be suitable additives when planning to conduct release experiments at shorter time scales. However, data obtained at higher surfactant concentrations has to be interpreted with care, as they seem to act not only by reducing the buffer contact angle but also by affecting interparticulate adhesion of the matrix material.

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Chapter 7

Effect of Protein and Dextran Molecular Weight on Release from Triglyceride Matrices

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Abstract

Release from triglyceride matrices is mainly controlled by diffusion of drug molecules through aqueous pores created by the release buffer upon penetration into the matrix and dissolution of water soluble drug or porogen. It was the aim of this study to investigate effects of molecular weight on this process. Therefore, glyceryl trimyristate was loaded with model proteins of different molecular weight, namely lysozyme (14 kDa), trypsin (24 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), catalase (232 kDa) and thyroglobulin (690 kDa). A PEG co-lyophilization method was employed to produce protein-loaded lipid powder, which was compressed into cylinders of 2 mm diameter, 2.2 mm height and 7 mg weight. The same method was applied for a second group of model substances, fluorescein isothiocyanate labeled dextrans (FITC-dextrans), ranging in size from 4 to 2,000 kDa. Release was tested with addition of Tween®20 and 81 to the release buffer at 0.1, 0.01 and 0.001 % (m/v) for FITC-dextrans, ensuring a sufficient wetting, which was not necessary in the case of proteins. In all groups, a general tendency for slower release at higher molecular weight was observed. Deviations of protein release from this relationship could be explained with the help of enzyme activity assays for lysozyme, trypsin, alcohol dehydrogenase and catalase as well as SDS-PAGE after 14, 30 and 60 days, suggesting that stability problems might led to slower release by aggregation (ovalbumin), faster liberation by incompatibility with the excipient PEG (catalase) or tryptic self-digest (trypsin). The impact of size was more pronounced for proteins than for FITC-dextrans, however, a direct comparison is not appropriate due to the different release conditions and better matrix wetting by surfactants for FITC-dextrans.

1. Introduction

Over the past decade, the area of controlled release science has been confronted with an increasing number of potential therapeutic biomacromolecules, comprising proteins with a wide range of sizes and molecule properties [1]. Apart from matrix biocompatibility, reliable, gentle manufacturing strategies and preservation of protein stability, the overall aim is to design controlled release systems with the desired release properties. The investigation of lipids under these aspects has revealed a great potential of triglycerides for the controlled release of protein drugs [2]. Microparticles and mini-cylinders have successfully been formulated with bovine serum albumin (BSA), hyaluronidase [3], interferon α-2a (IFN α-2a) [4], lysozyme, brain derived neurotrophic factor (BDNF) [5] and interleukin-18 (IL-18) [6]. So far, proteins have been chosen either for their therapeutic potential (IL-18, BDNF) or their good availability and generally accepted use as model drugs, irrespective of their individually different molecule characteristics, such as solubility, isoelectric point, structure and size. However, in the interest of device design, it is highly important to know, how deviations in molecule properties might influence the release profile of a drug delivery system. Very little effort has been dedicated to this problem so far, and first approaches to classify the effects of size on release behavior, attempted for polymeric carriers, have gained controversial results, probably due to differences in design, drug loading and release mechanism [7-9]. In parallel studies, we have demonstrated by the help of confocal microscopy, that release from triglyceride matrices is a diffusion controlled mechanism, with diffusing drug molecules following water filled pores created upon buffer penetration and dissolution of drug and excipients [10, 11]. As drug diffusivity is dependent on molecular weight [12], we strived to further characterize our delivery system by systematically investigating a wider range of model drugs. To this end, a choice of protein model substances differing in size from lysozyme (M_w 14 kDa), trypsin (M_w 24 kDa), chicken egg albumin (ovalbumin, M_w 45 kDa), bovine serum albumin (BSA, M_w 66 kDa), alcohol dehydrogenase (M_w 150 kDa) and catalase (M_w 232 kDa) to thyroglobulin (M_w 690 kDa) were incorporated into glyceryl trimyristate matrices by a PEG co-lyophilization method previously developed by our group [6]. As this choice comprises molecules, which do not only differ by their molecular weight, but also display different stabilities in solution, fluorescein isothiocyanate (FITC) labeled dextrans were evaluated as a second group of model substances. Their similarity to proteins concerning the phase separating behavior in the presence of PEG allowed to use

the same manufacturing procedure [10], while circumventing other potential impact factors such as degradation or aggregation in aqueous environment. A size range of 4 to 2,000 kDa was selected in order to fall into the same range of hydrodynamic radii as the proteins [13].

2. Materials and methods

2.1. Materials

2.1.1. Model drugs

Chicken egg lysozyme (Grade I, M_w 14 kDa), bovine pancreatic trypsin (Type I, M_w 24 kDa), chicken egg albumin (ovalbumin, Grade V, M_w 45 kDa), bovine serum albumin (BSA, Cohn fraction V, M_w 66 kDa), Bakers yeast alcohol dehydrogenase (ADH, M_w 150 kDa), bovine liver catalase (M_w 232 kDa), bovine thyroid thyroglobulin (M_w 690 kDa) and FITC-dextrans (M_w 4, 20, 40, 70, 150, 500 and 2,000 kDa) were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany).

2.1.2. Reagents

Sigma-Aldrich Chemical Company (Steinheim, Germany) supplied bicinchoninic acid disodium salt (BCA), *Micrococcus lysodeikticus*, N_α-benzoyl-L-arginine ethyl ester (BAEE), β-nicotinamide adenine dinucleotide (β-NAD) and hydrogen peroxide solution, 30% (w/v). Polyethylene glycol (PEG, M_w 6 kDa), tetrahydrofurane (THF) and Coomassie® Brilliant Blue G-250 were purchased from Fluka (Buchs, Switzerland). Copper-(II)-sulfate pentahydrate, sodium azide, ammonium sulfate, orthophosphoric acid 85%, isopropylic alcohol and all buffer salts in analytical grade were bought from Merck (Darmstadt, Germany). Fluorescein disodium salt hydrate was obtained from Lancaster Synthesis (Morecambe, England). Unstained SDS-PAGE protein marker (6.5-200 kDa), and all other reagents for SDS-PAGE were from Serva (Heidelberg, Germany). Tween®20 and 81 were obtained from ICI Specialty Chemicals (Essen, Germany). Glyceryl trimyristate (Dynasan®114) was a gift from Sasol (Witten, Germany).

2.2. Preparation of protein-loaded lipid matrices

Model drug-loaded lipid powder suitable for compression was obtained by a PEG co-lyophilization method previously described by our group [6]. In brief, 10 mg of the model substance (either protein or FITC-dextran) were dissolved in 2 ml of an aqueous solution of 10 mg PEG 6,000. After freezing the mixture in liquid nitrogen, it was lyophilized at 6°C and 0.12 mbar for 30 hours in a benchtop freeze-dryer (Beta 2-16 with LMC-2 system control, Christ, Osterode, Germany). The PEG fraction of the lyophilisate

was subsequently dissolved by addition of 1.5 ml THF after vacuum removal resulting in a dispersion of solid protein microparticles. PEG was either separated by centrifugation of the solid protein particles, removal of the supernatant and repeated washing with new THF, or left within the formulation. Glyceryl trimyristate (315 mg or 160 mg for formulations with and without PEG respectively) was added as a powder and dissolved by applying ultrasound for 5 seconds (5 W, Digital Sonifier 250-D, 3 mm microtip, Branson, Frankfurt, Germany). After freezing in liquid nitrogen, organic solvent was removed from the mixture under a vacuum of 6×10^{-3}mbar for 20 hours (Two stage High vacuum Pump E2M5, Edwards, Crawley, UK) and the resulting dry powder was ground in an agate mortar. Cylindrical matrices of 2 mm diameter, 2.2 mm height and 7 mg weight were prepared from the model drug-loaded lipid powder by manual compression in a custom-designed compression tool made of hardened steel [14]. Compression force was controlled at 250 N for 10 seconds by a Perkin-Elmer hydraulic press (Perkin-Elmer, Ueberlingen, Germany). Matrices were weighed on an analytical balance to determine their exact weight prior to release studies.

2.3. In vitro release study

All vials were treated with Sigmacote® prior to use to prevent the adsorption of protein. Matrices were incubated in 1.5 ml phosphate buffer pH 7.4 supplemented with 0.01% sodium azide at 37°C. Buffer was replaced completely after each sampling interval and stored at -20°C until further analytics.

2.4. Quantification of model substance release

2.4.1. Protein

100 µl bicinchoninic acid disodium salt (BCA) Micro-Working Reagent (mixture of 4% copper-(II)-sulfate pentahydrate, 4% BCA, and disodium tartrate dihydrate/ sodium carbonate buffer pH 11.25 in a volume ratio of 1:25:26) were added to 100 µl buffer containing released protein in a 96-well plate. The covered well-plate was incubated at 60°C for 1 hour as described in Smith et al. [15] and absorption was measured after cooling to room temperature at 562 nm using a 96-well plate reader (CS-9301 PC, Shimadzu, Duisburg, Germany). Protein concentration was calculated from standard curves obtained for known concentrations of each protein individually.

2.4.2. Polyethylene glycol

The use of an aqueous two-phase partitioning of fluorescein for the quantification of PEG in solution is described in Guermant et al. [16]. Briefly, a working solution was prepared from ammonium sulfate dissolved in 0.5 M sodium acetate buffer pH 5 (final concentration 31%, w/v), and fluorescein disodium salt hydrate (final concentration 77 µM). 300 µl of that working solution were added to 40 µl sample and allowed to stand in the dark for 30 minutes at room temperature before measurement of absorption at 475 nm. A known quantity of PEG 6000 was dissolved and diluted to obtain standard curves in the presence and absence of co-dissolved protein.

2.4.3. FITC-dextran

The amount of released FITC-dextran in the release buffer was determined fluorimetrically (spectrofluorophotometer RF-1501, Shimadzu, Duisburg, Germany) by measuring the emission of sample or standard solution diluted with water at 520 nm when excited at 490 nm. A standard curve was required for each molecular weight of FITC-dextran individually.

2.5. Protein characterization

2.5.1. Enzyme activity assays

Enzymatic activities of lysozyme, trypsin, ADH and catalase were determined in release buffer during the first 7 days of release and from encapsulated protein at selected time points. To that purpose, protein was extracted from freeze-dried matrices directly after preparation and after release for 14, 30 and 60 days by reversing the matrix preparation procedure: the model substance containing lipid cylinder was dissolved in 2 ml THF by shortly applying ultrasound (5 sec, 5 W). Protein was isolated by centrifugation (centrifuge 5415R, 12,000 rpm, 10 min, Eppendorf, Wesseling-Berzdorf, Germany) and removal of the supernatant containing triglyceride. The procedure was repeated twice and solvent traces were removed afterwards under vacuum for 2 hours (Two stage High vacuum Pump E2M5, 6×10^{-3} mbar, Edwards, Crawley, UK). Standard curves were freshly prepared from lyophilized enzymes dissolved shortly before assay performance.

Lysozyme activity was assessed as described by Shugar et al. [17] by monitoring the decrease in absorbance due to hydrolysis of a 0.015% (w/v) *Micrococcus lysodeikticus* suspension in 66 mM potassium phosphate buffer pH 6.24. 2.5 ml of that suspension were

added to 100 µl of lysozyme containing sample or standard and the absorption at 450 nm was measured for 2 minutes.

For assaying the activity of trypsin according to Bergmeyer [18], a solution of 0.25 mM BAEE in 67 mM sodium phosphate buffer, pH 7.6, was prepared as a substrate. Ester hydrolysis leads to the liberation of ethanol, and the resulting increase in absorption at 253 nm was followed for 2 minutes after mixing 200 µl sample or standard and 3 ml substrate solution.

Alcohol dehydrogenase catalyses the reduction of β-NAD by ethanol. A substrate mixture of 1.5 ml 15 mM β-NAD solution, 100 µl 95 % (v/v) ethanol and 1.3 ml 50 mM sodium pyrophosphate buffer, pH 8.8. was prepared according to Kagi and Vallee [19] and added to 100 µl enzyme solution. After mixing by inversion, the increase in absorption at 340 nm was observed for 2 minutes.

When catalase solution is incubated with 0.036% (w/w) hydrogen peroxide dilution in 50 mM potassium phosphate buffer pH 7, the formation of water and oxygen leads to an increase in absorption at 240 nm [20]. This was used to determine enzyme concentration of either 100 µl sample or standard by adding 2.9 ml substrate solution and UV measurement for 2 minutes.

The initial kinetic rate for all enzymes was calculated from the slope of the linear part of the curve for each sample and the concentration of active enzyme deducted from a fresh standard curve. Assay procedures were based on the protocols provided by the supplier and the given references.

2.5.2. SDS – polyacrylamide gel electrophoresis (SDS-PAGE)

Apparent molecular weight of incorporated protein was determined directly after matrix preparation and after 14, 30 and 60 days of release. Protein was extracted from matrices as described above. SDS-PAGE was performed under reducing and non-reducing conditions on discontinuous stacking (5%) and separating gels (7, 10 or 12.5% depending on molecular weight) using a Mighty Small II SE 250 unit (Hoefer Scientific Instruments, San Francisco, USA). Electrophoresis was run in a constant current mode of 30 mA and gels were stained by a procedure described in Westermeier [21] using colloidal Coomassie® Brilliant Blue G-250.

2.5.3. Size determination

PEG co-lyophilization was performed as described above; PEG was removed by dissolution in THF and solid particles were obtained by repeated centrifugation, solvent removal and exchange for isopropylic alcohol. Particle size was determined in isopropylic alcohol by laser diffraction (Mastersizer 2000, Malvern Instruments, UK).

2.6. Statistical analysis

All measurements were made in triplicate and expressed as means ± standard deviation (SD). Statistical significance was assessed by one-way analysis of variance (ANOVA) in conjunction with Dunnett's test (comparison versus control) at a level of $p < 0.01$ (fig. 4).

3. Results

3.1. Protein model drugs

Seven model proteins were chosen according to their different molecular weight and were incorporated into glyceryl trimyristate matrices by PEG co-lyophilization. This method is based on an aqueous phase separation step during freeze-drying [22], and led to the formation of protein microparticles, of which more than 85% were smaller than 10 µm. The release profile was different for each protein, with a clear tendency towards faster release at lower molecular weight (fig. 1A). The release profile of ovalbumin deviated distinctly from that trend, and a surprisingly low release was measured.

When half of the loading was composed of PEG, the same trend could be observed, however, release was slightly increased for all proteins except for thyroglobulin (fig. 1B). Again, ovalbumin release was lower than expected. In contrast, trypsin and catalase release were higher than would be expected from their molecular weight.

The velocity of PEG release from matrices with mixed loading was governed by the co-incorporated model protein (fig. 1C). While for lysozyme, trypsin, BSA and ADH, PEG was found to be released in parallel to the protein; release was slightly higher than thyroglobulin liberation and significantly exceeded release of ovalbumin and catalase (fig. 1, 7B). In the case of catalase, PEG- containing matrices started to show fissures after 2 days of release.

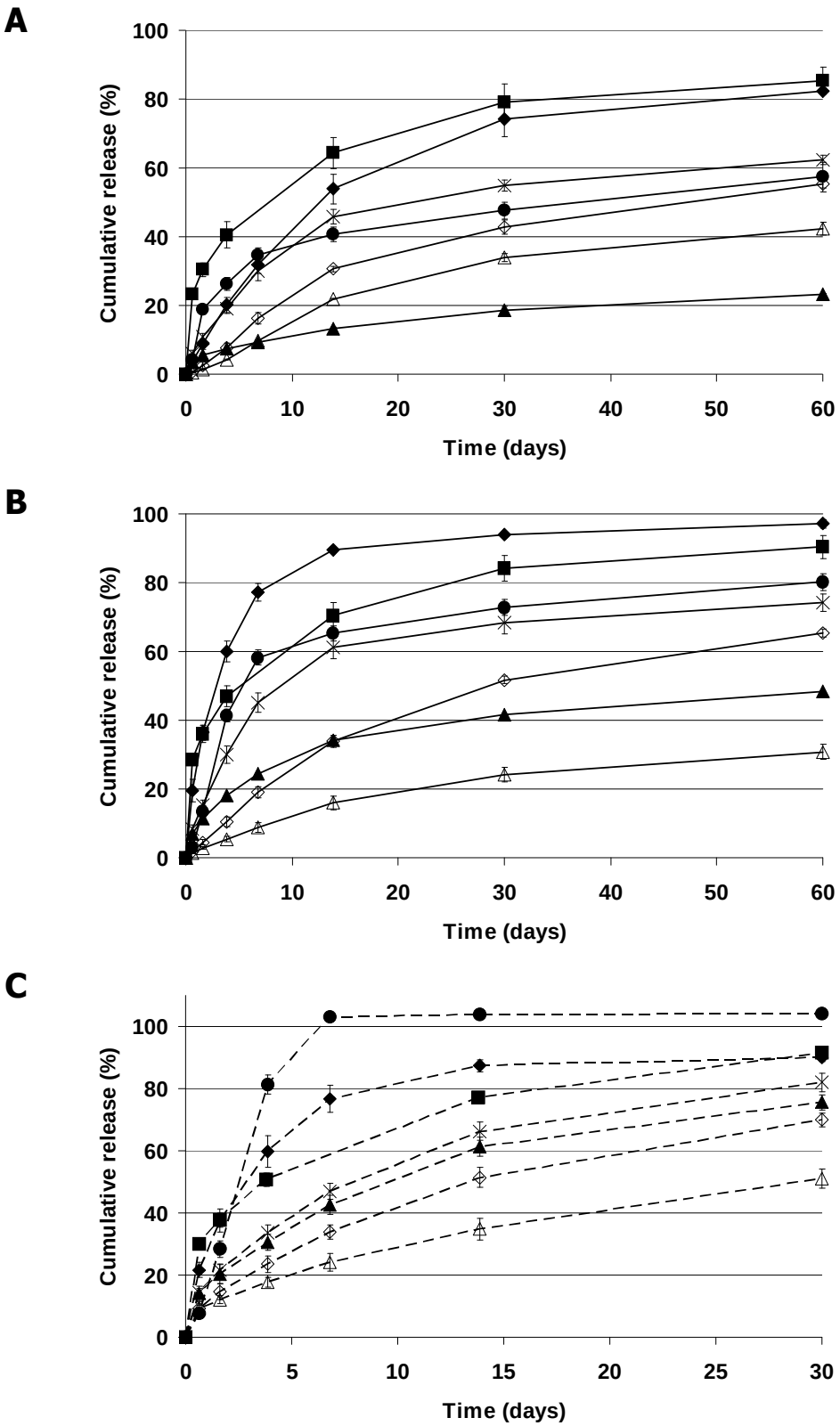

Fig. 1: ***(A)*** *In vitro release of protein from glyceryl trimyristate matrices loaded with 6% protein.* ***(B, C)*** *Matrices loaded with 3% protein and 3% PEG:* ***(B)*** *Protein release,* ***(C)*** *PEG release. Proteins incorporated were (■) lysozyme (14 kDa), (♦) trypsin (24 kDa), (▲) ovalbumin (45 kDa), (×) BSA (66 kDa), (◊) ADH (150 kDa), (●) catalase (232 kDa) and (Δ) thyroglobulin (690 kDa).*

At different time points of the study, the apparent molecular weight of incorporated protein was assessed by SDS-PAGE. Directly after preparation of the matrices, protein extracted from the matrices showed identical bands to untreated bulk substance. Only in the case of ADH, bands at lower molecular weight were slightly more distinct after preparation than for the bulk (fig. 2). No differences were observed between matrices containing only protein and matrices with mixed protein-PEG loading.

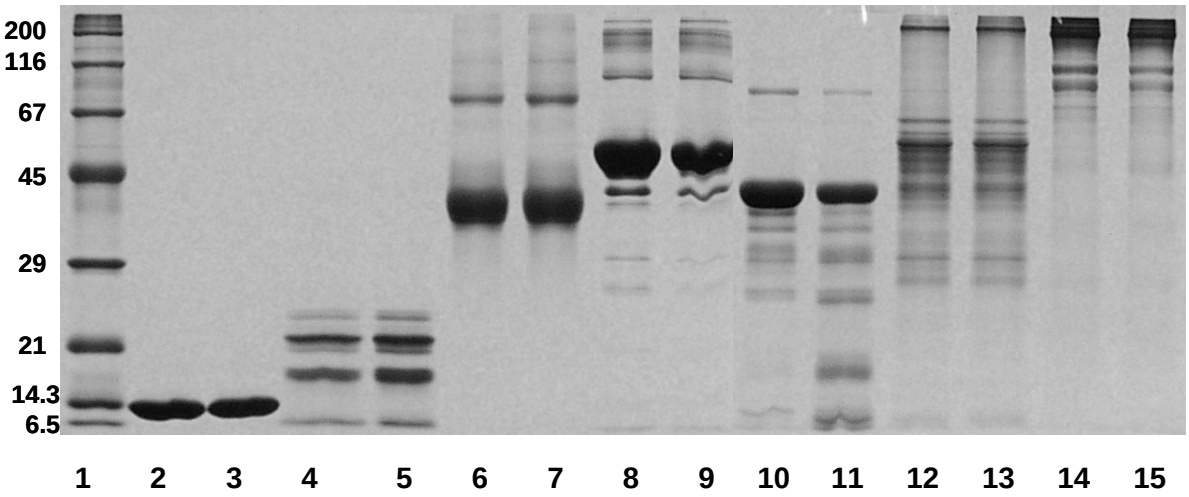

Fig. 2: SDS-PAGE comparison (12.5% separating gel, non-reducing conditions) of protein bulk (left lane) and protein after incorporation at 6% into glyceryl trimyristate (right lane). Lane 1: marker (M_w in kDa indicated on the left); lanes 2, 3: lysozyme; lanes 4, 5: trypsin; lanes 6, 7: ovalbumin; lanes 8, 9: BSA; lanes 10, 11: ADH; lanes 12, 13: catalase and lanes 14, 15: thyroglobulin.

While no changes could be detected for lysozyme extracted after 14, 30 and 60 days of release, a moderate tendency towards aggregation at longer incubation times was found for the other proteins, being most distinct for ovalbumin (data not shown). In addition, trypsin molecular weight was found to decrease over time (fig. 3). While after 14 days of release, bands for matrices with pure protein loading were identical to the ones found directly after preparation procedure (lane 3 and 7), trypsin from matrices containing PEG already showed a shift towards fragments with a molecular weight below 6.5 kDa (lanes 2 and 6). After 30 days of release, no bands were found in either preparation corresponding to the original composition of trypsin. In the presence of PEG, some faint bands indicating aggregates were found (lanes 4 and 8), while the rest of the extracted protein seems to have a molecular weight below 6.5 kDa for both preparations. In the case of ADH, catalase and thyroglobulin, also some lower molecular weight fragments appeared after incubation in release buffer for the said time periods (data not shown).

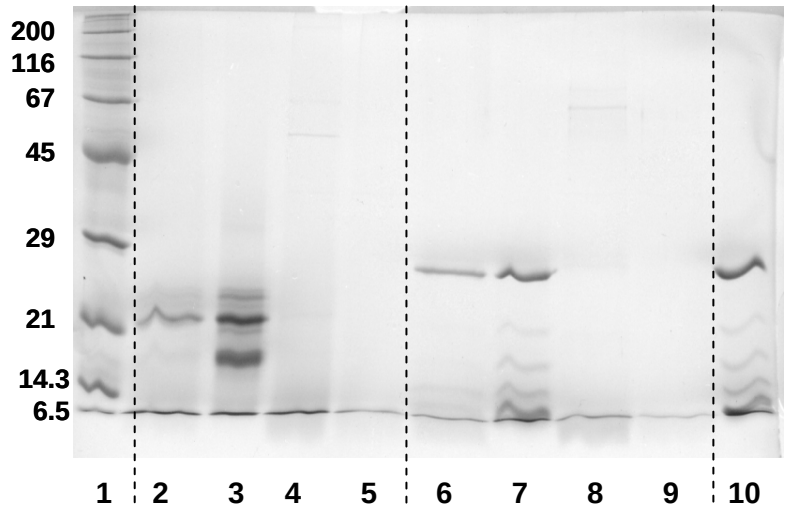

Fig. 3: SDS-PAGE analysis (12.5% separating gel) of trypsin under non-reducing (lane 2-5) and reducing (lane 6-10) conditions. Lane 1, marker (M_w in kDa indicated on the left), lane 10 trypsin bulk material. Trypsin was extracted from matrices after 14 days (lane 2, 3 and 6, 7) and 30 days (lane 4, 5 and 8, 9). Matrices contained either 3% protein/ 3% PEG (left lane, 2, 4, 6 and 8) or 6% protein (right lane, 3, 5, 7 and 9).

Enzyme activity was measured for lysozyme, trypsin, ADH and catalase in the release buffer during the first 7 days of release or directly after preparation of the matrices and after 14, 30 and 60 days of release by extraction of the matrices (fig. 4).

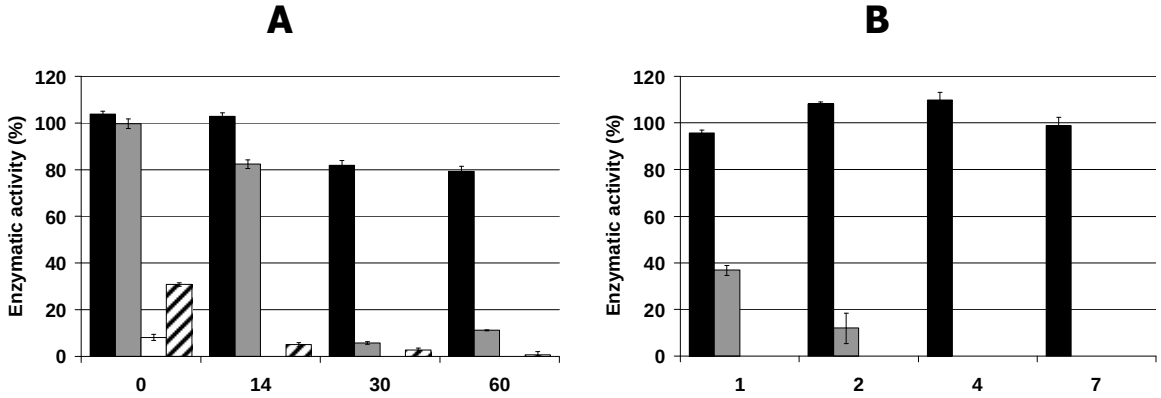

*Fig. 4: Enzymatic activity of lysozyme (black), trypsin, (grey), ADH (white) and catalase (hatched) **(A)** extracted from matrices after incorporation at 6% into glyceryl trimyristate matrices (0) and after incubation of matrices for 14, 30 and 60 days. **(B)** Enzyme activity in the release buffer after 1, 2, 4 and 7 days of incubation (decrease in activity statistically significant for all enzymes on a $p<0.01$ level except for lysozyme extracted after 14 days).*

While trypsin and lysozyme activity was completely retained after the manufacturing procedure, only 30 and 8% active enzyme were measured in the case of catalase and ADH, respectively (fig. 4A). Lysozyme released to the buffer was fully active, whereas trypsin

activity in the release buffer was diminished, and no activity was detectable for the two other enzymes (fig. 4B). Of the samples extracted from matrices after different time periods, again lysozyme and trypsin were most stable with more than 80% remaining activity after 14 days. Other proteins and later time points revealed less than 10% remaining activity with only lysozyme retaining more than 80% lytic potential (fig. 4A). The decrease of activity of trypsin, ADH and catalase in release buffer and extracted from the matrix after incubation was even more pronounced in the presence of PEG in the matrices resulting in approximately half the values measured in the absence of the excipient. Lysozyme, however was not influenced by co-incorporated PEG (data not shown).

3.2. Dextran model drugs

The performance of FITC-dextrans was assessed in the presence of surfactant. This was necessary as the matrices released no more than 5% during the first 4 days of the experiment, neither in the absence nor in the presence of co-incorporated PEG.. Afterwards, the loading remained trapped inside the matrices for the time period examined (57 days) independent of molecular weight (results not shown). With surfactant added to the phosphate buffer, FITC-dextran release was dependent on the Tween® type and concentration: at 0.01% and 0.001% (m/v) concentration, Tween®20 induced higher release rates than Tween®81 (fig. 5); at 0.1% both surfactants had a similar effect. The presence of surfactant did not suppress the influence of dextran molecular weight; still a tendency towards slower release at higher molecular weight was found. However, this trend was less distinct in the case of release buffer containing high (0.1%) and low (0.001%) amounts of Tween®81. In experiments with 0.1% Tween®81, matrices started to display fissures after 1 day of incubation in the release buffer.

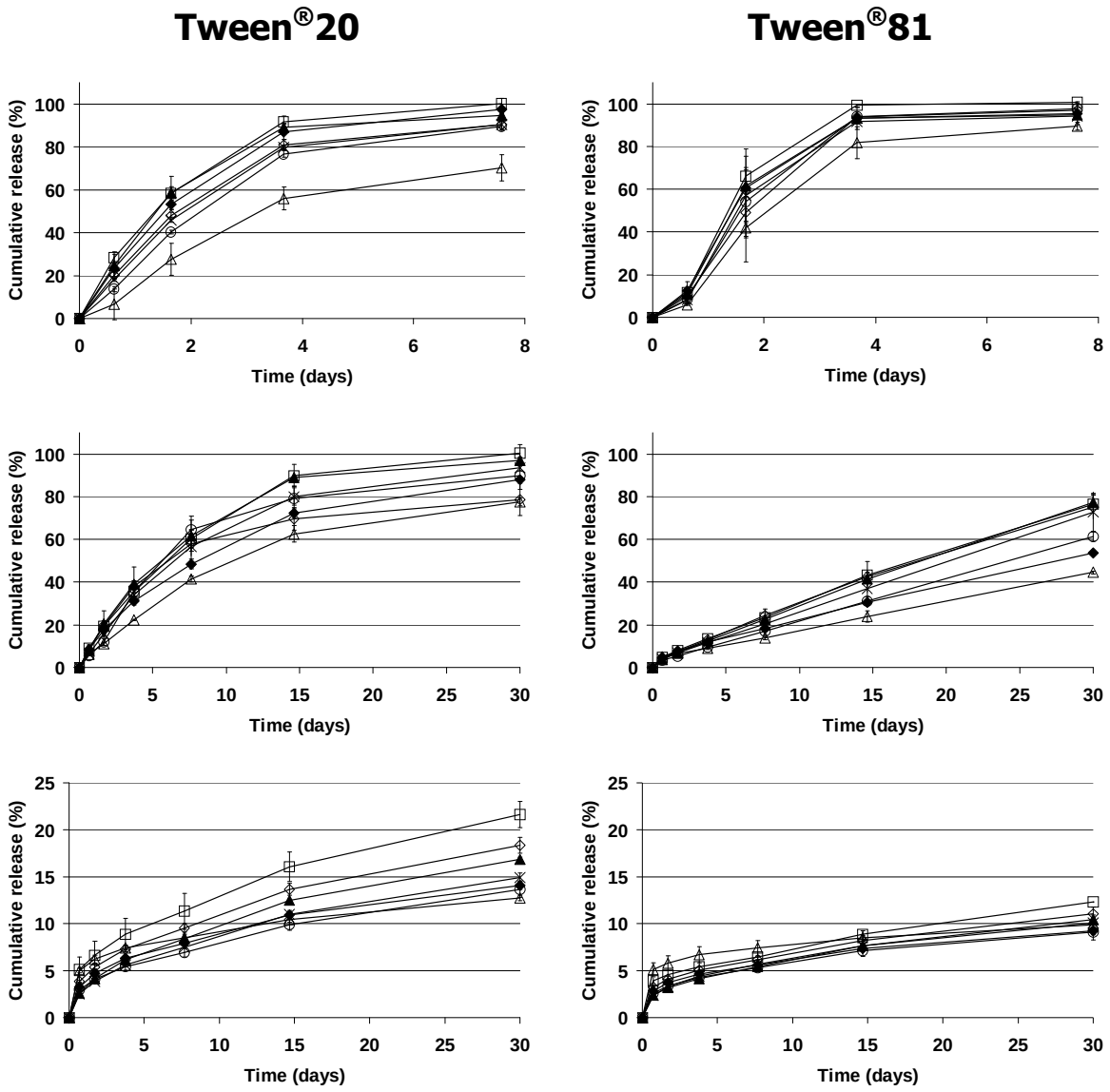

Fig. 5: In vitro release of FITC-dextrans with different M_w from glyceryl trimyristate matrices (6% loading) incubated in phosphate buffer pH 7.4 containing 0.1% (top), 0.01% (middle) or 0.001% (bottom) surfactant. Molecular weight was (□) 4, (♦) 20, (▲) 40, (×) 70, (◊) 150, (○) 500 or (Δ) 2,000 kDa.

3.3. Correlation of release data with size of model proteins and dextrans

As molecule size does not linearly increase with molecular weight for both proteins and dextrans (fig. 6), release data after 30 days was correlated with the hydrodynamic radius of the substances (fig. 7).

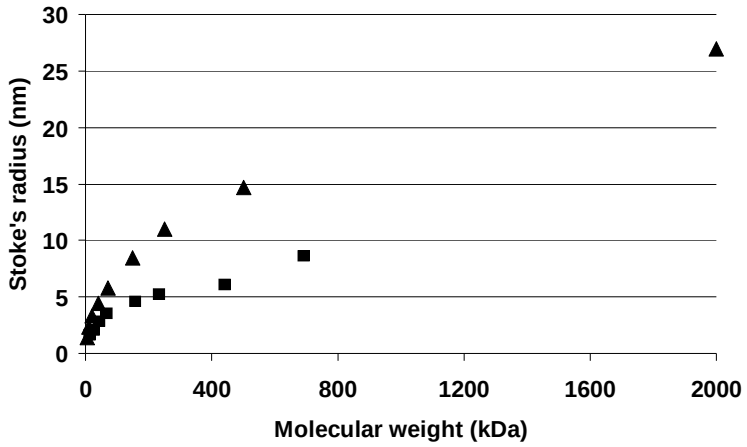

Fig. 6: Correlation between molecular weight and Stoke's radius for (▲) FITC-dextrans and (■) proteins [13, and data obtained from the supplier].

For both model drug categories, the impression of a direct relationship between released amount and molecule size is given. For proteins, the amount released in the absence of PEG (fig. 7A) was somewhat lower than in its presence (fig. 7B), as described above. In both cases, release of ovalbumin seemed to be extraordinarily low (symbol in brackets), but interestingly, PEG release from ovalbumin matrices with mixed loading was much higher than the amount of protein and fits well in the general trend of PEG release correlated to the size of co-incorporated protein (fig. 7B). For PEG, an outlier was encountered in catalase loaded matrices (symbol in brackets), where the complete amount had already been set free after 7 days, while catalase followed more slowly.

In figure 7C, an overview of the results for FITC-dextran released after 30 days in the presence of 0.01% Tween®20 and 81 in the buffer is shown. Figure 7C is representative of the experiments with FITC-dextran in the presence of other Tween® concentrations as well, at which also higher molecular weights generally resulted in a slower release. The decrease in release with higher molecular weight was less pronounced for FITC-dextrans compared to proteins, irrespective of type and amount of Tween® added.

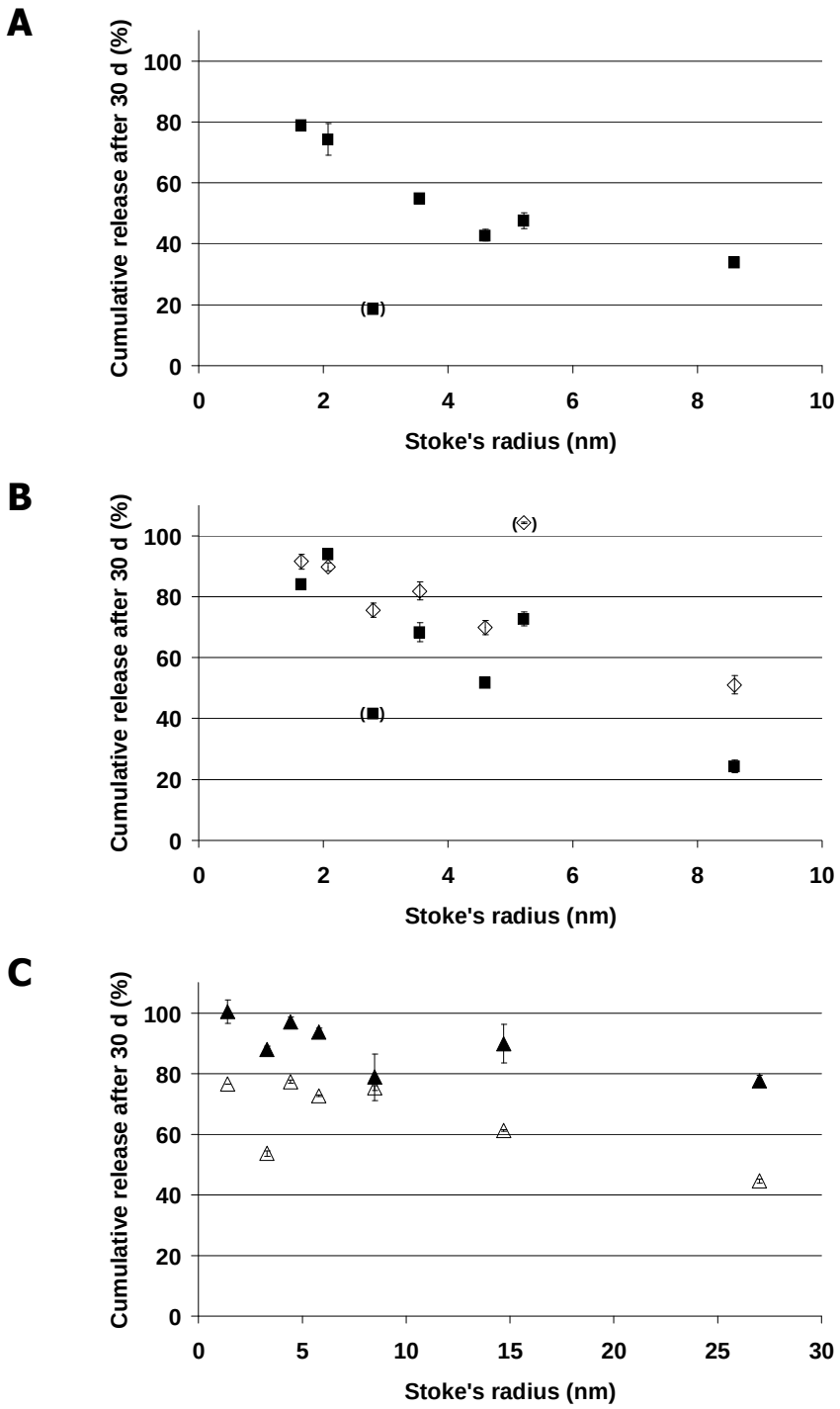

Fig. 7: Correlation between Stoke's radius of model proteins or dextrans and cumulative release after 30 days: ***(A)*** *Protein released from glyceryl trimyristate matrices loaded with 6% protein.* ***(B)*** *Protein (■) and PEG 6,000 (◊) released from matrices loaded with 3% protein and 3% PEG.* ***(C)*** *Matrices loaded with 6% FITC-dextrans; release buffer contained 0.01% Tween®20 (▲) or 81 (Δ).*

4. Discussion

Results for both groups of model substances impressively demonstrate in unprecedented clarity, that drug molecular weight is a crucial parameter in design of controlled release devices. For all protein and dextran model drugs, lipid cylinders could be manufactured by a PEG co-lyophilization method, which guarantees the homogeneous distribution of drug particles in the micrometer range within the matrix material as determined in a parallel study [5]. Although an influence of protein molecular weight on microparticle size resulting from PEG co-lyophilization has been reported [22], no effect was observed in this study (data not shown), probably due to the choice of PEG/protein ratio above the so-called bending point, where a particle size below 10 µm would be guaranteed [22]. This was a prerequisite for comparing release data, as different studies have shown, that particle size differences can influence the resulting release profile [23-26].

For polymer microparticles, similar investigations have been initiated, however, due to variations in manufacturing methods and the complexity of parallel processes of polymer degradation and release, controversial results have been reported. Whereas no relationship between size of model substance and release was observed for polyanhydride microspheres [9], similar effects as seen here have been reported for PLGA [7], partially loading dependent [8]. For lipid matrices on the contrary, release has been found to be clearly diffusion controlled [11, 27] and erosion takes place on a much longer time scale [28], thus not interfering with the release mechanisms. Both makes investigation of possible impact factors much easier than for polymeric systems and also enables better predictions for device design.

While the general conditions were constant for all proteins as to the manufacturing procedure, matrix material, loading and release medium, differences due to molecule properties other than the size cannot be ruled out and might explain the deviations. Isoelectric points of all model substances was either more than two units above (lysozyme, trypsin) or below that of the release medium, however variations in solubility might play a crucial role at the buffer penetration front, where saturated protein solutions must be expected.

Protein instability problems have been reported before to impact the release kinetics [29], therefore, protein integrity was monitored during the study. The most prominent deviations from the correlation of size versus release rate were found for ovalbumin, and for trypsin and catalase incorporated together with PEG (figs. 1 and 7). A slower release of ovalbumin

might be explained by formation of aggregates within the matrices, slowing release probably not only by lower diffusion coefficients, but also solubility problems [1, 30]. Also, increased matrix interactions might occur upon unfolding [31] and a general tendency towards better adsorption of larger molecules to hydrophobic surfaces has been reported [32, 33]. Co-encapsulated PEG was released at a rate actually expected for ovalbumin, indicating, that entering of release buffer and solute diffusion were not hindered. A faster release than expected was found for trypsin, pointing towards a lower molecular weight than assumed. Indeed, SDS-PAGE indicated that trypsin size might decrease due to its autolytic activity (fig. 3), paralleled by a reduction in enzymatic activity. A more distinct change was detected in the presence of PEG, probably due to a higher moisture content in the matrices, which was identified to increase the risk for protein stability problems [34].

The overproportionally high release of catalase and co-incorporated PEG could not be explained by protein stability aspects. Here, the matrix integrity itself was affected during incubation – after two days cylinders had started to display horizontal fissures, through which the PEG with its lower size might have escaped more quickly than the catalase. As no other protein had displayed such activity, a direct interaction between the co-incorporated PEG and catalase was assumed. Catalase action is directed towards peroxides resulting in the formation of oxygen, thus a reaction with peroxide traces in PEG might be imaginable, the originating gas overstraining interparticulate adhesion until rupture.

The loss in activity was most severe for ADH, without apparent effect on expected release behavior. Both catalase and ADH are well known for quickly loosing their enzymatic activity in unstabilized solution [8, 35] and should therefore be processed with extreme care and tested for release in stabilized buffer. The main focus of this study, however, was on release mechanistic aspects, therefore they had been treated with no special attention and under the same conditions as all other proteins. To preserve their stability, shorter times in the manufacturing procedure between dissolution and co-lyophilization with PEG and the optimization of the release buffer should be envisaged.

In order to avoid specific molecule properties and stability considerations, FITC-dextrans were chosen as a second group of model substances, varying mainly in the chain length. As for the proteins, the molecular weight had an impact on the resulting release profile (fig. 5). However, the range between release at highest and lowest molecular weight was less widespread as observed for proteins, although the size span of investigated dextrans

(1.4 to 27 nm) was larger than for proteins (1.6 to 8.6 nm). The dextrans' lack of surface active properties required the addition of surfactant to the release buffer, in order to induce sufficient pore wetting for the release buffer to enter the matrix [10]. The chosen concentrations were derived from a parallel study, in which lysozyme and FITC-dextran (4 kDa) were compared [10], and a direct relationship between contact angle of surfactant solution on lipid and the induced release rate was observed. The surfactant was claimed to facilitate buffer accession to the matrices, while the loading is released by diffusion. This hypothesis was supported by this study, where the molecular weight of the model substance still exerted an effect in the presence of both surfactants used.

Results for release in buffer supplemented with 0.1% Tween®81 should be interpreted with caution, as we have encountered the problem of fissures in the matrix due to interaction with the surfactant in this concentration [10], probably explaining the steep increase in the release profile irrespective of the dextran molecular weight. At 0.001% Tween®81, the impact of molecular weight could hardly be distinguished, which could be explained by the low wetting capacity of that surfactant concentration, generally resulting in a slow penetration of pores and thus probably being the rate limiting step for the overall release process.

As wetting effects seem to mask the influence of molecular weight on release to some extent, a comparison between data for FITC-dextrans and proteins does not seem appropriate due to the different release conditions of the experiments. The different impact of model substance size on release after 30 days might be artificially created by more favorable release conditions in the case of dextrans.

5. Conclusion

Independent of the substance class examined, a higher molecular weight in general resulted in a slower release from lipid matrices, which is in good agreement with the suggested diffusion controlled release mechanism. Experiments with FITC-dextrans confirmed the observations made for protein model substances. For proteins, the interpretation of the release data was complicated by stability difficulties. Especially the role of co-incorporated PEG should be re-examined with care. While it seems useful in replacing parts of the protein loading without changing the release mechanism, in some cases it also increased the risk for protein degradation by increasing the moisture content within the matrix. Apart from that, it was not compatible with all proteins tested in this study, showing undesirable interactions with catalase leading to matrix fissures. As an alternative to PEG, dextrans might be a suitable option as release modifier, fitting in the preparation procedure and showing a similar release behavior. Due to the lack of surface activity of dextrans, the protein might still be the governing factor of release behavior.

Acknowledgements

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Chapter 8

Summary and Conclusion

Summary and conclusion

It was the aim of this thesis to establish triglyceride matrices as a suitable alternative to polymeric carriers for the long-term controlled release of protein drugs. The success in comprehensively achieving this goal was to a great extent based on the parallel investigation into both mechanistic and application based aspects. Visualization of internal processes inside the matrices at all stages by confocal microscopy played a major role in advancing both fields of work and thoroughly understanding the system.

First, in a comparison with the so far used manufacturing methods [1-3], PEG co-lyophilization was developed as a new strategy with superior performance in finely and homogenously distributing lysozyme as a model protein in lipid matrix material with complete retention of activity (**chapter 3**). The method was designed as a one-pot procedure, where protein was micronized in a first step due to freezing–induced aqueous phase separation in the presence of PEG 6,000, and then dispersed in the solid state in a solution of the lipid in an organic solvent. Confocal microscopy pointed to the fine and homogenous distribution being the crucial prerequisite for long-term delivery. With this, a reduction in matrix size to dimensions of 1 mm diameter and 1 mm height was feasible, for the first time enabling their investigation in the animal brain, where they showed excellent biocompatibility. Material loss during manufacturing was practically excluded and the method could be down-scaled to batch sizes of 25 mg, allowing for the investigation of cost extensive therapeutic proteins. BDNF (**chapter 3**) and IL-18 (**chapter 4**) were incorporated by this method into glyceryl tripalmitate under complete retention of activity as demonstrated by BDNF-ELISA and a cell based IL-18 bioassay. Release from these matrices was measured for up to one month and could be tailored to the desired dosage regimen of 10 to 100 ng/day for IL-18 using BSA as an adjuvant supplement and 0.1 to 1.5 µg/day for BDNF with the help of PEG 6,000 as a porogen. At an incubation temperature of 37°C, activity loss during incubation was more pronounced than at 20°C and 4°C for IL-18, and BDNF showed a tendency to form more aggregates during release at 37°C than at 4°C. The higher release rates at elevated temperatures found in both studies suggested a diffusion controlled release mechanism.

A thorough understanding of the mechanisms underlying release from a drug delivery device is crucial for developing strategies to tailor the release profile and being able to react to drug stability problems. It could be demonstrated by diffusion studies of fluorescently labelled BSA, excipient PEG and release buffer visualized with the help of

confocal microscopy (**chapter 5**) that buffer could only penetrate into the matrices, where excipient or protein starts to dissolve and diffuses out. A linear concentration gradient of diffusing protein was formed between a buffer penetration front and the matrix surface until the buffer reached the centre of the device. After that time point, the concentration gradient gradually decreased and release slowed down. As a consequence of the preferential orientation of the lipid flakes during compression, tortuosity of the matrices was found to be higher in the axial orientation, leading to a faster penetration of buffer from the cylinders' side.

The power law [4] was an adequate mathematical model for the description of release profiles of FITC-BSA and lysozyme at loadings between 1 and 8%, with data being linear to time$^{0.45}$ for up to 60% cumulative release. At 5% protein loading, a percolation threshold could be identified, predicting the loading, above which complete release of the matrices is possible due to the formation of a continuous network of pores [5-7]. This value was reduced to 3-4 % when half of the protein loading was replaced by PEG 6,000 as a porogen. The value is comparatively low with regard to data published by Leuenberger et al. [5-7], which can be explained by the low drug/matrix particle size ratio [8, 9] resulting in a correlated percolation [7].

Buffer penetration and drug diffusion, the two crucial mechanisms for release identified by confocal microscopy, were further investigated for their dependence on matrix and drug properties. In **chapter 6**, it could be demonstrated, that wettability of the matrix governed the buffer penetration velocity. This could be employed to tailor the release profile in a time range of more than 14 months by varying matrix lipophilicity through changing the chain length of the fatty acid in the triglyceride. However, this was only possible when the incorporated drug was a protein, being able to reduce buffer contact angles via its own surface active properties. Non-surface active model substances, such as FITC-dextrans remained trapped within the triglyceride, due to their inability to induce wetting and buffer penetration. This, however, could be triggered by the addition of the surfactants Tween®20 and 81 at time points up to 57 days after initial incubation, leading to a complete release of the matrix loading. Thereby, the profile could be tailored by adjusting matrix wettability via the contact angle of the release buffer containing concentrations between 0.001 and 0.1% of the one of the two surfactants.

Apart from drug surface activity, another important characteristic was its molecular weight, being decisive for the diffusion step during release (**chapter 7**). An impact could be proven not only for seven different model proteins (lysozyme, trypsin, ovalbumin, BSA, ADH,

catalase, thyroglobulin) but also for FITC-dextrans of different molecular weights in the presence of surfactant in the release buffer.
Protein stability problems encountered during release were more pronounced in the presence of co-incorporated PEG in the case of trypsin, ADH and catalase, so that dextrans could be suggested as possible alternative fillers, available in different molecular weights, compatible with the preparation procedure and not influencing the release mechanism.

As a conclusion, with the work described in this thesis, lipid matrices could be established as a controlled release systems for long-term delivery of protein drugs. Concomitantly, it presents a fundamental insight into both release mechanisms and potential applications. It thus can provide a basis for a better understanding also for other kinds of matrix geometries and lipophilic materials, for which diffusion is the major release controlling factor.

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Appendices

Abbreviations

ADH	alcohol dehydrogenase
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
BAEE	N_{α}-benzoyl-L-arginine ethyl ester
BCA	bicinchoninic acid disodium salt
BDNF	brain derived neurotrophic factor
β-NAD	β-nicotinamide adenine dinucleotide
BSA	bovine serum albumin
Bq	Becquerel
CD	cluster of differentiation
Ci	Curie
CLSM	confocal laser scanning microscopy
CMC	critical micelle concentration
CNS	central nervous system
cpm	counts per minute
Da	Dalton
DAB	3,3'-diaminobenzidine tetrahydrochloride
DCM	dichloromethane
DIPEA	N-ethyldiisopropylamine
DMSO	dimethylsulfoxide
DSC	differential scanning calorimetry
DTT	dithiothreitol
Dynasan®112	glyceryl trilaurate
Dynasan®114	glyceryl trimyristate
Dynasan®116	glyceryl tripalmitate
Dynasan®118	glyceryl tristearate
ED_{50}	median effective dose
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immuno sorbent assay
EPC	egg phosphatidylcholine
EVAc	poly(ethylene-co-vinylacetate)

FDA	Food and Drug Administration (U.S.)
FITC	fluorescein isothiocyanate
GDNF	glial cell line derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte macrophage-colony stimulating factor
HCl	hydrochloric acid
HD	Huntington's disease
HLB	hydrophilic lipophilic balance
HPLC	high-performance liquid chromatography
HSPC	hydrogenated soybean phosphatidylcholine
IFN	interferon
IGIF	interferon-γ inducing factor
IL	interleukin
LHRH	luteinizing hormone-releasing hormone
MCP	monocyte chemoattractant protein
MGV	mean grey value
MIP	macrophage inflammatory protein
MOPS	3-[N-morpholino] propanesulfonic acid
mPEG-NH_2	methoxy-poly(ethylene glycol)-amine
M_w	molecular weight
MWCO	molecular weight cut-off
n.d.	not determined
NGF	nerve growth factor
NK cells	natural killer cells
o/w	oil/water
PBS	phosphate buffered saline
PEEK®	polyetheretherketone
PEG	poly(ethylene glycol)
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PVA	poly(vinyl alcohol)
rDNA	recombinant desoxyribonucleic acid
rpm	rounds per minute

SCG	sodium chromoglycate
SD	standard deviation
SDS-PAGE	sodium dodecylsulfate poly(acrylamid) gel electrophoresis
SEM	scanning electron microscopy
SHDP	sodium hexadecyl phosphate
SLN	solid lipid nanoparticle
s/o/w	solid/oil/water
SRH	sulforhodamine 101 hydrate
TAMRA	tetramethylrhodamine
TDC	taurodeoxycholate sodium salt
THF	tetrahydrofurane
US	ultrasonication
v/v	volume/volume
w/o/w	water/oil/water
w/v	weight/volume

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2004

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