The Development of Sustained Release Formulation for Pharmaceutical Proteins based on Vesicular Phospholipid Gels

Dissertation

zur Erlangung des Doktorgrades der
Fakultät für Chemie und Pharmazie der
Ludwig-Maximilians-Universität München

vorgelegt von

Weiwei Tian

aus Tianjin, China

München 2010

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Dr. G. Winter betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, den 01.12.2009

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Weiwei Tian

Dissertation eingereicht am: 02.02.2010

1. Gutachter: Prof. Dr. G. Winter
2. Gutachter: Prof. Dr. M. Brandl

Mündliche Prüfung am: 24.02.2010

ACKNOWLEDGEMENTS

The present work was carried out from Oct. 2006 to Dec. 2009 under the supervision of Prof. Dr. Gerhard Winter at the Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University, Munich.

Foremost, I would express my deepest gratitude to my supervisor, Prof. Dr. Gerhard Winter offering me the possibility to join his research group. I am extremely grateful for his inspiring encouragement, professional guidance and scientific support. Especially, I want to thank him for giving me chances to present the work in the international conferences.

I am also deeply grateful to Prof. Dr. Martin Brandl, my second supervisor, who introduced me the technological education and granted scientific advices and solutions. Thanks for his help I could always rely on during the last years.

Especially, I would like to thank the Chinese Scholarship Counsel for the financial support.

Moreover, I especially thank Dr. Sandra Schulze for the guidance over the second year.

Many thanks to all the past and present colleagues in the research group of Prof. Dr. Winter and Prof. Dr. Frieß for creating an outstanding working climate. I would especially thank for the relief they have granted me at the beginning of my study.

Furthermore, I am indebted to Dr. Matthias Hadesbeck, Ingrid Schmidt, Monique-Claudine Esnouf from the International Affairs of the university for supporting me so much with solving the problems outside the laboratory.

.

I would like to acknowledge Dr. Stefan Buchmann, from the Sport orthopaedic, TUM, Germany, for performing the animal experiments. Thanks are extended to his clinic research team.

Thanks are extended to the University of Tromso, Norway for offering the dual asymmetric centrifuge as well as to Lipoid GmbH, Ludwigshafen, Germany and PHOSPHOLIPID GmbH, Cologne, Germany, for providing various lipids.

Thanks are also extended to Prof. Dr. W. Frieß, Prof. Dr. F. Boeckler, and Prof. Dr. F. Bracher for serving as members of my thesis advisor committee.

Finally I would like to thank my parents and my friend Shen for their enduring love and constant encouragement. Thank you very much!

TABLE OF CONTENT

Chapter I: Introduction **1**

1. General consideration on pharmaceutical proteins **2**

2. Administration routes and existing problems **3**

2.1. Parenteral delivery 4

2.2. Oral delivery 4

2.3. Alternative routes 6

2.4. Controlled drug delivery system 7

3. Polymeric system **8**

3.1. Poly (Lactic-co-glycolic acid) system 9

3.2. Hydrogel 10

4. Lipidic systems **11**

4.1. Lipid implants 12

4.2. Multivesicular liposomes 14

4.2.1. Preparation method 14

4.2.2. Influence factors on the release 15

4.2.3. Mvls for controlled drug delivery 17

4.3. Vesicular phospholipid gels 21

4.3.1. Preparation methods 21

4.3.2. Characterization of vpgs 24

4.3.3. Application of vpgs for drug delivery 26

Chapter II: Aim of the thesis **30**

Chapter III: Materials and methods **32**

1. Materials **32**

1.1. Fluorescein isothiocyanate-dextran (FITC-dextran) 32

1.2. Erythropoetin (EPO) 32

1.3. Granulocyte colony-stimulating factor (G-CSF) 33

1.4. Monoclonal antibody immunoglobulin G (mAb) 33

1.5. Phospholipids 34

1.6. Chemicals and reagents 34

2. Methods **35**

2.1. Up-Concentration of proteins 35

2.2. Preparation of vesicular phospholipid gels 35

2.2.1. Preparation by high pressure homogenization 35

2.2.2. Preparation by dual asymmetric centrifugation 36

2.3. Microscopic studies 37

2.4. Rheology 37

2.5. Texture analysis 37
2.6. Dispersions of VPGs 37
2.7. Particle size analysis and zeta potential measurement 37
2.8. Turbidity 38
2.9. Extraction of proteins from the VPG matrix 38
2.9.1. Extraction of EPO 38
2.9.2. Extraction of G-CSF 38
2.9.3. Extraction of mAb IgG 39
2.10. Sodium dodecyl sulphate polyacrylamide gel electrophoresis 39
2.11. In vitro release tests 39
2.12. Quantification of released drugs 40
2.12.1. Determination of fitc-dextran concentration by fluorescence photometry 40
2.12.2. Quantification of protein by hplc 41
2.12.3. Quantification of protein by protein assays 42
2.13. Quantification of phosphatidylcholine by the enzymatic assay 43
2.14. Enzyme-linked immunosorbent assays of g-csf 43

Chapter IV: Tentative experiments 45

1. Preparation of VPGs using FITC-Dextran as drug model 45
1.1. High pressure homogenization 45
1.2. Dual asymmetric centrifugation 46

2. Homogeneity of VPGs 48

3. In vitro release tests 49

4. Summary and conclusion 51

Chapter V: Development of EPO loaded Vesicular phospholipid gels 53

1. Preparation of EPO loaded VPGs 53

2. Rheology and texture analysis 55

3. Evaluation of protein stability by SDS-PAGE 56

4. Development of quantification method of EPO 60
4.1. Sample preparation 60
4.2. Se-HPLC method 61
4.3. Rp-HPLC method 63
4.3.1. Validation of the HPLC method 63
4.3.2. Evaluation of detergents and organic solvents for EPO extraction 64
4.4. Validation of the developed quantification method 65
4.4.1. Calibration 65
4.4.2. Influence of centrifugation conditions 67
4.4.3. Influence of chloroform amount 67

4.4.4. Influence of lipid amount 68
4.4.5. Recovery of oxidized products 68
4.5. Summary of the quantification method 69
5. In vitro release behaviour of EPO loaded VPGs 69
5.1. Influence of the lipid content 70
5.2. Influence of the lipid composition 71
5.3. Release mechanism 73
5.4. Integrity of the released protein from VPGs 74
5.5. Particle size distribution 74
6. Adsorption study of EPO 75
7. Summary and conclusion 76
Chapter VI: VPGs for growth factor delivery 80
1. Preparation of G-CSF loaded VPGs 80
2. Texture analysis and rheology test of G-CSF VPGs 81
3. Evaluation of G-CSF stability by SDS-PAGE 83
4. Characterisation of the secondary protein structure within VPGs 84
5. Quantification method for released G-CSF 86
5.1. The fluorescamine assay 86
5.2. RP-HPLC method 87
5.3. Extraction method 87
5.3.1. Method development 88
5.3.2. Validation of the final extraction method with N,N-Dimethylformamid 90
6. In vitro release of G-CSF from VPGs 93
6.1. Influence of the lipid content 93
6.2. Influence of the G-CSF content 94
6.3. Daily release amount 95
6.4. Investigations on the imcomplete protein release 96
7. In vivo experiments 98
7.1. Providing back grounds 98
7.2. Experimental set-up 100
7.3. Elisa of human G-CSF 102
8. Conclusion 104
Chapter VII: MAB loaded VPGs 107
1. Preparation of MAB loaded VPGs 108
1.1. Concentrating MAB bulk 108
1.2. Formulation of MAB loaded VPGs 108
2. Texture analysis and rheology 109

3. Evaluation of MAB stability by SDS-PAGE 111
4. Quantification method for MAB in presence of lipids 113
4.1. CBQCA Assay 113
4.1.1. Calibration 113
4.1.2. Influence of the lipid content 114
4.2. Micro BCA assay 115
4.2.1. The method 115
4.2.2. Calibration of the standard 116
4.2.3. Extraction method with chloroform 116
4.2.4. Adding sodium deoxycholate 118
4.2.5. Summary of the quantification method 120
5. Stability study of MAB at 37°C 121
6. Adsorption of MAB to the release system 123
7. In vitro release behaviour of mAb (IgG) loaded VPGs 123
7.1. Influence of the lipid content 123
7.2. Influence of the mAb content 124
7.3. Quantification of phosphatidylcholine content 125
7.4. SDS-PAGE of the released proteins from VPGs 127
8. Summary and discussion 127
Chapter VIII: Final summary 131
Chapter IX: References 135
Publications 151
Curriculum Vitae 152

CHAPTER I: INTRODUCTION

Due to the great advances in recombinant DNA technology in the mid 1970s nowadays approximately 20 % of new drug applications involve a protein as active compound [1]. Unfortunately, proteins generally require parenteral administration for systemic delivery and the plasma half-lives of proteins are often very short, which is associated with frequent injections or administration via infusions. One way to overcome the related problems like poor patient compliance, increased occurrence of side effects, or cost consuming hospitalisation is the development of sustained release systems which should allow prolongated systemic or local delivery of the protein drug with the therapeutically desired dosing rate [2-3].

For that purpose research has been focused on the embedding of peptides and proteins in microspheres, or solid or in-situ forming implant systems based on synthetic biodegradable polymers, mainly poly(D,L-lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA). However, despite the early success of such systems for the delivery of small peptides no controlled release device for proteins is on the market. This limited success can be explained with the fragile, three-dimensional macromolecular protein structure rendering proteins susceptible to a variety of chemical and physical degradation pathways during the manufacturing, storage and release [2, 4]. In order to overcome this restriction lipid derived drug delivery systems have been proposed as an alternative [5-8]. Apart from matrix-like solid systems like lipid micro-particles and solid lipid implants liposomal drug carriers gained great interest offering the benefit of transporting the drug to certain target sites. However, especially traditional liposomes, such as unilamellar vesicles or multilamellar vesicles, often reveal an insufficient retention of the incorporated drug due to leakage of the phospholipid membrane. For instance, multilamellar liposomes loaded with granulocyte-macrophage colony stimulating factor (GM-GCSF) provided a sustained release of the cytokine over only 24 hours after subcutaneous administration [8]. Another potential problem might be that the generation of liposomes with a homogeneous size often comes along with low encapsulation efficiencies for hydrophilic drugs. Above these hurdles liposome processing methods often involve conditions that might induce protein denaturation and/or aggregation [5]. In order to prolong the release period very large (100µm), multivesicular liposomes or liposome

clusters have been proposed [9]. A further promising delivery concept, which has up to now not been evaluated for protein delivery, is the encapsulation of a macromolecular drug into vesicular phospholipid gels (VPGs). These gels display highly concentrated, semisolid, aqueous phospholipid dispersions, generated by a ‘forced hydration’ of phospholipids in the presence of relatively low amounts of water. The inner structure of the obtained semisolid pastes can be described as a matrix of tightly packed phospholipid vesicles. Depending on the phospholipid content and the preparation method the size and the morphology of these vesicles varies from small and unilamellar to large multivesicular or multilamellar [10-11].

So far it has been shown in literature that VPGs can serve as sustained release systems for various low molecular weight drugs, especially in the field of anticancer treatment [11-12]. First important success was achieved by loading of VPGs with the small peptide hormone cetrorelix: in vitro release experiments showed a wide range of cetrorelix release periods, ranging from 24 hours to a predicted sustained release of over more than 3 months [13]. However, still it was unclear if VPGs might be suitable for the delivery of high molecular weight drugs.

Compared to traditional liposomal formulations VPGs have been shown to facilitate a more sustained drug release and furthermore, difficulties associated with the low encapsulation efficiencies can be overcome, since even the fraction of drug which is not liposomally encapsulated during the preparation process is localized in-between the vesicles of the phospholipid matrix and thereby also retained in the gel. Moreover the manufacturing protocols are not only rather straightforward and easy to up-scale but also advantageous with regard to protein stability and safety as they avoid the usage of organic solvent.

1. GENERAL CONSIDERATION ON PHARMACEUTICAL PROTEINS

Peptides and proteins are polymers formed by amino acids’ joining with each other when the carboxyl group condenses with the amino group to eliminate water [14]. There are twenty natural amino acids to construct all physiological peptides and proteins. The distinction between proteins and peptides is normally defined by the number of amino acids and molecular weight. The proteins typically contain 50 or more amino acids resulting in a molecular weight above 5kD. For instance, Insulin

has a molecular weight of 5800, and is one of the smallest pharmaceutically relevant proteins available. Four levels of protein structure are principally described as the primary, secondary, tertiary, and the quaternary structures. Because proteins have higher numbers of amino acids within one molecule, only proteins can form the tertiary and quaternary structures, but not peptides.

Proteins have a uniquely important place in therapeutics for various physiological purposes due to the high specificity. Advances in biotechnology have led to significantly increasing production of biologically active proteins. However, the number of pharmaceutical proteins that reached the market is still limited despite the continuing trend of proteins to act as therapeutic agent. One of the most challenging tasks in the formulation of proteins into pharmaceuticals is to ensure the protein stability. Physical and chemical instabilities of proteins are broadly classified as shown in Table I-1. In most cases, protein degradation involves more than one pathway of physical or chemical instabilities, which are tied to each other [14-16].

Table I-1. Instability of proteins **[14-16]**

	Physical Instability	Chemical Instability
Referred to	Loss of secondary, tertiary, or quaternary structure	Covalent modification of the protein via bond formation or cleavage
Including	Denaturation Aggregation Precipitation Adsorption to surfaces	Hydrolysis Deamidation Oxidation Disulfide exchange B-elimination Racemization

2. ADMINISTRATION ROUTES AND EXISTING PROBLEMS

Proteins and peptides play a very important role in replacement therapy and are now well accepted for systemic treatment of certain diseases in medical practice. Parenteral injection is the most common route for proteins’ administration since the bioavailability of these therapeutic agents is poor when administered nonparenterally. Many other administration routes have been tried out, and various degrees of success have been achieved [14, 17].

2.1. PARENTERAL DELIVERY

The therapeutic peptide and protein drugs are mostly available for parenteral administration in the market [14]. However, frequent administration is normally required due to the often short half life for the delivery of peptides and proteins parentally. It results in poor patient compliance and high cost of the health care system. Intravenous injection or infusion is preferred when the peptide or protein drugs show significant degradation at the subcutaneous or intramuscular injection site. Thus intravenous injection may allow a smaller dosage as the drug is not lost by proteolytic degradation. However, high peak drug concentration occurs after intravenous injection, which might lead to local or systemic side effects [14, 18-19]. The intramuscular and subcutaneous routes of drug injection are often used when drugs cannot be injected intravenously because of their low aqueous solubility and/or when the high peak concentration creates a problem. Compared with intravenous injection, intramuscular and subcutaneous routes have fewer problems associated with the compatibility of the injection components with full blood in the circulation. A relatively longer duration of action is permitted resulting in less frequent administration. In addition, the subcutaneous route are mostly preferred by patients due to the feasibility of self administration and consequently improved compliance. However, these administration routes are also more problematic in terms of immunogenicity and local irritation. Moreover, the drug release after subcutaneous injection or intramuscular is known to be affected by many variables (e.g. drug lipophilicity , drug solubility. initial drug concentration, injection depth, body movement, blood supply at the injection site) [18]. Thus non parenteral formulations have been widely investigated to address these problems.

2.2. ORAL DELIVERY

Oral delivery of therapeutic proteins is theoretically the most preferable administration route due to its accompanying advantages of patient comfort, ease of administration, decreased medical costs, and improved patient compliance, but has been a challenge for decades. The relatively large size and hydrophilic nature of proteins limit the absorption of these molecules in the gastrointestinal tract. The gastrointestinal tract is rich in proteolytic enzymes, which are designed by nature to digest dietary proteins as well as therapeutic proteins with the same efficiency. Thus,

the major barriers for oral delivery of therapeutic proteins include poor intrinsic permeability, luminal and cellular enzymatic degradation, rapid clearance, and chemical and conformational stability of proteins [20-21].

Consequently, the bioavailability of therapeutic proteins by oral administration is poor. Conventional pharmaceutical approaches to address these barriers, which have been successful with traditional organic drug molecules, have not been effective for peptide and protein formulations. Meanwhile, various specific approaches are under investigation for oral delivery of proteins and peptides.

The adsorption of proteins and peptides varies from different regions of the intestine. For example, the preferred adsorption site is the duodenum for cyclosporine, and the upper GI tract for octreotide [22-23]. Hence site specific delivery may offer a solution to improve the drug transport and absorption. The stomach has harsh conditions for protein drugs due to the acidic environment and enzymatic activity while the colon offers a high population of bacteria and largely anaerobic species. Enteric coating is typically exploited to avoid dissolution in stomach and to target the proteins and peptides to the colon [24-25]. Saffran [25] has coated peptide drugs with polymers cross-linked with azoaromatic groups to form an impervious film to protect orally administered drugs from digestion in the stomach and small intestine.

Chemical modification is another approach to improve the oral absorption of proteins. In Milstei's study [26], low molecular weight compounds have been attached to protein molecules to form a conjugated molecule. The interaction of these carrier molecules with the proteins reversibly destabilizes the native state of the molecule favouring a partially unfolded conformation. These intermediate protein conformations are transport competent and are able to be absorbed through the intestinal tissue and into the bloodstream. However, this approach is generally more suitable for peptides than for proteins due to the structural complexity of proteins.

Protease inhibitors can reduce the proteolytic breakdown of therapeutic proteins in the GI tract, and may consequently promote oral adsorption [14, 27]. For example, the pancreatic trypsin inhibitor protected insulin in the intestine of rats. Some inhibitors that inhibit insulin-degrading enzymes, including N-ethylmaleimide, 1, 10-phenanthroline and pchloromercuribenzoate dramatically improved insulin transport across the ileum. The drawbacks of inhibiting agents, such as the risk of toxic side

effect, might be eliminated with advanced drug delivery systems. The introduction of delivery system containing mucoadhesive polymers with immobilized protease inhibitor, which provides associative binding contact with the mucosa, reduced drug degradation between the delivery system and the absorbing membrane [28].

The use of carrier systems such as nano-particles, microspheres and liposomes can also offer promising approaches to enhance protein oral delivery. Normally a combination of two or more approaches is required with the goal to improve the oral bioavailability of therapeutic proteins from less than 1% to 10% or higher. Kimura [29] has reported a formulation with insulin and protease inhibitor encapsulated in polymeric hydrogel spheres. In this case, insulin and inhibitor are released from the gel and provide a significant and prolonged reduction of blood glucose level.

2.3. ALTERNATIVE ROUTES

Because of the shortcomings of the parenteral and oral dosage forms, alternatively delivery routes are under active investigations.

The transdermal route of delivery is particularly attractive because it avoids protein degradation via the gastrointestinal tract and the hepatic first-pass effect, and the delivery can be interrupted by simply removing the device. However, the major problem is the low permeability of proteins across the skin which is an excellent barrier to hydrophilic, polar macromolecules [30]. The use of ultrasound energy can be applied to enhance the systemic delivery of protein molecules. In addition, other promising enhancement techniques have been developed, such as that applying micro-needles and jet propulsion can bypass the stratum corneum barrier to permit direct access to the viable epidermis [14]. For the topical delivery, growth factors have been applied for post surgery wounds showing accelerated healing effect [31]. Moreover, application of delivery systems, such as hydrogels [32]and liposomes [33] are tried to enhance the topical delivery of proteins.

The use of the pulmonary route may soon become one of the most promising routes for local and systemic delivery of therapeutic proteins. For non-invasive delivery of biologics, it was discovered that the large highly absorptive surface area of the lung could be used for local and systemic delivery of proteins such as insulin. Promising

results with an absolute bioavailability > 56% were achieved for alpha-Interferon by the pulmonary route [34].

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) has been prepared in powder formulations for inhalation [35]. The protein retained in vitro and in vivo bioactivity using rabbits as animal model. The market product Exubera® was developed for inhalation of insulin. Moreover, liposomes and microspheres have been widely investigated as the pulmonary delivery carrier for the controlled release of drugs to the lung. However, the major problem is that the lungs are capable of metabolizing proteins, of which the metabolic pathways may be different from those observed in the intestine. Thus, case-by -case investigations might be necessary to test the feasibility of the pulmonary administration.

In addition, other mucosal routes such as vaginal administration have been investigated for therapeutic proteins. Systemic delivery of insulin across the vaginal mucosa was studied in ovariectomized rats given subsequent estrogen treatment. Enhancers were necessary to achieve decreased blood glucose levels. However, variable and often severe histological changes were observed in the vaginal epithelium after treatment with the enhancer systems [36].

2.4. CONTROLLED DRUG DELIVERY SYSTEM

There are many challenges to develop an efficient controlled-release drug delivery system. Normally a high drug loading efficiency is desired and the delivery system should have the feasibility in drug release at various rates to meet different treatment schedules. Moreover, the formulation components should meet some basic biological, physical, and technological requirements as summarized in Table I-2 [37].

Table I-2. Optimal features of materials for protein delivery

Biological properties	Physical properties	Technological properties
Non-cytotoxic	Injectable	scalable
Non-immunogenic	Mechanical strength	Easy production or purification
Non-infectious	Suitable Viscosity	Retaining full protein stability
Biodegradable with non-toxic products	Suitable Elasticity	

Besides, for the therapeutic proteins, retention of biological activity is a big issue since the proteins are susceptible to proteolysis, chemical modification, and denaturation during formulation production, storage, administration, and release [38].

The research on synthetic biodegradable polymer systems such as poly(D,L-lactide) (PLA), and Poly(Lactic-co-glycolic acid) has gained first interests for sustained release formulations for therapeutic proteins [39]. However, limited success was achieved due to the inherent drawbacks of the polymeric systems. Protein instability occurs in the manufacturing process, as well as during the release and storage [4, 40]. Alternatively, hydrogels have been investigated for protein delivery, which exhibit more hydrophilic property and appear more suitable for proteins than the other polymeric system.

Recently, more interests were raised in the biocompatible natural materials (e.g. lipids [41]) as alternative to synthetic polymers. Lipids such as triglycerides and monoglycerides can be formulated into implants simply by compressing or extrusion. Importantly beside the good biocompatibility and easy manufacture process, lipid implants cause less detrimental effect on the incorporated proteins [42-43].

Besides the traditional use of liposomes as drug carriers which circulate in the blood stream upon intravesicular administration, some attempts have been made to establish liposome-based local depots for sustained drug delivery. One of these novel liposome based systems is the multivesicular vesicles which have a bi-liquid foam structure with a large particle size ranging from 1–100µm [44]. Multivesicular liposomes can be administered by subcutaneous, intramuscular, intra-tumoral or into cavities injection for sustained delivery of proteins and anticancer agents [45-49].

Moreover, vesicular phospholipid gels consisting of phospholipid vesicles were introduced as an intermediate to enhance the storage stability of liposomes. First investigation on small compounds and peptides showed its potential application as a depot for the controlled delivery over a prolonged period [50-51].

3. POLYMERIC SYSTEM

The desirable characteristic of polymer systems for drug delivery are: minimal effect on biological systems after introduction into the body, in vivo degradability at a well-

defined rate to nontoxic and readily excreted degradation products, absence of toxic impurities or residual chemicals e.g. cross-linking agents. Synthetic and naturally occurring polymer based systems have been developed in various forms, including nano-particles, micro-particles, films, and matrix devices, etc [52-53].

3.1. POLY (LACTIC-CO-GLYCOLIC ACID) SYSTEM

A frequently investigated polymeric system for controlled release is based on poly(lactic-co-glycolic acid) (PLGA) especially in the form of injectable micro-particles. PLGA is a linear polyester that hydrolyzes by an acid or base catalyzed reaction to form the natural metabolites, glycolic and lactic acids [54-55]. Thus these polymeric systems are biocompatible, biodegradable, and considered safe. Until now, liquid drug-polymer formulations generating (semi-) solid micro-particles (in situ forming micro-partices, ISM) on subcutaneous or intramuscular injection have grown exponentially. There were some market products developed with these PLGA systems, e.g. Nutropin Depot® developed by Genetech and Alkermes jointly as once or twice a month injection formulation by loading recombinant human growth hormone (hGH) into PLGA micro-particles.

The capability of the system in releasing therapeutically useful proteins in a controlled manner have been widely investigated [38, 55-56]. However, protein instability during preparation, storage and release resulting in incomplete release of native protein has become recognized as a major problem [4, 57-58].

In most cases organic solvents have to be utilized to prepare the polymeric system. Protein was loaded either by emulsifying an aqueous protein solution with the organic polymer phase or suspending the solid protein powder in the organic polymer phase. Denaturizing stresses were associated with this emulsion and the suspension loading [57, 59-60]. Moreover, the incorporated proteins are faced to a completely altered microenvironment [4, 58, 61] inside the matrix during degradation. The generated degradation products are accumulatively entrapped within the matrices and might consequently result in a low pH [62], an increased osmotic pressure [63], and accumulation of reactive species [64]. As a result, the structural integrity of the protein to be delivered can be adversely affected.

The use of various stabilization approaches has rendered some success in increasing protein stability, but, still, full preservation of the native protein structure remains a major challenge in the formulation of protein-loaded PLGA micro-particles [3, 65-70]. Moreover, individual proteins exhibit unfortunately large differences in sensitivity to stress factors. For a rational optimization of protein stability, one must first establish the stage at which degradation occurs and identify the stress factors compromising the stability of the particular protein. Then, a rational stabilization approach can be followed to ensure the safety and efficacy of these protein-loaded PLGA micro-particles [4].

3.2. HYDROGEL

As an alternative to the PLGA systems hydrogels were developed for protein delivery. Hydrogels are defined as cross-linked polymer matrices that exhibit the ability to swell in water without dissolving and to retain a large amount of water within its three-dimensional structure. Hydrogels have been proposed for local, sustained delivery of proteins and other biological pharmaceuticals because of their soft tissue biocompatibility, the ease with drug loading and the high degree of feasibility achieved by selecting the physical and chemical properties of the polymer network [71]. Moreover, the cross-linked polymeric networks can also protect proteins from the potentially harsh environment in the vicinity of the release site, such as the acidic environment of the stomach [38, 71-73].

Hydrogels are formulated by chemical cross-linking or physical cross-linking. Chemical linking results in a high mechanical strength of gels, while the incorporated drug or protein might be affected by the chemical reactions or by the cross-linking agents and loses the bioactivity consequently. Thus physical cross-linking gained more and more interests, which can be established by e.g. ionic, hydrophobic, or coiled-coil interactions [74].

A variety of synthetic or natural polymeric hydrogels (e.g. collagen) have been employed as the controlled release systems for drug delivery [72, 75-76]. Among the wide investigations, in situ forming hydrogels gained considerable interests. The in-situ cross-linking mechanisms render the system injectable and so that an invasive surgical placement can be avoided [77-78]. An aqueous mixture of gel precursors and bioactive agents were administered using a syringe into bodies. Cross-linking of

the gel precursors can happen either induced by the change in temperature or pH, or chemically by Michael type addition reaction or disulfide bond formation in the body environment [74, 79]. A temperature-sensitive and in-situ forming hydrogel system was developed by Chung [80-81] for the delivery of human growth hormone (hGH). A sustained release in a zero-order fashion was observed for 13 days controlled by a diffusion/erosion coupled mechanism.

However, the major drawback of the system is the slow gelation which is caused by the time lag between injection of a liquid mixture of gel precursors and protein drugs and the formation of a cross-linked gel network[38]. Thus, rapid initial burst and drug overdose were observed since a large amount of drugs diffuse away from the injection site after injection and before the hydrogels formed. Hence, injectable hydrogel systems were developed with a tunable gelation rate to achieve better therapeutic output [74, 82]. Delgado [82] has prepared a N,N-dimethylacrylamide-based hydrogels for the controlled release of bioactive agents including lysozyme, bovine serum albumin, and rabbit IgG. The rate of protein release showed a dependence on both the molecular weight of the protein and the amount of cross-linker utilized to prepare the hydrogels. Neither the toxicity against human dermal fibroblasts nor the immunogenicity in mice was observed for the hydrogels.

4. LIPIDIC SYSTEMS

Lipids are referred to a group of naturally-occurring molecules which includes fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E and K), as well as mono-glycerides, triglycerides, phospholipids. Lipids may be broadly defined as hydrophobic or amphiphilic small molecules; the amphiphilic nature of some lipids allows them to form structures such as liposomes, or membranes in an aqueous environment [83].

Bangham [84] was probably the first to recognize the potential of the lipid shells for delivering soluble materials for extended periods. In the past few decades, research into conventional liposomes with unilamellar and multilamellar vesicles played a major role in the area of injectable drug delivery systems. Significant efforts in basic and applied research institutions led to the clinical development and ultimate approval by regulatory agencies for human use of a lipid complex (Abelcet^{TM},

Amphotec™) and three liposome formulations, AmbiSome™, DaunoXome™ and a Stealth liposome DOXIL™ [85-86]. These products have been developed for intravascular administration. The advantages conferred by the lipid complex, liposome and stealth liposome technologies include enhancement of circulation times, and reduced toxicity by lipid encapsulation. For protein delivery, incorporation of IL-2 into liposomes seemed to prolong of the local (intra-and/or peritumoral) residence time of the cytokine and thereby to improve the antitumor effects [87-88]. However, an insufficient retention of the incorporated drug was often observed due to a leakage of the phospholipid membrane.

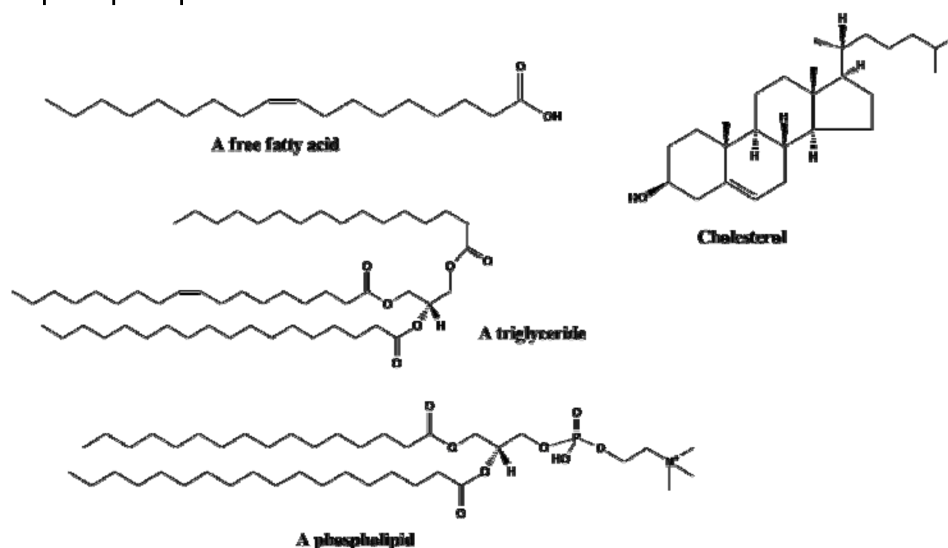


Figure I-1. Structures of some common lipids.

In order to address the issue, the most novel lipidic systems for protein delivery, e.g. lipid implants, multivesicular liposomes, vesicular phospholipid gels, will be discussed in detail below.

4.1. LIPID IMPLANTS

Lipophilic materials including cholesterols [89] as well as fatty acids, glycerides, and waxes can be used to form lipidic implant systems. In contrast to polymeric implants, the high compressibility of lipids allows the formation of solid matrices by traditional compression at mild conditions. Different techniques can be applied to generate the lipid protein blend: the protein in the solid form can be physically mixed with either the lipid powder or with the precipitated lipid powder, or alternatively the protein can be co-precipitated with the lipid powder [41, 90-96].

In the working group of Winter et al, many successes has been achieved on the study of this novel model depot system for the controlled release of proteins.

A promising platform for the delivery of rh-interferon-2α based on tristearin implants was developed by Mohl [90, 92, 97]. Polyethylene glycol 6000 was incorporated into the implants as release modifier. Varying amounts of polyethylene glycol 6000 and tristearin were mixed with the lyophilized protein and subsequently compressed to form the implants. Over 90% of the entrapped protein was released from the implants for over 1 month in virtually monomeric form in vitro. The implants using the preparation technique maintained the protein integrity both in the formation process, and in the release tests. The addition of hydroxypropyl-β-cyclodextrin (HP-β-CD) was applied to stabilize the protein and improve the storage stability of the matrix.

Herrmann [98-101] has further investigated the role of PEG on the protein release from the implants. Implants containing 5-20% PEG showed significantly changed release profiles of the entrapped protein. The underlying mass transport mechanisms were investigated by monitoring the release of IFN-α, HP-β-CD and PEG into phosphate buffer pH 7.4 simultaneously and fitting the results to Fick's second law of diffusion. It was suggested that the release of IFN-α from PEG-free implants was purely diffusion controlled while in contrast the reduced protein solubility and a reversible IFN-a precipitation during release in PEG-containing matrices were shown to control the protein release.

The in vivo release of the IFN-α implant was performed by Schwab [102] using rabbits as the animal model. Protein serum concentrations with the therapeutically relevant level maintained nearly constant for 9 days after implantation. However, the serum protein level abruptly weared off from the tenth day. It was assumed that IFN-α was continuously released from the implant after 9 days but the released protein in the serum was quantitatively captured by the rabbit antibodies. Good biocompatibility and little matrix erosion were observed for the implants. Moreover, a close in vivo–in vitro release correlation was revealed by computer modelling in the work.

4.2. MULTIVESICULAR LIPOSOMES

Multivesicular liposomes (MVLs, Trade name DepoFoam^{TM}) are spherical lipid vesicles with a size range of several micrometers. The particles are composed of multiple non-concentric aqueous chambers bound by a network of lipid bi-layers which appears like foam under microscopic examination (Figure 4) [103]. Compared with conventional liposomes, the unique structure of MVLs renders a higher aqueous volume-to-lipid ratio, which facilitates loading of water-soluble drugs and improves their encapsulation. The active ingredient is encapsulated within the non-concentric internal aqueous chambers so that a single breach in the external membrane will not result in a total release of the internal aqueous content. Moreover, MVLs are not rapidly cleared by tissue macrophages due to their large size [104-109].
MVLs can be applied as a drug depot, which has efficient entrapment of hydrophilic molecules including a variety of therapeutic proteins, providing slow release of drugs delivered through different routes of administration. Further, these particles are more stable than conventional liposomes due to the higher mechanical strength of the unique structure [103, 110-111].

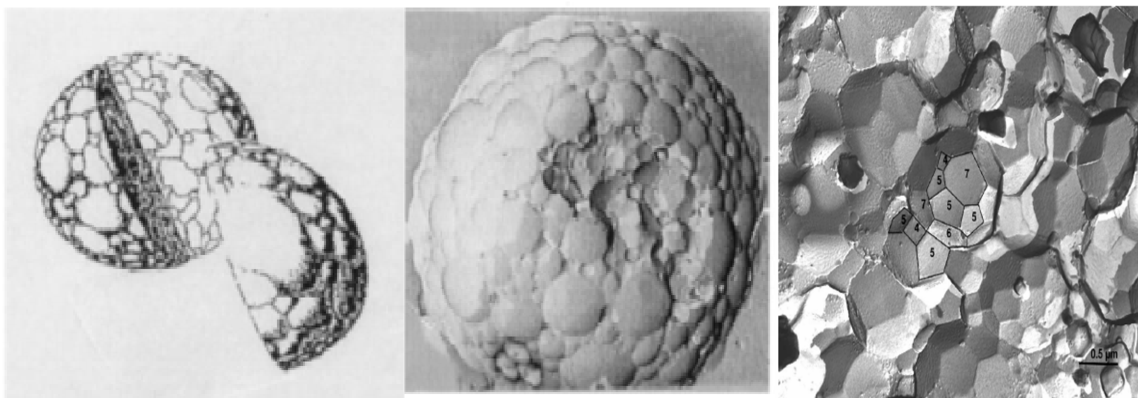


Figure I-2. Microscopy images of Multivesicular Liposomes [103, 110-111]

4.2.1. PREPARATION METHOD

In order to prepare MVLs, one neutral lipid (e.g. triglyceride) and at least one usual membrane forming lipids (e.g. phospholipids) must be included in the lipid compositions. When the neutral lipid is omitted, conventional multilamellar vesicles or unilamellar vesicles are formed instead of multivesicular liposomes [46]. The neutral lipid acts as hydrophobic space filler at bilayer intersection points and stabilizes these

junctions, and is also present as oil droplets dispersed in the encapsulated aqueous compartments.

Kim [44, 103, 112] has evaluated the influence of triolein and other lipid compositions on the capture volume and drug encapsulation efficiency of MVLs. In a mixture with phosphatidylcholine, cardiolipin and cholesterol (4.5:1:4.5 in mole ratio) maximal capture volume and encapsulation efficiency are obtained in the triolein mole fraction range of 0.01–0.08. At lower triolein content the capture efficiency is reduced markedly.

A two step emulsification process is critical to form MVLs whereas other types of smaller liposomes rather than MVLs are formed if other methods are used even with the same required mixtures of phospholipids and triglycerides The first step is making a ‘‘water-in-oil’’ emulsion: the lipids in one or more volatile organic solvents are emulsified with drug containing water phase under input of high mechanical energy. This emulsion is then mixed with a second immiscible aqueous component at less mechanical energy so that a water-in-oil-in-water (w/o/w) double emulsion is formed. The organic solvent is removed generally by evaporation, by reduced pressure or by passing a stream of gas over or through the suspension. Cross filtration is used to remove the un-entrapped drugs. The characteristics of prepared MVLs, such as particle size, viscosity, and conductivity are affected by many factors including mixing speed, mixing time, energy input, and temperature in the double emulsification process [46, 103, 113]. However, the preparations can not undergo terminal sterilisation and are too big for filtration through microbe-retentive filters. Therefore a GMP-conform process for commercial scale production was established with aseptic processing techniques containing a set of four sequential unit operations namely first emulsion, second emulsion, solvent extraction, and microfiltration [46].

4.2.2. INFLUENCE FACTORS ON THE RELEASE

The release rate of the biologically active compound from MVLs can be modified by utilizing a suitable neutral lipid component (triglycerides) in the preparation. Slower release rates are observed by incorporating long chain triglycerides in MVLs. Triglycerides having mono-unsaturated fatty acids containing about 14–18 carbons in the acyl chain and those with saturated fatty acid ester moieties containing about 10–

12 carbons in the acyl chain generally lead to slow release, among which triolein or tripalmitolein are mostly utilized. The neutral lipids inducing fast release are selected from triglycerides having saturated fatty acid ester moieties containing from about 6 to 8 carbons in the acyl chain with a molecular weight from about 387 to 471. Tricaprylin, and mixtures of tricaprylin and tricaproin, or mixed chain C6 to C8 triglycerides are most preferred. However, the use of neutral lipids with less than 6 carbons (e.g. tricaproin) in the acyl chain results in a rapid release of the encapsulated compound. The reason is assumed that the stabilizing effect on the structure of the particle by longer chain length is then missing. In mixtures, the concentration of the shorter versus longer chain triglycerides determines the release rate [111, 114-115].

The osmolarity of the aqueous phase containing the drug that is mixed with the immiscible solvent phase containing lipids is an important determinant of in vivo rates of release. The rate of release of the active substance into the surrounding in vivo environment can be decreased by increasing osmolarity of this solution. The decay half-life of the amount of cytarabine MVLs containing higher amount of cytarabin showed slower in vivo release rate than those containing lower content. Since the concentration gradient of drug between the inside of the particle and the peritoneal fluid for MVLs containing more drug is greater than those containing less, it is assumed that increased osmolarity of the drug containing aqueous solution (first aqueous solution) decreases the in vivo release rate of the active compound from MVLs [116]. Thus the in vivo release is suggested to be dominated by mechanisms other than simple diffusion [111].

Acids in the first aqueous solution are shown to have profound effect on the in vivo release rates. The half-life of cytarabine MVLs ranged from for 1.6 to 37.2 h depending on the acid added to a solution of cytarabine at a constant concentration, which consequently suggests the permeation of the salt complex is an important determinant of drug release in vivo [114].

It was suggested that the overall release mechanism of MVLs is composed of at least three components namely, diffusion, erosion and attrition [117-118]. For the diffusion component, particle number (measured by hemocytometry) remains constant but the amount of drug retained within the MVL particles decreases over time. Particle size

remains essentially constant, except when the particles act as osmometers sensing the loss of solute. For the erosion component, both particle concentration and internal drug concentration remain constant while particle size decreases. When the attrition part of the mechanism is operative, particle concentration decreases while internal drug concentration and size remain constant.

4.2.3. MVLS FOR CONTROLLED DRUG DELIVERY

Several hydrophilic molecules including a variety of therapeutic peptides and proteins have been efficiently entrapped in MVLs which offers a novel approach to sustained-release drug delivery [107, 119-121]. MVLs formulations can be administrated through injection into body cavities including intrathecal, epidural, intraperitoneal, subcutaneous, intramuscular but due to the particle size of up to 5µm, MVLs are not suited for intravascular injection [122-123].

The MVLs' particles are retained at the injection site and thus serve as a depot releasing the entrapped drug in a sustained manner over several days to weeks after non-vascular administration while drugs entrapped in conventional small liposomes normally last several hours to a few days after intravascular administration.

4.2.3.1. APPLICATION FOR SMALL COMPOUNDS

TOLIYAT [108] has reported MVLs for the delivery of desferrioxamine which is normally administered by the parenteral routes due to the poor absorption efficiency in the gastrointestinal tract. The results showed not only triolein in the lipid containing organic phase but also lysine in the second aqueous solution played significant effects on the captured volumes of the vesicles whereas cholesterol content in lipids has no significant effect. The effective concentration range for triolein is between 1.7 to 3.4 mg/ml and for lysine is is between 20 and 40mM. Large MVLs particles were obtained with a size between 15-35µm and a capture volume of about 27%. The in vitro studies in 0.9% NaCl at 37 ºC showed that 57% of desferrioxamine has released from MVLs in 9 days without a rapid initial burst.

Cisplatin [cis-dichlorodiamine platinum (II)] is one of the most effective antitumor agents in the treatment of testicular, ovarian, head and neck, and lung cancer. Due to the hydrophobic property, the loading efficiency of cisplatin was limited for

conventional liposomes (with unilamellar or multilamellar vesicle structure) and the pegylated liposomes [124-125]. Xiao [126] has investigated the cisplatin entrapped MVLs. The drug loading capacity of MVLs was improved to 0.148–0.444 mg per mg of lipid in contrast a drug to lipid ratio of 0.014 for pegylated liposomes. In vitro release of MVLs encapsulated cisplatin was over 7 days. The encapsulated drug showed much higher drug concentration in plasma as well as at the tumour site compared with unencapsulated drug after subcutaneous injection into mice inoculated with the murine carcinoma 180 (S180) tumour. Moreover, a longer circulation time was observed resulting in higher therapeutic efficiency to decrease tumor growth for MVLs cisplatin than uncapsulated cisplatin solution.

US FDA has approved two MVL formulations for clinical use. DepoCyt™ is the first clinically available MVL formulation, which contains the antineoplastic agent cytarabine for the treatment of malignant lymphomatous meningitis via intrathecal injection. MVLs encapsulated cytarabine is released in a sustained manner with cytotoxic concentrations in cerebrospinal fluid over 2 weeks after intrathecal injection. DepoCyt™ has good tolerability from the early efficiency data.The use of MVLs encapsulated cytarabine was expected to reduce the dosing frequency from twice a week to once every other week and improves the outcome compared with frequent intrathecal injections of unencapsulated cytarabine [127-129] .

DepoDur™ is a morphine loaded MVL formulation and it has heen approved for its single epidural injection in the treatment of postoperative pain. Animal experiments have confirmed the saftty, efficiency of encapsulated morphine after epidural administration. The half time in plasma and CSF were prolonged by different factors when administrating encapsulated morphine. However, no clinical studies suggest that the use of DepoDur™ decreases the amount of systemically administered analgesics needed for adequate postoperative pain control in humans. Due to the sustained release fashion of MVLs, it may provide superior pain control during the first 1-2 postoperative days compared with epidural administration of unencapsulated morphine or intravenous administration of an opioid. However, three to four times lower peak plasma concentrations were observed for the encapsulated morphine compared with administrating the same dose of unencapsulated drug. Moreover, some safety concerns remain because it is impossible to predict how long patients may be at risk of experiencing serious adverse effects [130-132].

4.2.3.2. APPLICATION FOR PEPTIDES AND PROTEINS

The versatility of the MVLs technique has also been demonstrated using peptide and protein drugs [49, 107, 110, 121, 133]. In general, as a sustained-release formulation of therapeutic proteins and peptides, MVLs have many advantages including high drug loading efficiency, low content of free drug in the suspension, little chemical change in the drug caused by the formulation process, narrow particle size distribution, and spherical particle morphology. Drug release assays of various protein loaded MVLs have been conducted in vitro in biological media such as human plasma. The results indicate that these formulations provide sustained release of encapsulated drug over a period from a few days to several weeks, and that the rate of release can be modulated. In vivo pharmacodynamic studies in rats also showed a sustained therapeutic effect over a prolonged period.

Progenipoietin is a dual receptor agonist of fetal liver tyrosine kinase-3 and granulocyte colony-stimulating factor receptors. The novel chimeric protein was entrapped into MVLs to develop a sustained delivery formulation. Various phospholipid blends containing DOPC, DEPC, and DPPG and two kinds of triglcerides including triolein and tricaprylin have been used to modulate the delivery profile. Examined by RP-HPLC, the encapsulated protein remained intact in the MVLs formulation. Pharmacodynamic studies were performed in rats using subcutaneous injection with a single dose of 1mg/kg (either the free protein or the MVLs entrapped protein). The same dose of progenipoietin doubled the count of neutrophil granulocytes in peripheral blood for 9 days after administering encapsulated drug, while the count was back to baseline within 3 days of injecting unencapsulated drug. Encapsulation of progenipoietin in MVLs led to retention of its structural integrity and maintenance of its biological activity in vivo over several days. Thus, a single dose of MVLs encapsulated progenipoietin was expected to improve hematopoietic recovery time after chemotherapy, and for other indications that require multiple daily doses of progenipoietin [134].

Langston [114, 135] has examined the efficiency of entrapped myelopoietins, a therapeutic grow factor, after subcutaneous injection in rats. A single injection of MVLs encapsulated myelopoietins results in elevated neutrophil counts for 10 days, which is a 5-fold increase in contrast to only 2 days for un-encapsulated drugs. The

duration of the pharmacodynamic effect of encapsulated drug can be modulated by varying the triglyceride (tricaprylin) content of the matrix from 10 days to 4 days, which offers the flexibility for controlled delivery of cytokine therapeutics. No increase in the extent of deamidation was observed during encapsulation and in vivo release.

A sustained release MVLs formulation of interferon (IFNα-2b) for subcutaneous administration was developed by Qiu [136]. Encapsulation efficiency of interferon was enhanced to about 60% by optimizing the protein-lipid ratio. Both the SDS-PAGE and HPLC analyses showed that the proteins were chemically intact. ELISA was performed and showed that the antibody binding affinity for the protein after encapsulation was only slightly reduced, indicating that substantial native structure remained after preparation. There was considerable protein detected in the circulation for more than 5 days using rats as animal models. The serum half-life was estimated to be approximately 30 h. Moreover, the in vivo release profile could be modified by varying the size of the MVL preparations. By the work of Bonetti [137], a linear release of encapsulated interferon was observed with a half-life time of 384hrs in human plasma.

In Katre's [107] work, Insulin-like Growth Factor I was entrapped into MVLs to achieve sustained therapeutic blood levels which would replace repeated administration. IGF-I was encapsulated in this system with good efficiency. The median size of MVLs particles ranged from 18 to 20μm. The integrity of the encapsulated protein was maintained, as characterized by physiochemical (HPLC, SDS-PAGE) and by biological methods (mitogenic activity). The in vitro studies in human plasma at 37 °C showed that the MVLs released 70-80% of the entrapped protein in 6-7 days without initial burst. In a pharmacokinetic in vivo study, after subcutaneous injections in rats, IGF-I levels were sustained for 5-7 days with entrapped protein formulation, whereas the free IGF-I was cleared in 1 day. Importantly, the full biological activity of the entrapped protein was maintained over at least 5 days at 37 °C, both for the protein released from the MVLs particles and for the drug retained in the particles. The protein in the MVLs and the lipid vesicle itself are assumed to have long-term shelf life stability at 4 °C for at least 12 months.

As a summary, MVL system was shown to be capable of efficiently encapsulating therapeutic proteins and peptides and effectively providing controlled delivery of these biologically active macromolecules.

4.3. VESICULAR PHOSPHOLIPID GELS

Vesicular phospholipid gels were first described as highly concentrated lipid dispersions, which normally consisted of 300mg/g-600m/g lipids [11, 138-140]. VPGs have a gel-like consistency formed by numerous densely packed vesicles. The aqueous compartments are both within the cores and in-between the vesicles so that the system is suitable for entrapping hydrophilic compounds including peptides and proteins. The gels are formulated by high-pressure homogenisation or dual asymmetric centrifugation. First investigations have shown that small water soluble compounds could be retained by vesicular phospholipid gels over extended periods of hours up to several weeks within an in-vitro set-up.

4.3.1. PREPARATION METHODS

4.3.1.1. HIGH PRESSURE HOMOGENIZATION

High pressure homogenization is basically used for preparing VPGs of small and unique vesicle sizes. The preparation approach is based on the one-step technique as originally described by Brandl, in which no organic solvent is involved [139, 141]. Briefly, dry lipids are mixed with water or buffer with gentle shaking. After a period of swelling, the raw lipid dispersions were processed with a high-pressure homogenizer. The device APV micro lab 40 can be normally used for lab scale production (with a batch size of 40ml).

A pressure of 70MPa with 10 processing times is usually appropriate to obtain finely dispersed gels which are available for spontaneous hydration (dispersion) leading to the formation of small liposome in the size range below 100nm.

The size distribution of the liposomes depends on the applied pressure, the number of homogenisation cycles, as well as the lipid type and content. Adding of cholesterol results in a slightly increased vesicle size of redispersed VPGs [123, 142-144].

4.3.1.2. DUAL ASYMMETRIC CENTRIFUGE

Dual asymmetric centrifugation has been known as a convenient technology for the rapid mixing of viscous components [145-146]. For example, by application of the mixer nano-$CaCO_3$ can disperse uniformly in polymer-composites (nano-$CaCO_3$ is dispersed as filler between the polymers. By adding a lower content of the fillers, the mechanical properties of the composites can be improved significantly) [145-146]. The working principle of this special centrifugation technique is that the sample is not only rotated around a central axis but also around a second axis in the centre of the sample container as shown in Figure I-3. The main rotation arm of the DAC forms an angle of about 40° with the rotation plane. At this angle, the rotating arm forces the content of the vial into the corner between the bottom and the vial wall [147]. The vial holder rotates in the opposite direction with approximately one fourth of the rotating arm's frequency. The combination of these two contra-rotating movements makes efficient homogenization of viscous materials possible [148-149].

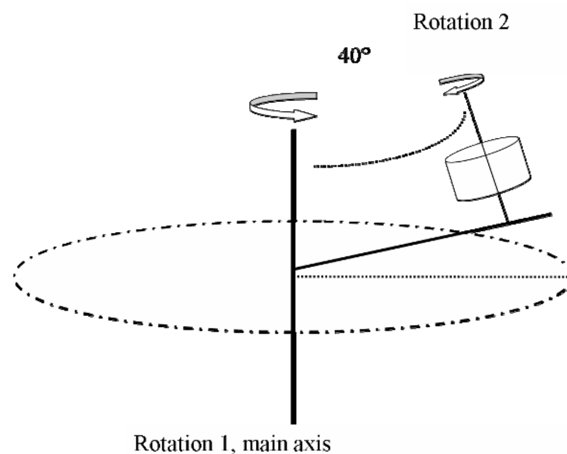


Figure I-3. Schematic drawing of the dual asymmetric centrifuge, SpeedMixer™ DAC 150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany [147].

DAC was recently applied for production of Vesicular phospholipid gels, which have previously been formulated by phospholipid/water blends using high pressure homogenizer. In contrast to the preparation technique with high pressure homogenizer, DAC is a fast, easy and straight forward method without specific need of know-how. Moreover, a small scale production for lab scale study is feasible using DAC, which directly lowers the cost. In addition, aseptic production is easily achieved with the method using a hermetically sealed sample vial.

In Massing's [147] study, the lipid blends consisting of hydrogenated egg phosphatidylcholine and cholesterol of a ratio of 55:45 (mole/mole) was homogenized with aqueous solution (Batch size: 0.25g-3.7g) by DAC (150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany) using brown injection vials as sample container. In their study, glass beads or stainless steel beads were added to the sample mixture as homogenizing aid. Multiples of 5 min-runs were performed until the desired mixing time was reached. After production, the VPGs were redispersed with a double volume of 0.9% sodium chloride solution to produce a liposome dispersion. The liposome size was used to evaluate the process parameters. When the lipid concentration in the blends varied from 350mg/g to 450mg/g, the liposome size did not significantly differ while an even higher lipid concentration of 500 mg/g led to a slightly higher mean liposome size and variability. Both the duration of DAC-homogenization and DAC-speed had influence on the size and the size distribution. A mixing period over 30 minutes at the speed of 3540 rpm was found necessary to obtain smallest liposomes and the lowest variability in mean size. Comparably small particles can be obtained from VPGs produced by High pressure homogenizer within a much shorter processing period. This contrast offered the hint that DAC generated a gentler shear force compared to High pressure homogenizer, which might be more applicable for entrapping sensitive compounds, e.g. proteins. Adding glass beads or stainless steel beads, regardless of the amount and size, all seemed to improve the mixing efficiency resulting in smaller liposome sizes (less than 100nm) and more homogenous distributions. Without adding the beads, the particle size was in the range from about 110nm to160nm. Moreover, the influence of the batch size (from 0.25g to 4g) was also evaluated and no significant influence on the liposome sizes was found. Hence, they have concluded an optimized process (Table. I-3) to prepare small liposomes (around 60nm) in a highly reproducible manner. However, a broad particle distribution was observed for VPG' dispersions prepared by DAC which was the same situation with those prepared by High pressure homogenizer. The trapping efficiency of the model compound calcein was higher in the case of VPGs prepared by DAC compared with HPH.

Table. I-3 Optimized parameters for VPGs' production with DAC process[147]

Lipid contwnt	Batch size	Duration of DAC	DAC-speed	Homogenizing aid
400 mg/g	0.5 g	30 min or longer	3540 rpm (maximum)	glass beads 1 mm, amount: 100% (equal amount as compared to batch amount)

As mentioned above, VPGs can been utilized as storage-stable intermediates for liposomes, as well as semisolid depot formulations for sustained release of entrapped drugs [143]. For the former purpose of developing a VPGs based liposomal formulation, DAC was fully demonstrated as a suitable method beside high pressure homogenizer for production of small liposomes which are applicable for parenteral administration. Moreover, autoclaving is no longer necessary since using hermetically sealed sample vial in addition with inert gas can easily provide a sterile production environment for entrapping sensitive compounds e.g. proteins in VPGs. The lysophosphatidylcholine generated in the sterile process conditions of DAC was lower compared with the process of High pressure homogenizer plus autoclaving (0.23% vs. 1.2%). In contrast to the High pressure homogenizer where a relatively large batch size is normally necessary, various batch sizes from below a gram up to several kilo grams can be processed by using different sizes of sample vials and DACs.

Moreover, it was predicted that sensitive drugs and bioactive compounds e.g. peptides, proteins could be entrapped within VPGs by DAC without significant degradation since the processing temperature could be maintained low by dividing the mixing into a series of short DAC-runs combined with sample cooling in between [147].

In summary, major problems for the VPG production by HPH and further liposome preparation (including overly large batch sizes, sterility and safety issues or the harsh conditions which limit the entrapment of sensitive compounds) may be overcome by using DAC.

4.3.2. CHARACTERIZATION OF VPGS

The morphology of VPGs was revealed by freeze-fracture electron microscopy [150-151]. The inner structure was described as a matrix of densely packed vesicles within the semisolid pastes. The influence of the mechanical stress, lipid content, and lipid type on the morphology was studied. When magnetic stirring was used for processing, very heterogeneous multilamellar vesicles are found whereas after high pressure homogenization, the pastes predominantly consist of homogenous small and unilamellar vesicles as long as the lipid content is less than 450mg/g (Figure I-4).

However, the characteristics of the vesicles within homogenized pastes depend on the lipid content. When a higher lipid content is applied, large multilamellar vesicles and planar lamellar stacks are found beside small unilamellar vesicles which leads to more heterogeneous in size extending to a range of several 10μm (Figure I-5) [140]. The gel pastes prepared with lipid blends containing DMPC, DPPC or mixtures thereof showed domains of ripple phases beside areas of vesicular character, which indicated incompletely hydration.

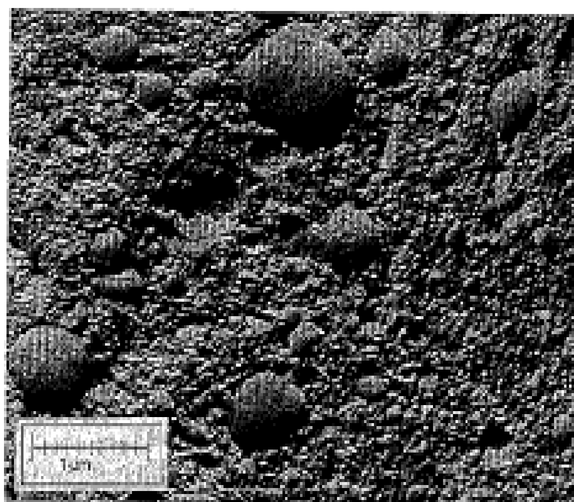


Figure I-4. Freeze-fracture electron micrograph of vesicular phospholipid gel based on 450mg/g lipids, soy PC, prepared by high pressure homohenization (10 cycles at 70MPa). Predominantly small unilamellar vesicles (SUVs) in the size range below 100nm, with some larger vesiclesin the several hundred nanometer range [50, 140, 143].

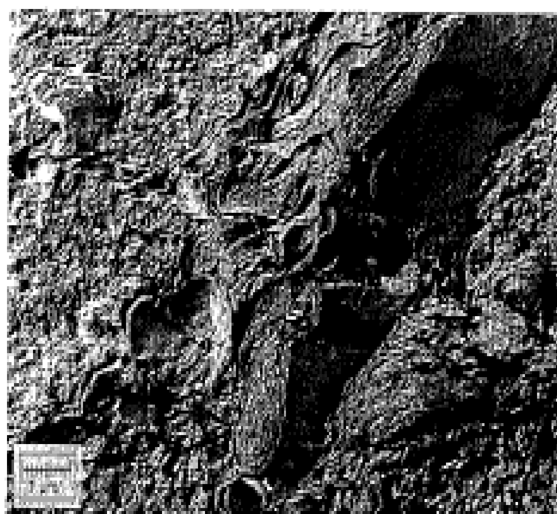


Figure I-5. Freeze-fracture electron micrograph of vesicular phospholipid gel based on 500mg/g lipids, soy PC, prepared by high pressure homohenization (10 cycles at 70MPa). Partly small unilamellar vesicles (SUVs) in the size range below 100nm, as well as larger vesicles in the micrometer-range [50, 140, 143].

4.3.3. APPLICATION OF VPGS FOR DRUG DELIVERY

4.3.3.1. VPGS BASED LIPOSOMES

VPGs have emerged both as intermediates for liposome production and as semisolid depot formulations for loco-regional sustained release of drugs upon implantation or injection.

By mixing with excess aqueous medium and mechanical agitation, VPGs can be transferred into conventional liposome dispersions [152]. Redispersing the VPGs by using a ball mill, the occurrence of larger particles could be widely suppressed, as indicated by photon correlation spectroscopy.

With the technique of VPGs based liposomes, encapsulation efficiencies of hydrophilic compounds into small unilamellar liposomes are improved compared with other liposome preparation techniques so that removal of unencapsulated drug may be avoided. Moreover, small and uniform vesicle sizes could be achieved at the same time. Improved storage stability is expected for the liposomes prepared this way.

Autoclaved VPGs have already been used in first animal experiments [153]. In general, steam sterilisation is considered applicable for VPGs which maintained their vesicular structure but showed a slight increase of vesicle size. This size drift was deemed irrelevant for safety despite the influence on the pharmacokinetics of liposomes redispersed from VPGs. For the application of VPGs as local-depot formulation, the bigger vesicle sizes and corresponding increase in packing density were assumed to slow down the erosion of the matrix and therefore lead to a slower release of the hydrophilic marker [11, 140, 154].

A liposomal formulation of gemcitabine based on VPGs was reported as a highly effective gemcitabine delivery system through intravenous administration [153]. The hydrophilic anticancer agent Gemcitabine can be entrapped in a stable manner into small liposomes through the VPG approach with a satisfactory drug entrapment efficiency and sufficient storage stability for potential clinical use. Redispersed VPGs consisted of 33% liposomally entrapped and 67% free gemcitabine. In vivo pharmacokinetics and antitumor activity showed efficiency compared to the conventional formulations due to the sustained release and passive tumor targeting

of the liposomes. Using the VPG concept, another antitumor agent Vincristine [155] was passively entrapped in small liposomes with efficient entrapment and good retention without using deleterious acidic pH values. Furthermore, such a liposomal formulation allowed application without removing non-entrapped drug, and provided a prolonged shelf-life of the liposomes. By redispering VPGs, it was possible to develop liposomal formulation of Bendamustine [156] which was difficult with conventional liposome preparation techniques because of the insufficient stability of Bendamustine in aqueous solutions. A sterile DAC process with optimized processing parameters was performed to achieve small particles within VPGs. The gel pastes were subsequently redispersed into liposomes with an average size of about 62.5 nm and an entrapment efficiency of around 41.5%. Entrapped Bendamustine showed a better stability in aqueous solution. Moreover, an enhanced antitumor efficacy was observed in cell culture for liposomal Bendamustine.

4.3.3.2. VPGs as local depot systems

Local delivery is frequently the only feasible strategy to achieve locally high concentrations of therapeutic substances. Some proteins, such as chemokines, cannot be delivered systemically because their mode of action requires a concentration gradient. Local rather than systemic delivery may be the preferred route of protein delivery in the case of that: (i) the protein can not reach the site of action; (ii) the protein acts undesirably on other organs; (iii) high local concentrations are needed; or (iv) local concentration gradients need to be established [157-158].

The use of the undiluted VPGs as implants for the local administration of drugs was applied to meet the requirement. The multivesicular morphology of VPGs offers the potential as local depot as the pastes appeared to retain entrapped drugs when in contact with excess aqueous medium [50, 138, 143].

Tardi [11] has used Calcein (Mw 623) as a hydrophilic model to evaluate the in vitro release behaviour of VPGs. The small water-soluble compound could be retained within the matrix over extended periods of hours up to several weeks depanding on the lipid content within the various formulations of VPGs. The released calcein and released phosphatidylcholine were both quantified so that two mechanisms controlling the release were found occurring at the same time: (1) erosion of the matrix with release of liposomal compounds and the compounds trapped in between

(2) diffusion through the matrix. In addition, increasing the lipid content of lipids decreases matrix erosion rate and consequently slows down the release of the entrapped model, whereas no influence was observed of the entrapped calcein.

Kaiser [159] has prepared different formulations of 5-fluorouracil loaded VPGs with hydrogenated soy phosphatidylcholine and cholesterol by high pressure homogenization. The entrapment efficiency of liposomal 5-fluorouracil redispersed from VPGs was around 40%. A prolonged half-life time of the antitumor agent released from VPGs was found to be in the order of 4–5 h and the kinetics was typical for matrix-controlled drug diffusion. Thus it was suggested VPGs were applicable as implants with controlled release properties or, after redispersion, as intravenously injected liposomal formulations.

Another potential application concerns prolonged systemic delivery of peptide hormones after implantation [13, 160-161]. VPGs were prepared with 300mg/g-500 mg/g egg phosphatidylcholine and 0.5–10 mg/g cetrorelix acetate (CXA) using High pressure homogenization. The in vitro release behaviour of the peptide loaded VPGs were influenced by the lipid content, the drug content within the formulations, and also the interactions between the two factors. A fast complete release within 24hrs was observed for the formulation consisting of 0.5 mg/g CXA and 400 mg/g lipids while a sustained release of therapeutically relevant CXA doses over up to 6 weeks also appeared feasible with the formulation containing 8.5 mg/g and 360 mg/g lipids. Erosion of the phospholipid matrix was found to be the main release mechanism, following zero order or first order kinetics depending on the composition of the VPGs. CXA-concentration dependent drug–drug or drug–lipid interactions were assumed to be responsible for the change in release kinetics and the decrease of CXA release at high concentrations of the peptide. VPGs were thus considered as a promising new approach for the sustained release of peptide hormones.

In addition, Farkas [162-163] has incorporated β-sitosterol into VPGs of hydrogenated phospholipids and chlorhexidin digluconate, which led to the phase transition of the gels to the less ordered fluid vesicular state. The membrane deformability and the system dispersity were increased as a result of increasing the β-sitosterol ratio in the lipid blends. Moreover, the release extent of chlorhexidine from the matrix was significantly increased.

As a summary, current work has shown promising results of VPGs as controlled delivery system. The feasibility of the system has been demonstrated by varying the components of VPGs to modulate the in vitro release behaviour.

CHAPTER II: AIM OF THE THESIS

Lipid based carriers have attracted increasing scientific and commercial attention during the least few years for controlled delivery of proteins concerned with stability issues [164].

Vesicular phospholid gels have emerged as a novel lipid based formulation with a three-dimensional network. The gel formulations can be applied both as intermediates for liposome production and as semisolid depot formulations for loco-regional sustained release of drugs upon implantation or injection [50, 123, 143, 152].

As a loco-regional application, the sustained release character of vesicular phospholipid gels has been demonstrated using the small water soluble compounds [152, 165]. First important success was achieved by loading of VPGs with the small peptide hormone cetrorelix: in vitro release experiments showed a wide range of cetrorelix release periods, ranging from 24 hours to a predicted sustained release of over more than 3 months [13]. However, still it was unclear whether VPGs might be suitable for the delivery of proteins due to the inherent instabilities of the macromolecules. Therefore, the first major aim of the present work was to develop various protein-loaded VPGs.

Second, dual asymmetric centrifuge was introduced as an alternative for the traditional preparation method of VPGs with high pressure homogenisation [147]. Thus lab scale preparation became feasible. Our second part of work was to develop a suitable preparation method for loading protein into VPGs.

Moreover, morphology and rheological behaviour of VPGs have been investigated in previous work [151-152]. For the intro release tests, Grohganz [160, 166] has developed a RP-HPLC method to quantify released cetrorelix from the gel matrix. Various organic solvents and surfactants have been tried out to extract the peptide from the release fractions since liposomes in the release fractions got strong interference if they are not disrupted. However, for the protein loaded VPGs, we have to develop suitable characterization methods both for the gel matrix and for the entrapped protein.

As a brief, the project was to focus on:

- Preparation of VPGs with various phospholipid contents and compositions, to evaluate their influence on the physical character of VPGs, as well as the influence on the in vitro release behaviour.
- Preparation of VPGs with various proteins, for each protein to evaluate the influence of protein concentration on the physical character of VPGs, as well as the influence on the in-vitro release behaviour.
- Establishment of analytical methods for various proteins and phospholipids.
- Adaption of the release system.
- In vitro release tests of the various VPG formulations and analysis of the released fractions.
- Investigation on the in vitro release kinetics of the formulations.
- Establishment of an ELISA method for the in vivo experiment.
- Preparation of VPGs for intra-articular application in the animal models.

CHAPTER III: MATERIALS AND METHODS

1. MATERIALS

1.1. FLUORESCEIN ISOTHIOCYANATE-DEXTRAN (FITC-DEXTRAN)

FITC-dextran (FD-40, with an average molecular weight of 40kDa) was supplied from Sigma-Aldrich, Steinheim, Germany. The molecular structure is shown in Figure III-1 [167]. FITC-dextran is a water-soluble yellow powder. It has good stability at pH 4-10 at room temperature and excellent biocompatibility in vivo. FITC-dextrans have been used as tracers for studying microcirculation or membrane permeability in vivo [168-170]. Besides, FITC-dextrans were also applied as hydrophilic macromolecular models to evaluate drug delivery systems [171-172].

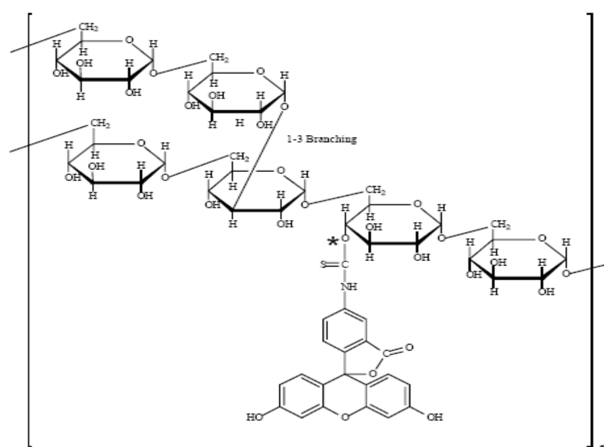


Figure III-1. Structure of FITC-dextran.

1.2. ERYTHROPOETIN (EPO)

EPO is a glycoprotein with a molecular weight of 30.4kDa, 40% of which is ascribed to carbohydrates [172]. The protein molecule contains a single-chain with 165 amino acids [173]. EPO is produced primarily by the kidney and to a lesser extent in the liver. The physiological function of EPO is to regulate the red blood cell production [174-176]. Besides, the protein is also involved in the wound healing process [177] as well as the brain's response to neuronal injury [178]. The iso-electric point of EPO is around a pH value from 4.2 to 4.6 [179].

The protein bulk used in this work had a concentration of either 0.65 mg/ml or 0.75mg/ml in 20 mM phosphate buffer pH 7.2, containing 20 mM sodium chloride. However, further concentrating of these protein bulks was needed for the study.

1.3. GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF)

Recombinant human (rh-) G-CSF was provided with a concentration of 4.04 mg/ml. The protein buffer consisted of 10mM sodium acetate buffer, 0.004% Tween and 5% Sorbitol with a pH of 3.9.

Mouse granulocyte colony-stimulating factor (G-CSF) was first recognized and purified by Rafael Duarte in 1983 [180]. and the human form was cloned later in 1986 [181]. G-CSF is a single, non-glycosylated, polypeptide chain containing 175 amino acids and having a molecular mass of 18.8 kDa. G-CSF is a cytokine which naturally regulates the survival, proliferation, and differentiation of hematopoietic cells [182-183]. rh G-CSF has been therapeutically utilized to increase the production of neutrophils, as well as to mobilize the hematopoietic stem cells [183]. However, due to the short human plasma life (ca. 3.5hr) and limited therapeutic efficiency, multiple injections are normally required to achieve the desired therapeutic outcome [184].

1.4. MONOCLONAL ANTIBODY IMMUNOGLOBULIN G (MAB)

The antibody used in the study is a monoclonal recombinant human IgG antibody. The monoclonal antibody is produced by a type of immune cells that are all clones of a single parent cell. The IgG antibodies are composed of 4 peptide chains with molecular weight of about 150 kDa. There are two identical heavy chains (ca. 50kDa) linked to each other and to a light chain (ca.25kDa) each by disulfide bonds. The resulting tetramer forms the Y-like antibody. Each IgG has two antigen binding sites. IgG is the most abundant immunoglobulin constituting 75% of serum immunoglobulins in humans. IgG antibodies are predominately involved in the secondary immune response. The presence of specific IgG generally corresponds to maturation of the antibody response [185-188].

The monoclonal IgG antibody bulk was provided with a concentration of 17.6mg/ml in 50 mM phosphate buffer pH 6.4, containing 150mM sodiudm chloride and 0.1% BSA.

1.5. PHOSPHOLIPIDS

The following phospholipids were kind gifts from Lipoid GmbH (Ludwigshafen, Germany): Egg lecithin (E 80) containing 80%-85% phosphatidylcholine (PC) was used as the basic liposome forming phospholipid. The other major compounds in E 80 are specified (certificate of analysis) as: phosphatidylethanolamine (7.0–9.5%), lysophosphatidylcholin (less than 3%), sphingomyelin (2–3%) and triglycerides (less than 3%). DOTAP (1, 2-dioleoyloxy-3-trimethylammonium propane chloride) was used as a cationic, DPPA (dipalmitoylphosphatidic acid) as an anionic phospholipid.

The other phospholipids used in the work were generously provided by PHOSPHOLIPID GmbH (Cologne, Germany).

1.6. CHEMICALS AND REAGENTS

Acetic acid (100 %), p.A., VWR International GmbH, Darmstadt, Germany
Acetonitril, CH_3CN, gradient grade, VWR International GmbH, Darmstadt, Germany
Basic fibroblast growth factor, bFGF, PROSPEC, Israel
Chloroform, $CHCl_3$, p.A., Merck KGaA, Darmstadt, Germany
Dipotassium hydrogen phosphate, K_2HPO_4, p.A., VWR International GmbH, Darmstadt, Germany
Disodium hydrogen phosphate dihydrate, $Na_2HPO_4 \times 2\ H_2O$, p.A., VWR Int. GmbH, Germany
Ethanol, p.A., Merck KGaA, Darmstadt, Germany
FLuorescamine, Sigma-Aldrich, Steinheim, Germany
Hexane, p.A., Merck KGaA, Darmstadt, Germany
Lyophilised bovine serum albumin, 66kDa, Fraction V, Sigma-Aldrich, Steinheim, Germany
Methylene blue, VWR International GmbH, Darmstadt, Germany
N, N-Dimethylformamide, DMF, Merck KGaA, Darmstadt, Germany
Octyl ß-D-glucopyranoside, ODG, Sigma-Aldrich, Steinheim, Germany
Potassium chloride, KCl, p.A., VWR International GmbH, Darmstadt, Germany
Sodium azide, NaN_3, p.A., VWR International GmbH, Darmstadt, Germany
Sodium borate, Sigma-Aldrich, Steinheim, Germany
Sodium chloride, NaCl, p.A., VWR International GmbH, Darmstadt, Germany
Sodium deoxycholate, DOC, Thermo Fischer Scientific Inc
Sodium dihydrogen phosphate monohydrate, $NaH_2PO_4 \times H_2O$, p.A., VWR Int. GmbH, Germany
Sodium dodecyl sulphate, $C_{12}H_{25}NaO_4S$, VWR International GmbH, Darmstadt, Germany
Sodium hydroxide, NaOH, p.A., VWR International GmbH, Darmstadt, Germany
Trifluoroacetic acid, TFA, p.A, Merck KGaA, Darmstadt, Germany
Triton X-100, Sigma-Aldrich, Steinheim, Germany
Tween®80, Polysorbate 80, Serva, Heidelberg, Germany
Ultra pure water, taken from Purelab Plus®, USF GmbH, Ransbach-Baumbach, Germany

2. METHODS

2.1. UP-CONCENTRATION OF PROTEINS

The protein bulk solutions were concentrated to the desired concentrations by ultrafiltration using Vivaspin tubes (Sartorius Stedim Biotech GmbH, Goettingen, Germany). According to the molecular weight of the proteins, various Vivaspin tubes were used as shown in Table III-1.

The concentrated proteins were quantified by UV photometry at 280nm and calculated with the extinction co-efficiency. For each protein, the integrity proved to be maintained from the ultrafiltration process by HPLC.

Table III-1. Concentrating of proteins with Vivaspin tubes

Protein	Concentrating fold	Centrifugal concentrators	Centrifugal conditions	Extinction coefficiency at 280nm.
EPO	Ca. 10 x	Vivaspin 20ml, 5kDa membrane cut-off.	Swing Bucket Rotor, Centrifugation at 3000rpm, 15°C, multiple runs of 30minutes till desired concentration achieved	0,743ml/mg.cm [189]
G-CSF	Ca. 2-8 x	Vivaspin 2ml, 3kDa membrane cut-off.		0,860ml/mg.cm [190]
mAb-IgG	Ca. 2-4 x	Vivaspin 20ml,100kDa membrane cut-off.		1,499ml/mg.cm

2.2. PREPARATION OF VESICULAR PHOSPHOLIPID GELS

In the first steps, FITC-dextran loaded VPGs were formulated either by High pressure homogenization or Dual asymmetric centrifugation to compare the preparation methods. Proteins were loaded into VPGs by DAC.

2.2.1. PREPARATION BY HIGH PRESSURE HOMOGENIZATION

The preparation method of VPGs using High pressure homogenization was first described by Brandl [50, 139, 143, 151-152]. FITC-Dextran was used as the macromolecular model instead of proteins to evaluate the suitability of the preparation method. Briefly, FITC-dextran was dissolved in destilled water. Phospholipids were subsequently added to the FITC-dextran solution. The blends

were allowed to swell for 10 minutes and then fed into the high-pressure homogeniser (APV Micron Lab 40, APV Homogeniser, Luebeck, Germany). The mixture was homogenised 2-7 times at 70 MPa at room temperature. The batch size was between 40 and 45 g. After homogenization the gels were stored in capped glass vials at 2–8°C.

2.2.2. PREPARATION BY DUAL ASYMMETRIC CENTRIFUGATION

The preparation of VPGs using Dual asymmetric centrifugation was based on the method described by Massing et al [191]. Modifications were made in order to avoid potential heat-stability problems with the proteins. Briefly, FITC-dextran or proteins were incorporated by direct loading, i.e. EPO solution (formulated in 20mM PBS buffer, pH 7.2) was added to the accurately weighed lipids and homogenized by means of a dual asymmetric centrifuge (SpeedMixer^{TM} DAC 150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany) (Figure III-2.) in a 25ml cylindrical container (PP, Duerrmann GmbH, Hohenlinden, Germany). For homogenization a process speed of 3500 rpm was utilized in all experiments. According to the first report on the utilization of DAC and the fact that a continuous long-time centrifugation run causes a significant temperature increase in the product, multiple centrifugation steps of 1.5-minutes were added up to a total mixing time of 45 mins with interrupts for cooling at 2-8°C. In addition, methylene blue loaded VPGs were prepared to demonstrate the homogeneity distribution of VPGs.

Figure III-2. The dual asymmetric centrifugation, SpeedMixer^{TM} DAC 150 FVZ

2.3. Microscopic studies

Microscopic studies of protein loaded VPGs were carried out using an Olympus microscope. Magnifications at 400 x was applied in order to visualise the surfaces of the VPGs. Microscopic pictures were collected with a TK-C1380 colour video camera (camera digital – ½ inch CDD, JVC Professional Products GmbH, Friedberg, Germany) coupled with Lucia software.

2.4. Rheology

The rheologic behavior of VPGs was studied on the rotational rheometer (Physica MCR 100, Anton Paar GmbH, Ostfildern, Germany) with cone and plate geometry. The shear rate was set to 10-100 s^{-1}. About 1g of VPGs were used for each measurement. The temperature was set to 25°C. The viscosity of VPGs was compared at the fixed shear rate of 37.9 s^{-1}.

2.5. Texture analysis

Texture analysis was performed to evaluate the gel strength of VPGs. The measurement was performed on a texture analyzer (TA.XT plus, Stable Micro Systems, UK) with a micro-probe of 4mm in diameter. The test speed was set to 0.50mm/sec. A gel volume of 1.5ml (in u-bottom eppendorf tubes) was used for each measurement. The value given by the texture analyzer is the gel strength expressed in gram versus time plot according to the standard "Bloom test" for determination of the gelatine's gel strength.

2.6. Dispersions of VPGs

VPGs were accurately weighed and dispered with excess aqueous buffer solutions to form the liposomes. Vigorous vortex (Heidolph REAX Top, Heidolph GmbH, Germany) was applied to offer a mechanical agitation.

2.7. Particle size analysis and zeta potential measurement

Particle size analysis and zeta potential measurement was conducted on the Zetasizer nano ZS (Malvern Instruments, UK). The vesicle size of liposomes dispersed from VPGs and from release fractions were analyzed and represented as intensity-weighted averages.

The Zeta potential is the electric potential at the shear plane of a particle [191]. The ionic interaction between proteins and lipids was evaluated by measuring the zeta potential of dispersed VPGs. The test temperature was set to 25 °C.

2.8. TURBIDITY

The turbidity of the release fractions was determined to reflect the release degree of the VPGs matrix. The Nephla turbidity photometer (Dr Lange GmbH-Berlin, Germany) used in the study has a measurement range of 0.001-1000 FNU at the working wavelength of 860 nm.

2.9. EXTRACTION OF PROTEINS FROM THE VPG MATRIX

Liposomes in the dispersions of VPGs or in the release fractions from VPGs were found to have strong interference with the entrapped proteins in characterization and quantification. Therefore, extraction processes had to be applied, which allows the quantitative recovery of the proteins from the liposome fractions without having detrimental effects on the protein integrity. Since individual proteins exhibit. large differences in extraction process, a case-by-case investigation was performed testing several organic solvents and surfactants.

2.9.1. EXTRACTION OF EPO

Chloroform was found suitable for EPO extraction. For disperions or release fractions containing uncharged and negatively charged lipids, 0.3 ml of chloroform were added per ml of release medium sample volume. In the case of samples containing cationic lipids, 200mM sodium chloride was added to reduce the ionic interaction between EPO and cationic lipids before 0.3ml chloroform was added per release sample volume. Afterwards the mixtures were vortexed and centrifuged (at 3000rpm, 15°C, 30 mins). The upper aqueous phase was analyzed by reverse phase-HPLC to quantify the EPO content. The protein stability was evaluated by gel electrophoresis (SDS-PAGE).

2.9.2. EXTRACTION OF G-CSF

In order to extract G-CSF from liposome containing samples, the following steps were performed: 1.5mg/ml phospholipids (E 80) were added to the sample solutions.

The pH was adjusted to 3.5. 0.4ml/ml DMF was then added to the dispersions. Lipids were removed by centrifugation (3000rpm, 15°C, 30mins). Protein concentration was determined by using a Reverse phase-HPLC, and protein stability was evaluated by gel electrophoresis (SDS-PAGE).

2.9.3. EXTRACTION OF MAB IGG

0.015% sodium deoxycholate (DOC) was added first to reduce the interaction between the antibody and lipids. After incubating at 37°C for 20mins, 1ml/ml chloroform was added to dissolve the lipids. The protein remains in the aqueous phase after centrifugation at 3000rpm for 30mins (15°C). Protein concentration was determined by using a micro BCA assay (Pierce, Bonn, Germany).

2.10. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

The integrity of proteins after incorporation was analyzed by SDS-PAGE with non-reducing conditions. The proteins were extracted by applying the suitable method as described in 2.10. The protein containing samples were diluted with Tris-buffer pH 6.8 containing 2% SDS and incubated for 20min at 90 °C. Unstained molecular standard (Mark12 ™, Invitrogen, Groningen, Netherlands) was used as the molecular weight marker. For EPO and G-CSF, 20µl of the samples were loaded into the 10% Bis-Tris gel wells (NuPAGE® Novex 10 % Bis-Tris Pre-Cast Gel 1.0 mm, Invitrogen, Groningen, Netherlands). Electrophoresis was performed at a constant current mode of 40 mA in a MES running buffer (Invitrogen Groningen, Netherlands). The gels were stained either with silver staining kit (SilverXpress® Stain Kit, Invitrogen) or a coomassie blue kit (Colloidal Blue Staining Kit ® Stain Kit, Invitrogen) , and dried using a DryEase ® Gel Drying System (Invitrogen). For the monoclonal antibody, the electrophoresis was performed on the 7% Tris-acetate gels (NuPAGE® Novex 7% Tris-acetate Pre-Cast Gel 1.0 mm from Invitrogen, Groningen, Netherlands) in a 7% Tris-acetate/SDS running buffer (Invitrogen Groningen, Netherlands).

2.11. IN VITRO RELEASE TESTS

Flow-through release cells were made of Teflon material (Figure III-3, by the workshop of the Department of Chemistry and Pharmacy, LMU, Munich) based on the design described by Tardi [11]. The donor compartment (lower compartment) had

a rectangular cross-section in order to ensure a relatively constant contact area even if some portions of the VPG were lost due to erosion. The acceptor compartment (upper compartment) on the other hand showed a semicircular cross-section, which was beneficial for the facilitation of a laminar flow.

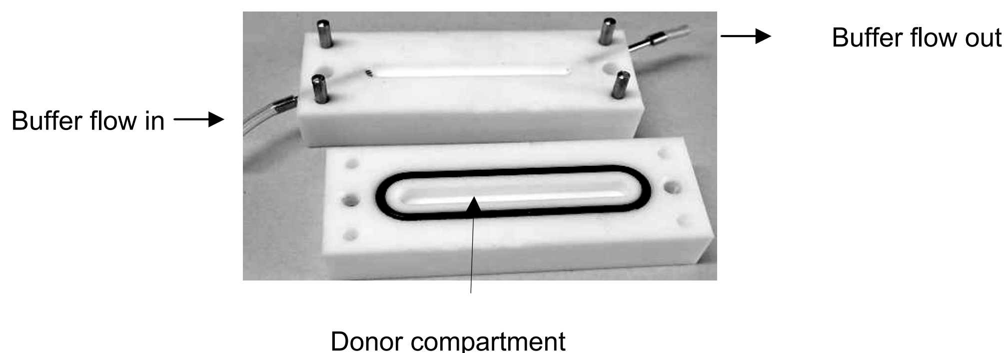


Figure III-3. The custom-made release cells for the in vitro tests, made by the workshop of the Department of Chemistry and Pharmacy, LMU, Munich

Teflon tubing was used in order to minimize the surface adsorption of the released proteins. In order to study the erosion of VPGs, no membrane was used between the donor and acceptor compartment. The cells were filled with 1g accurately weighed VPGs and kept at 37°C by a water bath (Thermomix; B. Braun Biotech International). The pumping rate was set to 1ml/hr by using a multi-syringe pump (KDS 220, KD Scientific Inc). For EPO and mAb IgG, 40mM PBS buffer pH 7.4 containing 80mM sodium chloride was used as acceptor medium while 20mM PBS buffer pH 7.4 was used for G-CSF release.

2.12. QUANTIFICATION OF RELEASED DRUGS

2.12.1. DETERMINATION OF FITC-DEXTRAN CONCENTRATION BY FLUORESCENCE PHOTOMETRY

The FITC-dextran concentration upon release fractions was determined by using Varian fluorescence spectrometer Cary eclipse. 1.8ml ethanol was added to 2ml samples to dissolve the liposomes. The excitation wavelength was fixed at 488 nm, and the emission was collected at 517nm.

2.12.2. QUANTIFICATION OF PROTEIN BY HPLC

Size exclusion-HPLC (SE-HLC) and Reverse phase-HPLC (RP-HPLC) are the two standard methods for protein analysis. SE-HPLC is often used in protein quantification and aggregation monitoring. RP-HPLC can be applied to assess the protein concentration and the amount of chemically modified protein e.g. oxidized species

2.12.2.1. SE-HPLC OF EPO

SE-HPLC of EPO was performed on a TSKgel size exclusion column (G3000SWXL, 7.8mm x 30.0mm, Tosoh Biosep, Stuttgart, Germany). The mobile phase consisted of 100 mM disodium hydrogen phosphate dihydrate and 200mM sodium chloride with a pH of 7.0. The flow rate was set to 0.7 ml/min and the injection volume was 100µl. EPO was detected at 215nm (UV detector, Spectra Series 1100, San Jose, CA, USA) with a retention time of about 12.5mins.

2.12.2.2. RP-HPLC OF EPO

RP- HPLC of EPO was performed on a Spectra-Physics HPLC system (San Jose, CA, USA), equipped with a ternary gradient pump (Spectra-Physics SP 8800), an autosampler (Spectra Series AS 100) and a UV detector (Spectra Series 1100). The system was controlled by HPLC ChromQuest software. The analysis was conducted on Phenomenex C18 Jupiter columns (5µm, 250 x 4.6mm, Aschaffenburg, Germany) with a flow rate of 0.5ml/min. The UV detector was operated at 215nm. The injection volume was 100µl. The eluents were: (A) 0.1% TFA in ultrapure water and (B) 84% CH_3CN (in ultrapure water) with 0.08% TFA. The gradient started with 80% eluent A, over a period of 10min decreased to 40% A and then lasted for 5min. Afterwards, eluent A decreased to 0% in 5 min, and finally returned to 80% over a 5min period.

2.12.2.3. RP-HPLC OF G-CSF

The ProSwift^{TM} RP-2H column (ProSwift^{TM}, 4.6 x 50mm, Dionex) was used for RP-HPLC of G-CSF. The UV detector was operated at 215nm. The injection volume was 100µl. Gradient elution was performed as shown in Table III-2. The eluent A consisted of 0.12% TFA in destilled water, and the eluent B consisted of 90% CH_3CN (in ultrapure water) and 0.1% TFA.

Table III-2. Gradient elution for RP-HPLC of G-CSF

Time/min	0	5	10	25	30	35	40	45
Eluent A%	100	80	60	60	40	20	0	100
Eluent B%	0	20	40	40	60	80	100	0
Flow rate					1.5ml/min			

2.12.3. QUANTIFICATION OF PROTEIN BY PROTEIN ASSAYS

2.12.3.1. FLUORESCAMINE PROTEIN ASSAY

Fluorescamine assay was assessed for the quantification of G-CSF in the lipid containing samples. The assay was based on the principle that fluorescamine reacts rapidly with primary amines in proteins, to yield highly fluorescent products [192-193]. A modified process using the 96 well-microplate as described by Lorenzen [194] was performed. Briefly, 150µl of the protein containing samples (in PBS buffer pH 7.4) and standards were pipetted into microplate wells (Nunc MaxiSorp™, Roskilde, Denmark). Fluorescamine was dissolved in acetone resulting in a concentration of 3mg/ml. The microplate was placed on a microplate shaker and 50µl of the fluorescamine solution was added to each well. After shaking for a minute, the fluorescence was determined by using an excitation wavelength of 400nm (bandwidth: 20nm), and an emission wavelength of 460nm (bandwidth: 20nm).

2.12.3.2. CBQCA ASSAY

The CBQCA Protein Quantitation Kit (Molecular Probe, Invitrogen) was evaluated for the quantification of MAB. The assay utilizing the ATTO-TAG CBQCA reagent (3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde) has proven a rapid and highly sensitive method for quantitating amines in solution, including the accessible amines in proteins [195-197].The protein samples were diluted into 0.1 M sodium borate buffer pH 9.3. 5 µL of 20 mM KCN (supplied in the kit) was added and the reaction was started by adding 10µl of 5mM CBQCA (in 0.1 M sodium borate buffer pH 9.3). After incubating for 1 hour (room temperature, protected from light), the samples were measured with the FLUOstar Omega multifunctional micro-plate reader (BMG LABTECH GmbH. Offenburg, Germany). The excitation wavelength was set to 485nm, and the emission was collected at 580nm.

2.12.3.3. MICRO BCA ASSAY

The micro BCA protein assay is based on the following reactions: First, the working reagent (Cu^{2+}) is reduced by protein in an alkaline environment [198-199]; Bicinchoninic acid (BCA) subsequently chelates with the reduced (Cu^{1+}) cation to form the intense purple-colored reaction product. The water-soluble product exhibits a linear behaviour with increasing protein concentrations.
The procedure for microplate mode followed instructions from the manufacture. Briefly, 150µl of the working reagent was added to 150µl sample solutions in the microplates (Nunc MaxiSorp™, Roskilde, Denmark). After incubating at 37°C for 2hrs, the samples were measured at 562nm. Prior to assay the lipids containing samples, protein extraction (as described in 2.10) was needed to eliminate the interference of lipids. The concentration of the mAb IgG from release fractions or from VPGs' redispersions was determined by using this micro BCA assay. It was a major consideration in developing the extraction method for the antibody that some often used organic solvents and surfactants including Triton, DMF, and Tween were reported to have interference with the assay at a certain amount [200-201]. The micro BCA assay was used for the quantification of MAB in absence and in presence of lipids in the context.

2.13. QUANTIFICATION OF PHOSPHATIDYLCHOLINE BY THE ENZYMATIC ASSAY

An enzymatic assay (LabAssay Phospholipid, Wako, Neuss, Germany) was used to quantify the phospholipid content in the release medium following the manufacturer's instruction [202]. Shortly, phospholipids in the samples are hydrolyzed to choline in a reaction catalyzed by phospholipase D. The subsequent reaction of choline produces hydrogen peroxide, which causes the quantitative production of a blue pigment. The assay was performed in 96-well microplates (Nunc MaxiSorp™, Roskilde, Denmark). The amount of phospholipids E 80 in the samples were determined by measuring the absorbance at 600nm using the Fluostar Omega multimode microplate reader (BMG LABTECH, Offenburg, Germany).

2.14. ENZYME-LINKED IMMUNOSORBENT ASSAYS OF G-CSF

In order to assess the protein concentration in the serum/plasma samples, G-CSF was assayed by the enzyme-linked immunosorbent assays (ELISA). For example,

the Quantikine (DCS50, R&D Systems) Human G-CSF Immunoassay used in the study was a solid phase ELISA designed to measure G-CSF in cell culture supernates, serum, and plasma. It contains E. coli-expressed recombinant human G-CSF and antibodies raised against the protein. It has been shown to accurately quantitate recombinant human G-CSF without obvious cross reactivity with rabbit serums [203]. The detailed steps of the assay followed the manual by the supplier.

Another two ELISA kits: RayBio Human G-CSF ELISA from RayBiotech., Human G-CSF Instant ELISA Kit from Bender MedSystems, have also been assessed in the work, but were not chosen finally due to insufficient results.

CHAPTER IV: TENTATIVE EXPERIMENTS

In a first approach, FITC-dextran (40KDa) was applied as hydrophilic macromolecular model to establish the preparation and characterization methods.

1. PREPARATION OF VPGS USING FITC-DEXTRAN AS DRUG MODEL

The preparation of VPGs can be performed either by high pressure homogenization (HPH) [11, 140] or by dual asymmetric centrifugation (DAC) [147]. Using a HPH, it is suitable for the production of VPGs which can be redispersed into very small (less than 100nm) and homogeneous liposomes [139, 143, 151-152]. However, a relatively large batch size is necessary (tens of grams) even for a lab scale HPH. In the early stages of development of VPGs formulations there is a clear need to produce batch sizes of about several grams or even less.

With the introduction of the DAC, production of VPGs with small batch sizes (about several grams) has become feasible, which is especially useful for entrapment of expensive biological materials such as proteins [147].

1.1. HIGH PRESSURE HOMOGENIZATION

The HPH method to prepare VPGs was first described by Brandl [10]. Briefly, FITC-dextran (40KDa) was dissolved in destilled water. Phospholipids were subsequently added to the FITC-dextran solution. The blends were allowed to swell and then fed into the high-pressure homogeniser (APV Micron Lab 40, Luebeck, Germany).

Based on the previous findings [11, 152], a lipid content of 400mg/g (egg PC, PHOSPHOLIPON) was used for VPGs' preparation by HPH. The concentration of FITC-Dextran was 2mg/g for basic research. The mixture was homogenized at 70MPa for 2-7 cycles. The batch size was between 40g and 45g.

All the obtained VPGs showed a semi-solid consistency. The viscosity of the VPGs increased with the increasing homogenization cycles judged by visual examination. The gel strength of VPGs was determined by texture analysis (The texture analysis method used here is according to the "Bloom test" for determination of the gelatine's gel strength which is normally expressed in the mass values).

The gel strength value of VPGs was increased from 18g to 86g as the homogenization cycles increased from 2 to 7 times (Figure IV-1).

VPGs were redispered with excess aqueous buffer solutions (PBS buffer pH 7.4) to form the liposomes by applying vigorous vortex. Over 90% of the particles were in the range of 87nm-129nm for all the VPGs prepared by HPH. The vesicle size was slightly decreased when the process cycle was increased from 2 to 6 times while the particle size was much smaller for the VPGs homogenized after 7 cycles (Figure IV-2).

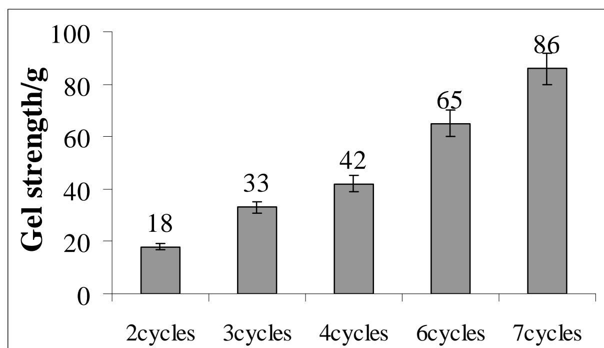


Figure IV-1. Gel strength (mean±SD, n=3) of VPGs prepared by high pressure homogenization. VPGs were processed by various homogenization cycles (as indicated in the figure).

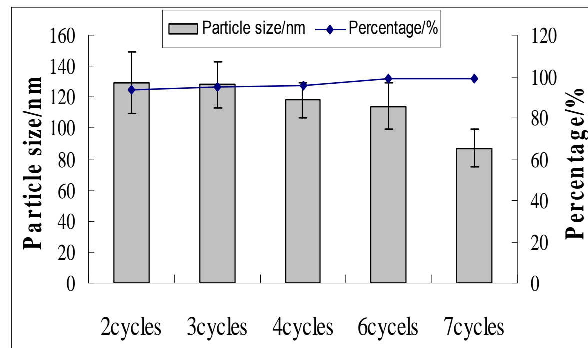


Figure IV-2. Particle size of VPG dispersions (mean±SD, n=3), influence of homogenization cycles (as indicated in the figure).

1.2. DUAL ASYMMETRIC CENTRIFUGATION

As described in the Chapter I, dual asymmetric centrifugation (DAC) is a double centrifugation technique where the sample is not only rotated around a central axis but also around a second axis in the centre of the sample container. Due to this combination of two contra-rotating movements efficient homogenization of viscous materials is possible. Compared with HPH, the manufacturing process using DAC can be easily modulated to control the system temperature which might be an essential factor for protein entrapment. Moreover, sterile production can be easily realized by performing the centrifugation with a hermetically sealed sample container [147].

DAC (SpeedMixerTM150 FVZ, Hauschild) was used for the preparation of VPGs. The preparation process was based on the method described by Massing et al [147]. FITC-dextran was incorporated by direct loading: the FITC-dextran solution was added to accurately weighed lipids resulting in a semisolid dispersion.

Homogenization was performed with a centrifugation speed of 3500rpm. In previous studies, glass beads were used as homogenization aid. However, in our work we omitted the use of beads in order to avoid potential heat-stability and protein adsorption problems. Since a long time run of centrifugation causes the temperature increasing of the system which might have interference on the bioactivity of the protein, multiples of 1.5-minute runs were set up to a total mixing time of 45 minutes. Using this procedure an average process temperature of below 40°C could be maintained during the production, which appeared appropriate for the incorporation of sensitive proteins.

The VPGs were prepared with various contents of lipids (egg PC, PHOSPHOLIPON) in the range from 400mg/g to 550mg/g. The concentration of FITC-dextran was 2mg/g in all the formulations. The gel strength was determined by texture analysis. As it can be seen in Figure IV-3, increasing the lipid content resulted in higher gel strength. The gel strength value was 5g for VPGs based on 400mg/g lipids while it was 60g for VPGs containing 550mg/g lipids.

VPGs were also loaded with various contents of FITC-Dextran in the range from 1mg/g to 3mg/g with the lipid content of 550mg/g in all the formulations. In general, the gel strength was increased as incorporating more FITC-Dextran within the VPGs. The gel strength value was 46g for VPGs containing 1mg/g FITC-Dextran while VPGs with 2mg/g FITC-Dextran got a value of 60g and VPGs with 3mg/g FITC-Dextran had a gel strength value of 70g (Figure IV-4).

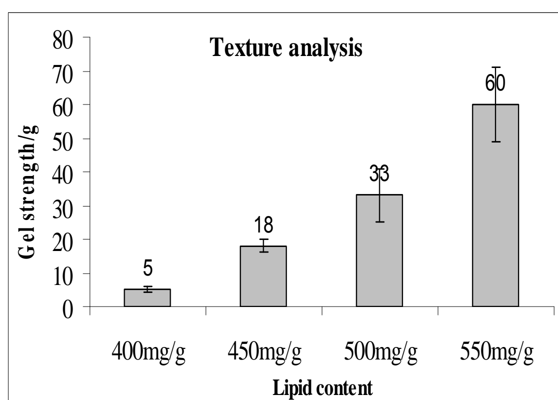


Figure IV-3. Gel strength (mean±SD, n=3) of VPGs formulations with various lipid contents (as indicated in the figure). All gels were based on egg PC and loaded with 2mg/g FITC-Dextran.

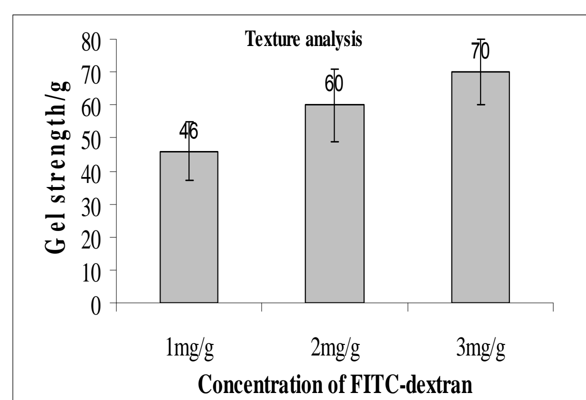


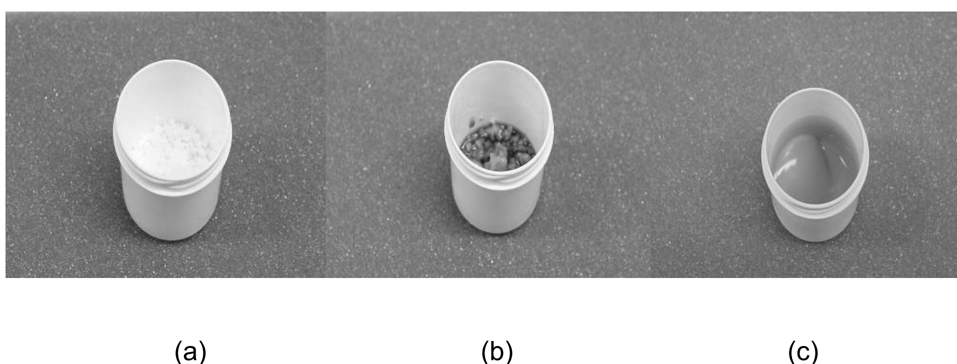
Figure IV-4. Gel strength (mean±SD, n=3) of VPGs formulations with various FITC-Dextran contents (as indicated in the figure). All gels were based on egg PC.

Comparing the VPGs prepared by HPH with the VPGs prepared by DAC, the VPGs prepared by HPH had a higher gel strength valule (ranging from 18g to 86g depending on the homogenization cycle) than the VPGs prepared with DAC (5g) based on the same contents of lipids (400mg/g egg PC) and FITC-dextran (2mg/g). It was assumed that the difference between the properties of VPGs prepared by HPH and those by DAC were due to the preparation technology of the different devices. In agreement with previous report [147], this comparison clearly shows that the shear forces generated by DAC are much lower than those generated by HPH, which on the other hand might be advantageous when sensitive compounds are entrapped within VPGs.

2. HOMOGENEITY OF VPGS

It might be argued that a non-uniform drug distribution of the VPGs could be caused by the manufacture process by dual asymmetric centrifugation. Consequently variations of drug loading could be possible.

Methylene blue was used as model drug compound for homogeneity evaluations of the manufactured VPGs due to its colour enabling a convenient visual control. Methylene blue was dissolved in destilled water and subsequently added to the lipids in a cylindrical container (Figure IV-5a-5b). VPGs were formulated with dual asymmetric centrifugation as described previously. No obvious darker or lighter areas were visible (Figure IV-5c), being a result of homogeneous methylene blue contents in the gels.



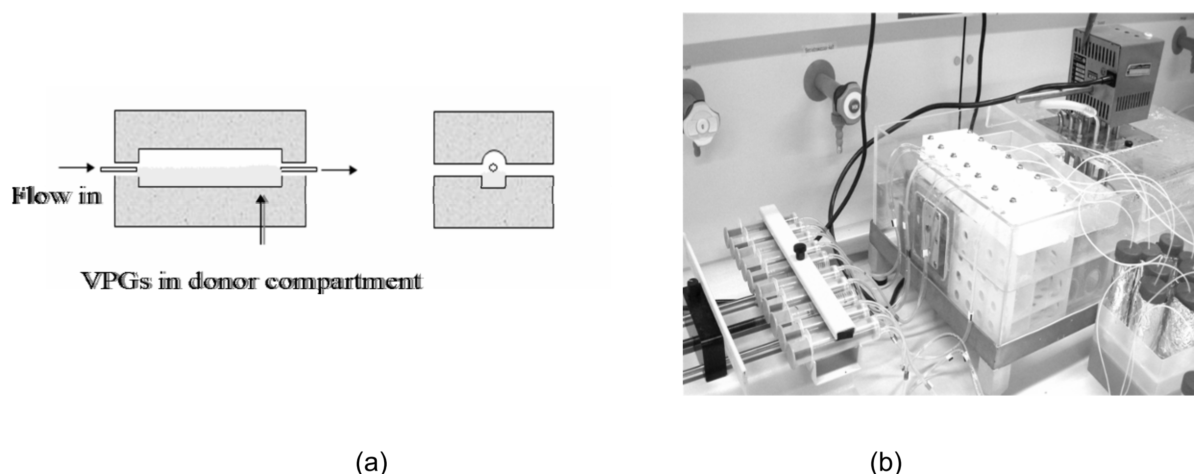
(a) (b) (c)

Figure IV-5. Images of VPGs loading with methylene blue: (a) the addition of lipids in the cylindrical container; (b) methylene blue and lipid mixture before dual asymmetric centrifugation; (c) VPGs with loading of methylene blue (the mixture after dual asymmetric centrifugation).

3. IN VITRO RELEASE TESTS

Flow-through cells were made of Teflon material based on the design described by Tardi (Figure IV-6a) [11] for in vitro release tests. The donor compartment (lower compartment) had a rectangular cross-section in order to ensure a relatively constant contact area of about 250 mm^2 (50mm x 5mm) even when portions of the VPG were lost due to erosion. The acceptor compartment (upper compartment) on the other hand showed a semicircular cross-section, which was beneficial for the facilitation of a laminar flow [3, 9].

Teflon tubing was used in order to minimize the surface adsorption of the released protein. In order to allow the erosion of VPGs, no membrane was used. The cells were filled with 1g accurately weighed VPG and kept at 37°C by a water bath. PBS buffer (40mM, pH 7.4) was used as acceptor medium and the flow rate was set to 1ml/hr by using a syringe pump (Figure IV-6b). The concentration of FITC-Dextran upon release fractions was determined by fluorescence photometry (Excitation wavelength: 488nm, Emission wavelength: 517nm). 0.2ml of a release fraction was diluted to 2.0ml by adding ethanol to dissolve the lipids. The results were represented as the average of triplicates.



(a) (b)

Figure IV-6. In vitro release set-up: (a) Schematic drawing of the release cell; (b) Release system.

VPGs were prepared with various concentrations of lipids (egg PC, PHOSPHOLIPON). The concentration of FITC-Dextran was 2mg/g in all the formulations. As it can be seen in Figure IV-7, the release behavior could be adjusted by the variation of the phospholipid concentration. Slower release rates were observed with increasing the lipid concentrations. For instance, the release of VPGs

prepared with 550mg/g lipids lasted more than 288hrs, whereas the release of FITC-Dextran was terminated after 50hrs for VPGs based on 400mg/g lipids. This confirmed that it should be possible to achieve a desired release profile for proteinaceous substances by optimizing the formulation of VPGs with respect to its gel properties. Moreover, it was importantly found that the release of the macromolecules from various VPG-formulations all followed a close to zero-order kinetics.

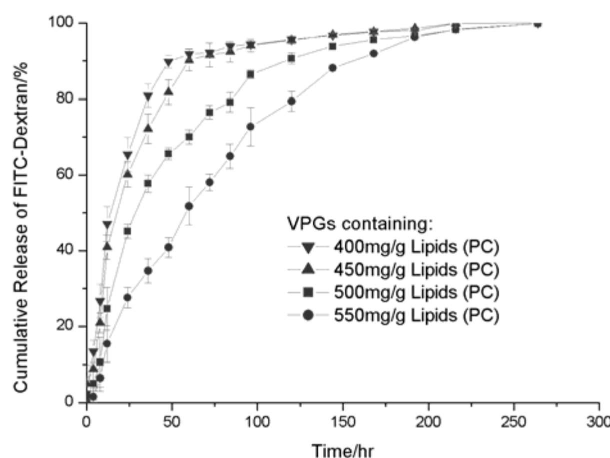


Figure IV-7. Cumulative release of FITC-Dextran from VPGs based on various concentrations of egg PC (Mean± standard deviation, n = 3 each).

The turbidity of the release fractions was determined to reflect the erosion of the VPG-matrix. It was found that the turbidity of the release fractions was in parallel with the release content within the whole release period (Figure IV-8 showed the VPGs based on 550mg/g lipids and 2mg/g FITC-Dextran for example). Since the turbidity of the release fractions was mostly from the lipids eroded from the matrices, it was assumed that erosion of VPGs is the controlling mechanism for the release of FITC-Dextran as a macromolecular weight model. This is in agreement with former observations which showed that mainly matrix erosion governed the delivery of small molecular weight drugs [11] and peptides [51] from VPGs.

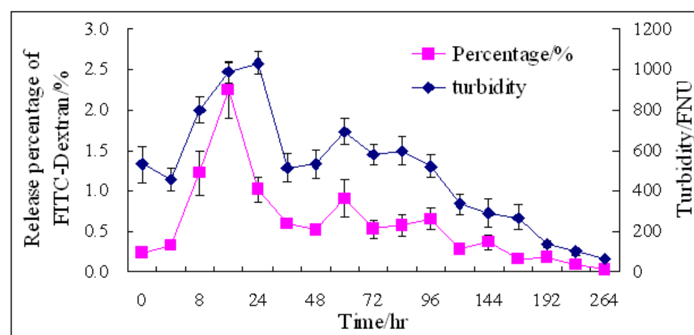


Figure IV-8. Release percentage of FITC-Dextran (left Y-axis) and turbidity of the release fractions (right Y-axis) at each time point. VPGs were based on 550mg/g lipids (egg PC, PHOSPHOLIPON) and 2 mg/g FITC dextran.

The vesicle size of the release fractions was also determined at each time interval by PCS. As summarized in Table IV-1, the release fractions from various VPG-formulations mainly consisted of small liposomes,

Table IV-1. Particle size of release fractions from VPGs based on various contents of lipids.

VPGs	Based on 400mg/g lipids	Based on 450mg/g lipids	Based on 500mg/g lipids	Based on 550mg/g lipids
Particle size of release fraction	170nm-232nm	185nm-258nm	184nm-250nm	199nm-290nm

4. SUMMARY AND CONCLUSION

In this chapter, FITC-Dextran was used as drug model for the loading of macromolecules. Various VPG-formulations were prepared using high pressure homogenization and dual asymmetric centrifugation respectively. Texture analysis was performed to characterize the gels. The gel strength was also found influenced by the variation of lipid content and drug content within the VPGs prepared by DAC.

In order to demonstrate the homogeneity of the drug distribution, methylene blue was entrapped within the VPGs. Upon visual and quantitative examination, a sufficient homogeneity was revealed of the drug loading.

In vitro release tests were set up using flow-through cells as described by Tardi[11]. Sustained release of FITC-Dextran from various VPG-formulations was observed following a close to zero-order kinetics. Moreover, the release rate can be modified by incorporating various amounts of lipids within VPGs. Slower release was observed by the addition of more lipids within the system. Turbidity of the release fractions was determined, which reflect the erosion of the matrices. Accordingly, it was consequently found erosion is the controlling mechanism for the release of the

macromolecular drugs as the determined turbidity was in parallel with the released amount of FITC-Dextran at each sample point. This result was in agreement with the previous work that matrix erosion governed the delivery of small molecular weight drugs [11] and peptides [51] from VPGs. The vehicle size was determined and was shown small liposomes (less than 300nm) released from the matrices. As summary, based on the obtained results it could be stated that VPGs possessed great potential as alternative system for the controlled delivery of proteins.

CHAPTER V: DEVELOPMENT OF EPO LOADED VESICULAR PHOSPHOLIPID GELS

VPGs represented a promising approach for the sustained in-vitro release of peptide drugs [13]. As shown in Chapter IV, we used FITC-dextran as a drug model to demonstrate the suitability of VPGs for sustained delivery of macromolecular compounds. However, due to the sensitivity of the three dimensional structure and inherent instability of proteins, it was still unclear whether the system can be applied to proteins.

Therefore, it was the aim of the following work to assess the potential of Vesicular phospholipid gels as alternative delivery system for therapeutic proteins. Erythropoietin, the first model protein, was incorporated into various VPGs formulations by using dual asymmetric centrifugation. The protein stability and in vitro release behaviour were used as main criteria to evaluate the suitability of the system. In order to characterize and to quantify the incorporated protein, different extraction protocols were investigated. Physicochemical characterizations were performed for the EPO loaded VPGs.

1. PREPARATION OF EPO LOADED VPGS

In this work production of protein loaded VPGs was performed with a DAC (SpeedMixerTM150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany). The preparation process was based on the method described by Massing et al [147]. In previous studies, glass beads were used as homogenization aid. However, in our work, we omitted the use of beads in order to avoid potential heat-stability problems with the protein. Then, EPO was incorporated by direct loading, i.e. EPO solution (formulated in 20mM PBS buffer, pH 7.2) was added to the accurately weighed lipids and homogenized by means of a dual asymmetric centrifugation in a cylindric container of 25 ml total volume. The phospholipids' (egg phosphatidylcholine, E 80) content ranged from 300mg/g to 550mg/g in total weight of the gels. The formulation contained a total protein drug load of 3.8 mg/g gel. Lipid blends consisting of egg phosphatidylcholine and charged lipids (egg PC: DPPA/ 9:1; egg PC: DOTAP/ 8:2, in total 500mg/g) were used to evaluate the influence of the lipid charge. For homogenization a process speed of 3500 rpm was utilized in all experiments. According to the first report on the utilization of DAC [147] and the fact that a

continuous long-time centrifugation run causes a significant temperature increase in the product, multiple centrifugation steps of 1.5-minutes were added up to a total mixing time of 45 mins with interrupts for cooling at 2-8°C. Using this procedure aprocess temperature of below 40°C could be maintained during the production, which appeared appropriate for the incorporation of temperature sensitive proteins.

The inner structure of VPGs has been revealed by freeze-fracture electron microscopy in previous work [151, 165], which was described as a matrix of densely packed vesicles within the pastes. Upon visible examination it was observed that all the VPGs obtained exhibited a viscous semi-solid consistency. With increasing the lipid content in the formulations, the obtained VPGs showed increased viscosity and behaved less fluidity. Upon optical microscopic inspection all the formulations exhibited a homogeneous appearance (Figure V-1). Increased packing density was observed for the VPGs containing more lipids which might lead to the decreased fluidity.

(a). VPGs containing 300mg/g lipids; (b). VPGs containing 400mg/g lipids; (c). VPGs containing 450mg/g lipids

(d). VPGs containing 500mg/g lipids; (e). VPGs containing 550mg/g lipids

Figure V-1. Light micrograph of VPG formulations based on various contents of egg PC (LIPOID E80) and 3.8mg/g EPO at 400X magnification. (a). 300mg/g, (b). 400mg/g, (c). 450mg/g, (d). 500mg/g, (e). 550mg/g.

2. RHEOLOGY AND TEXTURE ANALYSIS

Bender [150] has investigated the complex viscosities of VPGs formulated with various types of lipids with oscillation rheometry. The viscoelastic character in the gel state increased due to decreased fluidity in the membrane bilayers and in the VPG structure.

In our study, the rheologic behavior of VPGs was studied by a rotational rheometry (Physica MCR 100, Anton Paar). The measurement was performed at 25°C. The viscosities of VPGs were compared at a fixed shear rate of 37.9 s^{-1} (Figure V-2). Increasing the lipid content resulted in increased viscosity whereas adding a certain amount of charged lipid DOTAP or DPPA had little influence on the gel viscosity (Figure V-3).

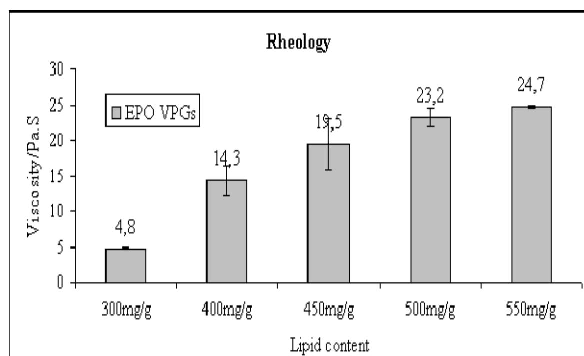


Figure V-2. Viscosity (mean±SD, n=3) of VPGs formulations with various lipid contents (as indicated in the figure). All gels were based on egg PC (LIPOID E80) and loaded with 3.8mg/g EPO (formulated in PBS buffer 20mM).

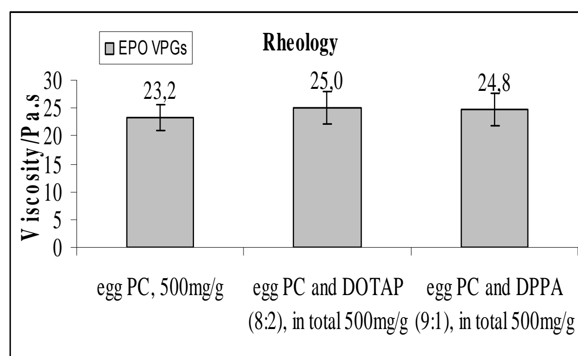


Figure V-3. Viscosity (mean±SD, n=3) of VPGs formulations with various lipid compositions (as indicated in the figure). All gels were based on 500mg/g lipids or lipid blends and loaded with 3.8mg/g EPO (formulated in PBS buffer (20mM).

Texture analysis was performed with the TA.XT plus texture analyzer (Stable Micro Systems, UK) to determine the gel strength (represented as the maximal force value). More rigidity of VPGs was observed by increasing the lipid content of the formulations (Figure V-4). Incorporating charged lipid DOTAP or DPPA slightly increased the gel strength compared with the VPGs containing egg PC only (Figure V-5).

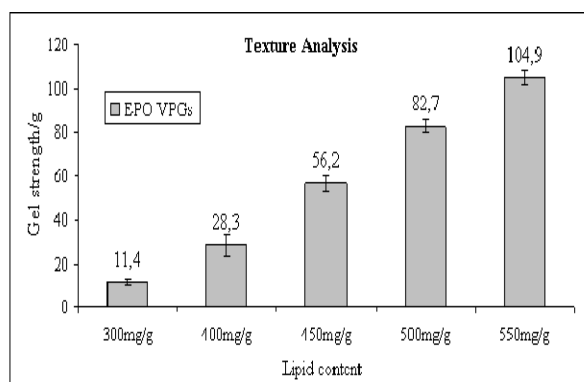


Figure V-4. Gel strength value (mean±SD, n=3) of VPGs formulations with various lipid contents (as indicated in the figure). All gels were based on egg PC or and loaded with 3.8mg/g EPO (formulated in PBS buffer (20mM).

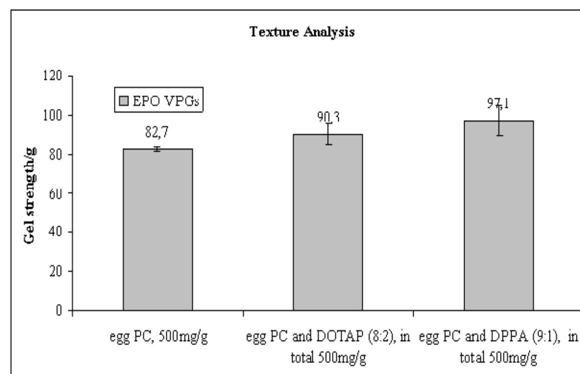


Figure V-5. Gel strength value (mean±SD, n=3) of VPGs formulations with various lipid compositions (as indicated in the figure). All gels were based on 500mg/g lipids or lipid blends and loaded with 3.8mg/g EPO (formulated in PBS buffer (20mM).

3. EVALUATION OF PROTEIN STABILITY BY SDS-PAGE

SDS-PAGE was performed to determine whether the manufacture process e.g. the mechanical shear force on the protein integrity. Since organic solvents or surfactants were applied in the protein extraction process from VPGs' dispersions or release fractions, it was also necessary to evaluate their effect on the protein integrity. The samples were analysed by non-reducing SDS-PAGE with subsequent coomassie blue staining or silver staining. Electrophoresis was performed on the Bis-Tris Pre-Cast Gel (Invitrogen, Groningen, Netherlands) in the MES running buffer (Invitrogen).

Since the presence of lipids had a strong interference with the protein in SDS-PAGE analysis (data not shown), it was necessary to apply extraction agents. As the first step various extraction agents (as indicated in Table V-1) were applied to the lipid-free EPO samples. The protein solutions were emulsified with organic solvents by vortex, and EPO was subsequently recovered from the aqueous phase after centrifugation. If Triton X-100 was applied, incubation with the protein solutions was needed at 37°C for 30 minutes.

Table V-1. Organic solvents and surfactants applied for EPO extraction

	Organic solvents			Surfactants
Extraction agent	Ethanol	Dichloromethane	Chloroform	Triton X-100
Applied amount to 1ml VPGs' dispersions	1ml/ml	1ml/ml	1ml/ml	5%

The obtained protein samples were analysed by gel electrophoreses with subsequent coomassie blue staining (Figure V-6). Only the band of monomer EPO was detectable irrespective of the extraction agents. Hence it can be stated that applying the extraction agents induced no instability of EPO.

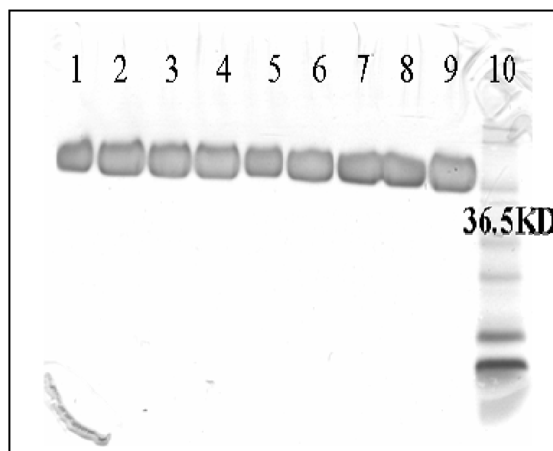


Figure V-6. Influence of extraction agents on the EPO stability. Lane 1/ lane 5, adding ethanol to EPO; lane 2 / lane 6, adding Triton X-100 to EPO; lane 3 / lane 7, adding dichloromethane to EPO; lane 4 / lane 8, adding chloroform to EPO; lane 9, EPO standard solution; lane 10, molecular weight maker. SDS-PAGE with subsequent blue staining.

Further investigations applied these organic solvents and surfactants to extract EPO from freshly prepared VPGs (500mg/g egg PC and 3.8mg/g EPO). The formulation process was performed with DAC as described above in Chapter III. The protein had a concentration of 3.8mg/g in the final VPGs formulations. Aliquots of 0.2 g gel were dispersed in 10ml PBS buffer with vigorous vortex. EPO was extracted and analysed by SDS-PAGE with subsequent coomassie blue staining. No band was observed when ethanol was used to dissolve the lipids (Figure V-7, lane 1/ lane 5) indicating low protein recovery from extraction. As shown in lane 2 and lane 6, adding Triton X-100 caused bands' tailing, which was probably caused by the lipids remained in the samples. Only monomeric bands were detectable by applying chloroform (as indicated in lane 4/ lane 8) or dichloromethane (as indicated in lane 3/ lane 7) for lipid extraction and subsequently recovering EPO from the aqueous phase. Due to the higher protein recovery chloroform was used in the further investigations for extraction of EPO from lipid containing samples for the SDS-PAGE analysis.

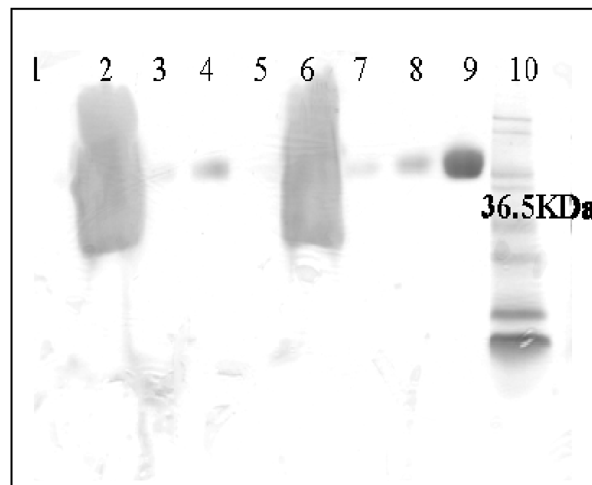


Figure V-7. Extraction of EPO from VPGs. Lane 1/ lane 5, applying ethanol to extract EPO from VPGs; lane 2 / lane 6, applying Triton X-100 to extract EPO from VPGs; lane 3 / lane 7, applying dichloromethane to extract EPO from VPGs; lane 4 / lane 8, applying chloroform to extract EPO from VPGs; lane 9, EPO standard in PBS buffer 20mM; lane 10, molecular weight maker. SDS-PAGE with subsequent blue staining.

However, it was important to exclude the possibility that the protein aggregates or fragments which were probably induced either in the manufacture process or in the extraction process might not be extracted and then not detected by the SDS-PAGE analysis. Further investigations were carried out by stressing the protein to generate aggregation or degradation products. In brief: EPO solutions were incubated at 60°C for 24hrs and on the other hand the protein was oxidized with hydrogen peroxide. Lipids (egg PC, 1.5mg/ml) were dispersed into these stressed-protein solutions. Chloroform was applied and the extracted proteins were analysed by SDS-PAGE with subsequent silver staining (To improve the sensitivity of the PAGE method, silver staining was used instead of coomassie blue staining in further work). Denatured protein samples without adding lipids or applying extraction process were analysed directly as the control. As shown in Figure V-8, it could be concluded that: (I) Denatured EPO could be extracted from lipids using chloroform and detected by the SDS-PAGE (lane5, lane 6, and lane 8); (II) Applying chloroform for EPO extraction did not create more denatured protein fractions (No additional band was observed for extracted proteins, see: lane 3 and lane 5; lane 4 and lane 6; lane 7 and lane 8); (III) As expected, the detection of aggregate and fragment fractions was dependant on the loading content of the protein. Some denatured fractions became invisible at lower concentrations (comparing lane 3/ lane 5 with lane 4/ lane 6). Consequently, it

could be stated that the developed extraction protocol with chloroform and the SDS-PAGE were suitable for evaluation the stability of entrapped EPO from VPGs.

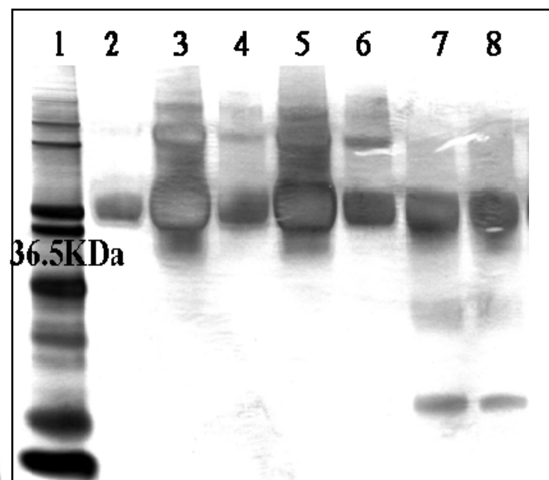


Figure V-8. Analysis of denatured EPO samples. SDS-PAGE with subsequently silver staining:

Lane 1, molecular weight marker

Lane 2, EPO standard in PBS buffer 20mM

Lane 3, denatured EPO by incubating at 60°C for 24hrs with a total protein concentration of 750µg/ml.

Lane 4, denatured EPO by incubating at 60°C for 24hrs with a total protein concentration of 50µg/ml.

Lane 5, denatured EPO (by incubating at 60°C for 24hrs) extracted from lipids containing samples, with a total protein concentration of 750µg/ml.

Lane 6, denatured EPO (by incubating at 60°C for 24hrs) extracted from lipids containing samples, with a total protein concentration of 50µg/ml.

Lane 7, oxidized EPO, with a total protein concentration of 150µg/ml.

Lane 8, oxidized EPO extracted from lipids containing samples, with a total protein concentration of 150µg/ml.

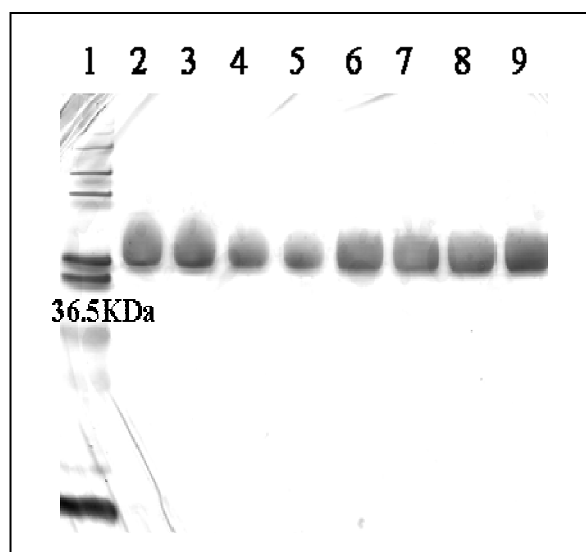


Figure V-9. Proof of EPO integrity within VPGs formulations. Lane 1, molecular weight maker; lane 2 to 5: EPO extracted with chloroform from various VPG formulations based on 500mg/g egg PC and 3.8mg/g EPO, lane 6 to 9, EPO standard in PBS buffer 20mM. SDS-PAGE with subsequently silver staining.

Consequently, the influence of the manufacturing process on the integrity of EPO was studied by SDS-PAGE with subsequent silver staining. Chloroform was applied to extract EPO from freshly prepared gels (containing 500mg/g egg PC and 3.8mg/g protein). As shown in Figure V-9, no aggregation or fragmentation bands of EPO were observed. It could be concluded that EPO was maintained stable within VPGs after production, which encouraged further investigation of the in-vitro release tests.

4. DEVELOPMENT OF QUANTIFICATION METHOD OF EPO

As described previously, VPG formulations offer the unique property that drug incorporation is achieved either within the tightly packed lipid vesicles or in-between them. Consequently, it can be expected that EPO is released from the gel matrix both in free form and in liposomal form. Therefore it is essential to develop an extraction method that allows the quantitative recovery of the protein from the liposome fraction without having detrimental effects on the protein integrity. In previous work, Qi [172, 204] used chloroform to extract EPO from liposome solutions prior to quantitative analysis by RP-HPLC. In a comparative study on the extraction efficiency of the decapeptide cetrorelix from liposome dispersions 2.5% Triton X-100 and hexane were the best choices among several detergents and organic solvents. However, it should be also noticed that the Triton X-100 containing samples featured creation of multiple peaks [160]. Also the detergent 30 mM octyl ß-D-glucopyranoside (ODG) has been suggested for protein extraction [205].

4.1. SAMPLE PREPARATION

Since the released amount of VPGs at each time interval was unknown, it was important to develop an extraction protocol and a subsequent quantification method which featured a high robustness against variations of the lipids as well as against variations in the protein amount which make this method suitable for protein quantification during in-vitro release studies.

Initial screening of the extraction methods was done with a constant EPO concentration of 10µg/ml. The extraction agents and analytical methods for initial screening were used as in Table V-2. Surfactants were accurately weighed and added to lipid containing EPO samples and incubated for a short period before

injection to HPLC. Alternatively, the lipids were extracted by organic solvents and EPO was subsequently recovered from the water phase.

To investigate the influence of variation in the lipid content on the analysis method, various amounts of lipids were dispersed by applying vigorous vortex in PBS buffer pH 7.4 (release medium) and diluted to the concentration of 0.5mg/ml, 1.5mg/ml and 5.0mg/ml respectively (These concentrations were assumed to be around the concentrations which would be expected in the release fractions during the release tests.).

Table V-2. Extraction and quantification protocol

Applied surfactants or organic solvents	Volume/Concentration	HPLC-Method	Reference
Triton X-100	1.25%- 5.0%	SEC, RP	Grohganz, 2004 [160]
Tween 80	0.03%	SEC, RP	Hermeling, 2003 [206]
ODG	10 mM - 50 mM	SEC,RP	Gonzalez, 1981[205]
Hexane	0.3 m l – 4 ml per 1 ml sample	RP	Grohganz, 2004 [160]
Chloroform	0.3 ml – 4 ml per 1 ml sample	RP	Qi,1995[172]
Acetonitrile	1 ml per 1 ml sample	RP	-

4.2. SE-HPLC METHOD

A classical size exclusion HPLC approach using SEC column (TSK-Gel G3000Wxl, 7.8 mm x 30.0 cm) with a flow rate of 0.7 ml/min and UV detection at 215 nm was applied for EPO analysis as the start point. The mobile phase consisted of 100 mM disodium hydrogen phosphate dehydrate and 200 mM sodium chloride, adjusted with hydrochloric acid to a pH of 7.0. EPO solutions of known concentrations (3.0 – 37.5 µg/mL) were used to generate the calibration curve (Figure V-10). As shown in Figure V-11a, dimers (5.7± 0.4%) were detected in the protein bulk solutions.

The next step under investigation was to evaluate possibilities for protein quantification in the presence of various amounts of phospholipids.

Applying Triton X-100 caused generation of additional peaks and baseline shift. Moreover, EPO could not be totally separated from the Triton (Figure V-11b) Adding 0.03% Tween 80 produced a peak around the retention time of EPO, which could not be baseline separated from the EPO peak (Figure V-11c). It caused problems in quantification especially for the proteins with low concentrations. Applying either 30mM (Figure V-11d) or 50mM ODG no protein peak could be identified. Whether

there possibly occurs aggregation or degradation of EPO could not be clarified. Consequently, judged by the obtained results no suitable extraction protocol for SE-HPLC could be developed and it could be stated that a SE-HPLC method might not be suitable for the determination of EPO in presence of lipids due to the lack of sensitivity and high cost.

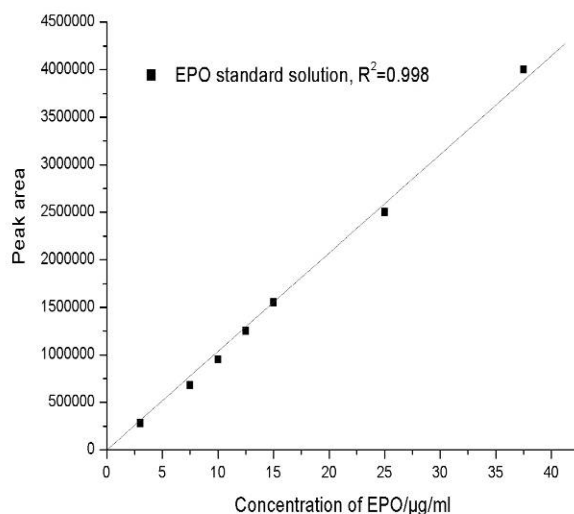
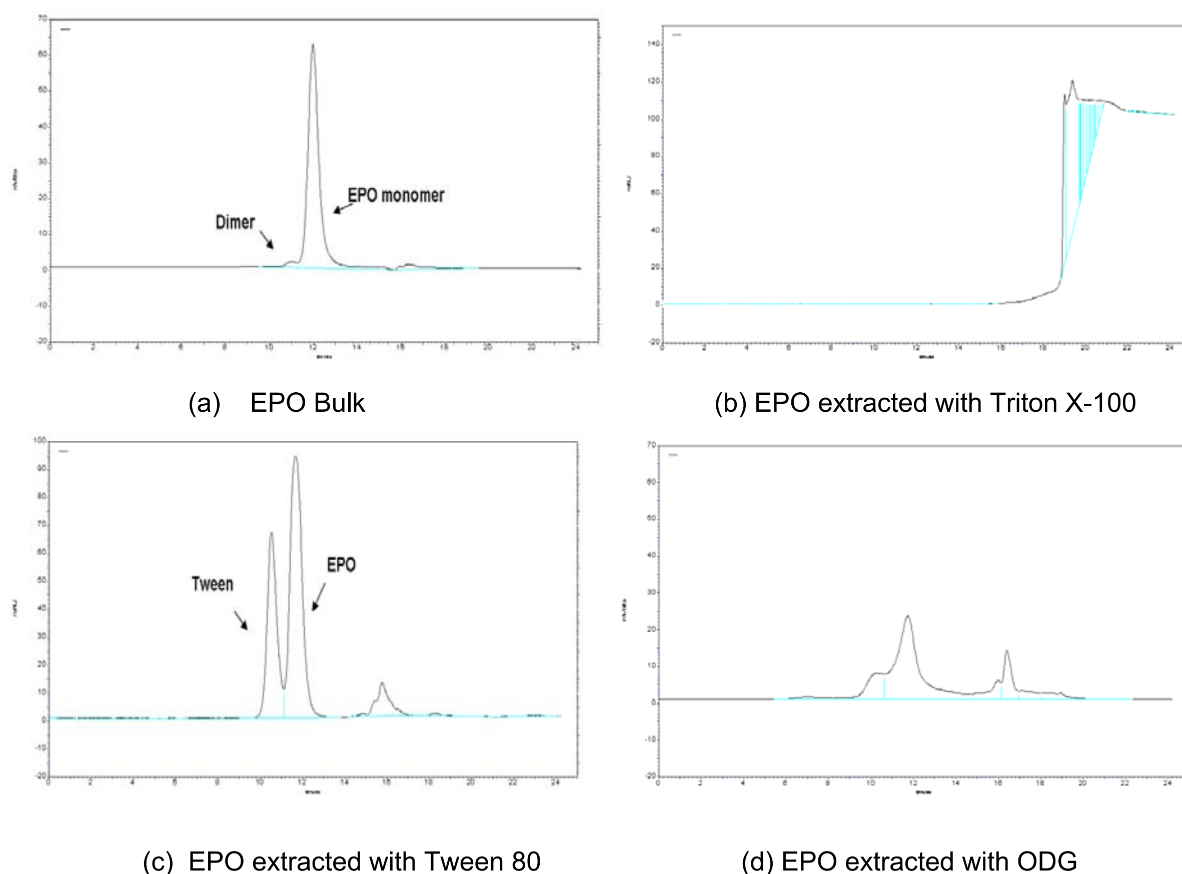


Figure V-10. Response curve of EPO standard solution by SE-HPLC, n=3

The next step under investigation was to evaluate possibilities for protein quantification in the presence of various amounts of phospholipids.

Applying Triton X-100 caused generation of additional peaks and baseline shift. Moreover, EPO could not be totally separated from the Triton (Figure V-11b) Adding 0.03% Tween 80 produced a peak around the retention time of EPO, which could not be baseline separated from the EPO peak (Figure V-11c). It caused problems in quantification especially for the proteins with low concentrations. Applying either 30mM (Figure V-11d) or 50mM ODG no protein peak could be identified. Whether there possibly occurs aggregation or degradation of EPO could not be clarified. Consequently, judged by the obtained results no suitable extraction protocol for SE-HPLC could be developed and it could be stated that a SE-HPLC method might not be suitable for the determination of EPO in presence of lipids due to the lack of sensitivity and high cost.



(a) EPO Bulk

(b) EPO extracted with Triton X-100

(c) EPO extracted with Tween 80

(d) EPO extracted with ODG

Figure V-11. SE-HPLC chromatograms of EPO. (a) EPO bulk, detection of aggregated specimen; (b) EPO extracted from lipids containing samples by applying 2.5% Triton; (c) EPO extracted from lipids containing samples by applying 0.03% Tween 80; (d) EPO extracted from lipids containing samples by applying 30 mM ODG.

4.3. RP-HPLC METHOD

Compared with SE-HPLC, reverse phase HPLC method has many advantages in our case, such as the flexibility in choosing extraction agents and robustness against the variation of lipids in the samples from the release fractions.

4.3.1. VALIDATION OF THE HPLC METHOD

For EPO quantification a RP-HPLC method using a Phenomenex C18 Jupiter column (5 µm, 250 x 4.6 mm) together with UV detector (215nm) was developed. Gradient elution was carried out (Table V-3) with eluent A containing 0.1% TFA (in ultrapure water) and eluent B containing 84% CH_3CN and 0.08% TFA. EPO has a retention time around 15 minutes (Figure V-11). No obvious oxidized specimen was observed for the EPO bulk material. Calibration of lipid-free protein samples was established with the protein concentration ranging from 3.0µg/ml to 15.0µg/ml.

Table V-3. Gradient elution for RP-HPLC of EPO

Time/min	Eluent A	Eluent B	Flow rate
0	80%	20%	
10	40%	60%	
15	40%	60%	0.5ml/min
20	0%	100%	
25	80%	20%	

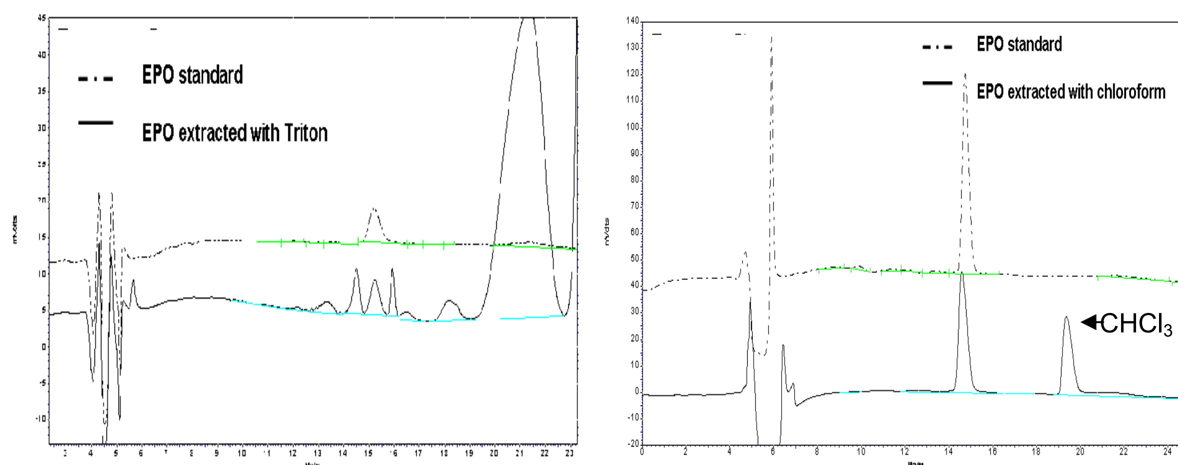
4.3.2. Evaluation of detergents and organic solvents for EPO extraction

The next step under investigation was to evaluate possibilities for protein quantification in the presence of lipids. To simulate the conditions expected during in-vitro release EPO solutions were spiked with lipid dispersions (with a final lipid concentration of 1.5mg/ml) and the recovery of EPO was determined by the aforementioned RP-HPLC method and compared to the HPLC-response of lipid-free EPO solutions after applying a detergent or an organic solvent. Initial screening of the extraction methods was done with a constant EPO concentration of 10µg/ml.

The ability of several extraction strategies to recover EPO from VPG dispersion is summarized in Table V-4. Among the detergents only Triton-X featured an acceptable protein recovery of around 90.7 % (as shown in Figure V-12a). In the organic solvent group the extraction of the lipids by chloroform resulted in a recovery of 84.6 % (Figure V-12b) whereas with hexane and acetonitrile a disastrous protein loss was observed. As the extraction involves the generation of water organic solvent interfaces proteins tend to adsorbs onto this interfaces which usually accounts for protein unfolding and/or aggregation [55, 207-209]. Presumably these processes explain the large quantities of EPO lost due to the extraction with hexane. Compared to the liposome disruption process caused by Triton-X the recovery of EPO by extraction with chloroform was slightly lower. Nevertheless, the extraction of the lipids by chloroform and subsequent recovery of EPO from the water phase was chosen for further investigations as subsequent injections of Triton X-100 containing samples strongly reduced the column efficiency.

Table V-4. Recovery of EPO determined by RP-HPLC (mean± standard deviation, n = 3 each). For protein extraction a VPG dispersion containing 1.5 mg/ml lipid (egg PC, LIPOID E80) and an initial protein concentration of 10 µg/ml was treated with several detergents or organic solvents.

Applied agent for EPO recovery	$CHCl_3$	Triton	Tween	ODG	Hexane	AcCN
Applied concentration per ml VPG dispersion	0.3ml	2.5%	0.03%	30mM	0.3ml	0.3ml
Recovery/%	84.6±5.3	90.7±2.2	43.0±7.6	48.2±12.8	8.7±0.2	0



(a) EPO extracted with Triton X-100, EPO bulk was shown as standard.

(b) EPO extracted with chloroform, EPO bulk was showed as standard

Figure V-12. RP-HPLC of EPO. (a) EPO extracted with Triton, EPO bulk was shown as standard; (b) EPO extracted with chloroform, EPO bulk was shown as standard.

4.4. VALIDATION OF THE DEVELOPED QUANTIFICATION METHOD

4.4.1. CALIBRATION

The influence of various protein concentrations on the extraction efficacy was evaluated. Figure V-13a shows the AUC of extracted EPO by adding chloroform in comparison to the calibration curve in the absence of any lipids. Obviously, a good correlation was achieved between the detected AUCs of extracted and untreated EPO solutions. Based on these data and an acceptable linearity the extraction by chloroform was used as the standard method for EPO quantification for the in-vitro release studies.

However, as the release studies will also include investigations on the influence of the lipid charge, the extraction efficacy in the presence of negatively and positively charged lipids was determined. For that purpose EPO solutions were spiked with VPG dispersions based either on the lipid blend of negatively charged DPPA and egg PC in a ratio of 1 to 9 or on a mixture of the cationic lipid DOTAP and egg PC in a ratio of 8 to 2. For the negatively charged lipid formulation the response curve revealed no differences compared to the uncharged lipid formulation (Figure V-13a). However, the recovery of EPO from cationic lipid dispersions was dramatically reduced (Figure V-13b). This poor extraction efficiency can presumably be explained by electrostatic interaction between the positively charged lipid vesicles and the

negatively charged protein (EPO has an isoelectric point of 4.2 to 4.6. At pH 7.4, EPO is negatively charged.). Furthermore, to confirm this, zeta potential measurements were performed and the results are shown in Table V-5.

Table V-5. Zeta potential measurements. EPO was formulated in PBS buffer with a pH of 7.4. VPGs were dispersed by the same PBS buffer resulting in a lipid content of 1.5mg/ml.

Samples	Zeta potential ζ/mV
EPO without any lipids, in PBS buffer pH 7.4	-7.8±0.4
uncharged gel vesicles, dispersed in PBS buffer, pH 7.4	-3.4±0.7
negatively charged gel vesicles (Egg PC/ DPPA 9:1) dispersed in PBS buffer, pH 7.4	-6.0±0.8
positively charged VPGs (Egg PC/ DOTAP 8:2) dispersed in PBS buffer, pH 7.4	+14.0±0.9
EPO + uncharged gel vesicles, redispersed in PBS buffer, pH 7.4	-2.8±0.4
EPO + negatively charged gel vesicles (Egg PC/ DPPA 9:1) dispersed in PBS buffer, pH 7.4	-4.5±0.4
EPO + positively charged gel vesicles (Egg PC/ DOTAP 8:2) dispersed in PBS buffer, pH 7.4	+7.7±0.7

To reduce these interactions various amounts of sodium chloride were added to the protein containing VPG formulation before the standard extraction protocol using chloroform was carried out. As shown in Figure V-13b the addition of at least 200 mM sodium chloride resulted in a suitable response curve similar to that of uncharged lipids.

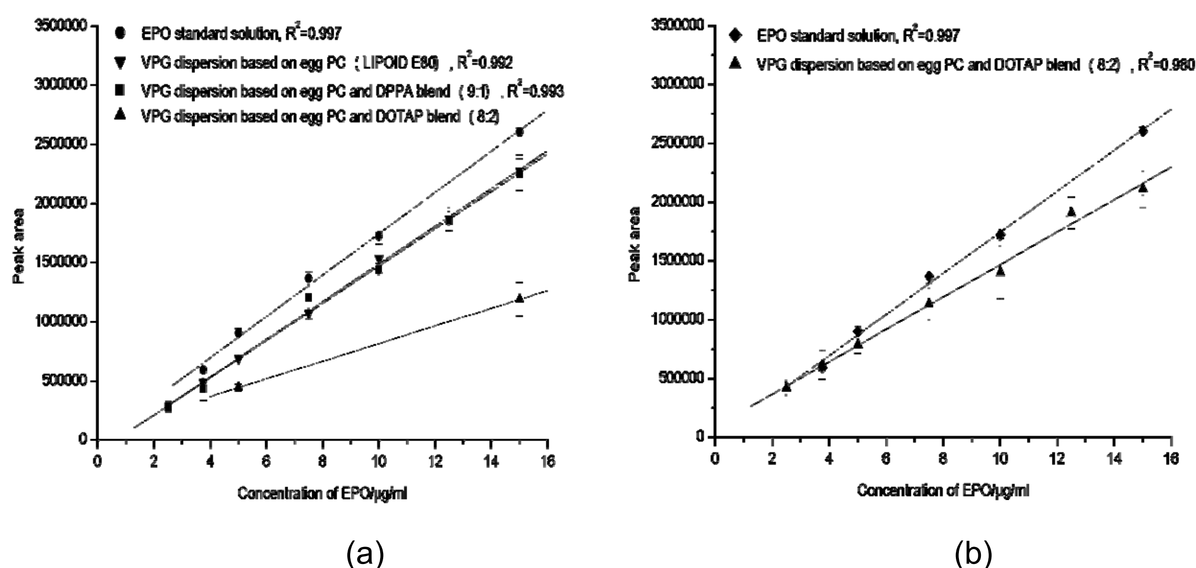


Figure V-13. Response curve of EPO standard solution in comparison to the recovery after extraction from VPG dispersion based on uncharged lipids/charged lipid blends. Extraction was performed with $CHCl_3$ either by the standard protocol (a) or after the addition of 200mM sodium chloride (b).

4.4.2. INFLUENCE OF CENTRIFUGATION CONDITIONS

A centrifugation process was involved in the extraction protocol using chloroform. Thus it was necessary to evaluate the effect of centrifugation conditions on the protein recovery. The samples contain a lipid content of 1.5mg/ml and 10µg/ml of EPO. The centrifugation duration was varied from 15 minutes to 30 minutes and the centrifugation speed was set to either 1500rpm or 3000rpm. The centrifugation was performed at 0°C or 15°C.

As shown in Table V-6, in general the centrifugation conditions have marginal effect on the protein recovery. Low temperature at 0°C was preferred concerning the protein instability. Best recovery was achieved when centrifugation was performed at 3000rpm for 30 minutes. So this condition was applied in the standard extraction protocol for EPO extraction.

Table V-6. EPO recovery: effect of centrifugation conditions (n=3). The samples contain a lipid content of 1.5mg/ml and 10µg/ml of EPO.

Temperature	Centrifugation speed	Recovery of EPO (centrifugation for 15mins)	Recovery of EPO (centrifugation for 30mins)
0°C	1500rpm	82.9±1.0%	87.0±0.8%
15°C	1500rpm	84.4±1.9%	84.4±1.4%
0°C	3000rpm	89.1±1.3%	90.5±0.7%
15°C	3000rpm	81.9±0.8%	86.8±2.8%

4.4.3. INFLUENCE OF CHLOROFORM AMOUNT

Further investigations were carried out in order to exclude the possibility that adding more chloroform might induce the protein aggregation or degradation and consequently reduce the protein recovery. An increasing amount of chloroform ranging from 0.3ml to 4ml per ml was added to 1ml lipid containing protein samples respectively. The samples were centrifuged and the extracted EPO was quantified by RP-HPLC as described above. Table V-7 showed that the amount of chloroform had little influence on the EPO recovery. It was suggested that the extraction method featured the flexibility in dissolving larger amount of lipids if in the release fractions by applying more chloroform. For the current study, 0.3ml Chloroform per ml sample was preferred in the standard extraction protocol.

Table V-7. Protein recovery (n=3): Effect of chloroform amount. The samples contain a lipid content of 1.5mg/ml and 10µg/ml of EPO. The protein was extracted by applying various amount of chloroform.

Volume of chloroform per ml sample	0.3ml	1ml	2ml	4ml
Recovery of EPO	87.8±1.9%	90.1±2.3%	86.0±2.2%	89.4±2.3%

4.4.4. INFLUENCE OF LIPID AMOUNT

It was important to evaluate the robustness of the extraction and quantification method against the variation of the lipid content in the samples for the release tests. Various amounts of lipids (egg PC) were dispersed into PBS buffer resulting in concentrations of 0.5mg/ml, 1.5mg/ml and 5.0mg/ml respectively. These concentrations were assumed to be around the concentrations which were expected in the release fractions during the release tests. The proteins were extracted from all the samples by applying the same standard protocol using 0.3ml/ml chloroform. As a result, varying the lipid content in the samples had little influence on the EPO recovery (Table V-8). Since the amount of eroded lipids was unknown in the released fractions, this result allows concluding that the extraction of EPO from released fractions will be fine in the range up to ca. 5mg/ml lipid in the release samples.

Table V-8. EPO recovery: Effect of the lipids content (n=3). The samples contain 10µg/ml of EPO.

Lipid content in the sample	0.5mg/ml	1.5mg/ml	5.0mg/ml
Recovery of EPO	86.7±1.4%	89.7±2.4%	88.7±2.9%

4.4.5. RECOVERY OF OXIDIZED PRODUCTS

In addition, it was also necessary to survey whether denatured EPO could be recovered from lipids using the developed extraction protocol and detected by the RP-HPLC. EPO solutions were incubated with hydrogen peroxide to generate oxidized products. Lipids were dispersed to these oxidized protein containing solutions and resulted in a concentration of 1.5mg/ml. The samples were extracted and quantified by RP-HPLC. The recoveries of these oxidized products containing EPO samples (Table V-9) were comparable to those non-oxidized samples. As a conclusion, the extraction method with chloroform and RP-HPLC are capable for the quantification of oxidized products containing EPO.

Table V-9. Recovery of the oxidized products containing EPO samples (n=3). The samples contain 1.5mg/ml of lipids. The protein was extracted by applying chloroform.

Concentration of EPO	Percentage of the oxidized products in the samples	Recovery of the oxidized product containing proteins in percentage/%,
150µg/ml	7.3±0,5%	82,7±4,4%
30µg/ml	10.4%±0,7%	76,2±3,1%

4.5. SUMMARY OF THE QUANTIFICATION METHOD

It can be expected that EPO is released from the gel matrix both in free form and in liposomal form. Therefore it is essential to develop an extraction method that allows the quantitative recovery of the protein from the lipid containing samples without having detrimental effects on protein integrity. In the work of this chapter, several organic solvents and surfactants have been tried out. Using chloroform to extract lipids and subsequently recovering EPO from the water phase was found suitable in our case. A reverse phase HPLC method with gradient elution was developed for quantification of extracted proteins. Calibration of the extracted proteins was established with acceptable linearity and moreover it was in comparison to the calibration curve of the lipid-free proteins. The developed extraction and quantification method featured a high robustness against variations of the lipid as well as against variations in the protein amount. It was also possible to recover oxidized protein products from lipid containing samples with a comparable recovery. Hence it could be stated that the developed method was suitable for protein quantification during in-vitro release studies, which made it possible for the further investigations on the in vitro release of VPGs.

5. IN VITRO RELEASE BEHAVIOUR OF EPO LOADED VPGS

In vitro release behaviour is the most important characteristic for VPGs. So far it has been shown in literature that VPGs can serve as sustained release systems for various low molecular weight drugs, especially in the field of anticancer treatment [11,12] Grohganz [13] has entrapped the small peptide hormone cetrorelix into VPGs: in vitro release of the peptide from VPGs ranged from 24 hours to a predicted sustained release of over more than 3 months. The release mechanism of VPGs has been demonstrated by Tardi [11] using calcein as the model compound: the entrapped drug released via erosion of the lipid matrix as well as diffusion out of the swollen VPGs. This holds true for the small molecular weight substances. In the case of the peptide, it was demonstrated that free diffusion of the peptide through the tightly packed lipid matrix could not be expected due to its missing lipophilicity and the larger molecular size. Hence it could be assumed that erosion was also the dominating factor for protein release from VPGs. It was the aim of our work to reveal the protein release behaviour from VPGs and proof the hypothesis. In vitro release

tests were performed in flow-through cells (as described in Chapter IV) with PBS buffer pH 7.4 at 37°C. The influence of the lipid content and the composition of the VPG on the release behavior was evaluated. The released EPO was extracted by the developed protocol using chloroform and subsequently quantified by RP-HPLC. To investigate the erosion of the matrix, lipid content in the release fractions was quantified by an enzymatic assay. Moreover, the protein stability during release was examined by SDS-PAGE. The vesicle size of the release fractions was analyzed with PCS. Protein adsorption to the release system was also studied.

5.1. INFLUENCE OF THE LIPID CONTENT

The influence of lipid concentration on the release of VPGs was the first factor to be evaluated. VPGs were prepared with a constant EPO content of 3.8mg/g and increasing lipid concentration from 300mg/g to 550 mg/g egg PC (E 80). As it can be seen in Figure V-14 increasing the lipid concentration from 300mg/g to 550 mg/g decreases the release rate of EPO from the VPGs. From 400mg/g to 500 mg/g lipid the system was rather robust and lipid concentration did not have much effect on the release kinetics. For instance VPGs consisting of 400mg/g, 450mg/ml and 500 mg/ml lipid all delivered about 60% of the incorporated EPO within 280 hours. In contrast, VPGs based on 550mg/g lipid released only 40% of the drug and VPGs with 300mg/ml about 75% of the drug load within this time period. Importantly, EPO was released from all formulations in a linear manner with no burst effect. This trend of the influence of the lipid content was in good agreement with the earlier studies on the calcein and the peptide CXA.

The amount of phospholipid content in the release fractions was analyzed by an enzymatic assay (LabAssay Phospholipid, Wako, Neuss, Germany). The assay was performed in 96-well plates. The adsorption of the reaction product was measured at 600nm. Figure V-15 showed the lipid release profiles from VPGs based on various contents of uncharged egg PC. As a result, low lipid concentration facilitated fast erosion of lipids while higher lipid concentration delayed matrix erosion. A closer investigation on the release mechanism is discussed later.

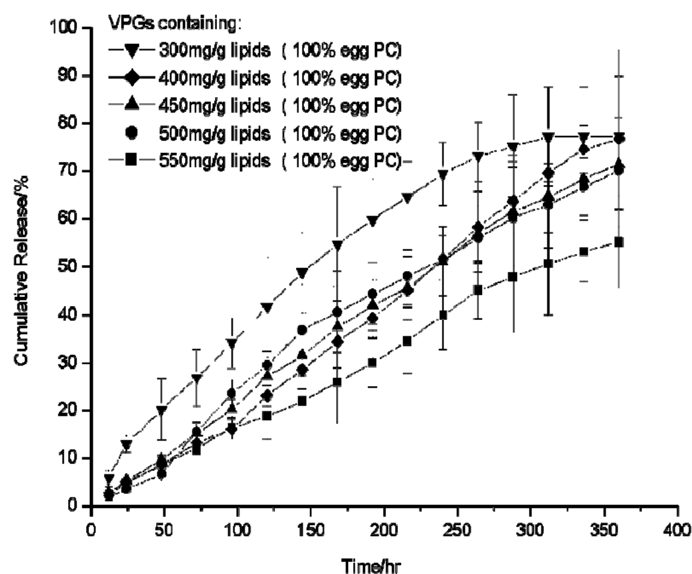


Figure V-14. Cumulative release of EPO from VPGs based on various concentrations of egg PC (LIPOID E80) (Mean± standard deviation, n = 3 each). The protein concentration in all formulations was 3.8mg/g.

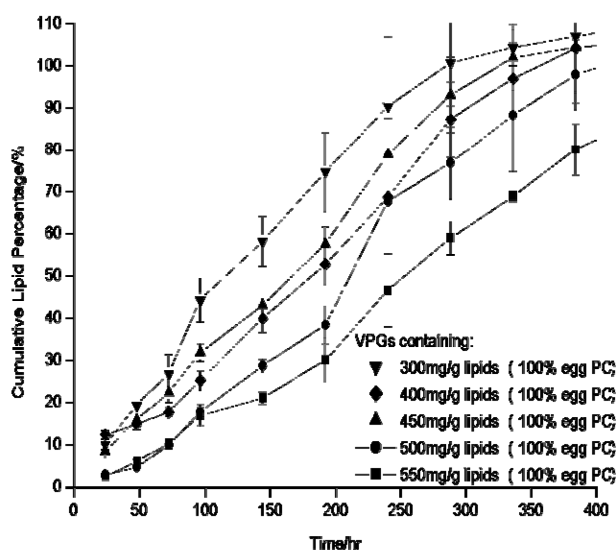


Figure V-15. Lipid release (Erosion) from VPGs based on various concentrations of egg PC (LIPOID E80) (Mean± standard deviation, n = 3 each). The protein concentration in all formulations was 3.8mg/g.

5.2. INFLUENCE OF THE LIPID COMPOSITION

In order to investigate the influence of lipid composition, release tests of two VPG formulations with charged lipids (E 80/ DPPA 9:1, E 80/ DOTAP 8:2, respectively, total lipid concentration 500mg/g) were performed. A constant protein concentration of 3.8 mg/g was used in these formulations. As it can be seen in Figure V-16 the negatively charged gel vesicles (Egg PC/ DPPA 9:1) delivered the incorporated

protein in a comparable manner to the uncharged formulation whereas the positively charged VPGs (Egg PC/ DOTAP 8:2) revealed a strongly accelerated release rate. In agreement with protein release the erosion of lipids from VPGs containing positively charged DOTAP was faster than from uncharged or negatively charged gel formulations (Figure V-17).

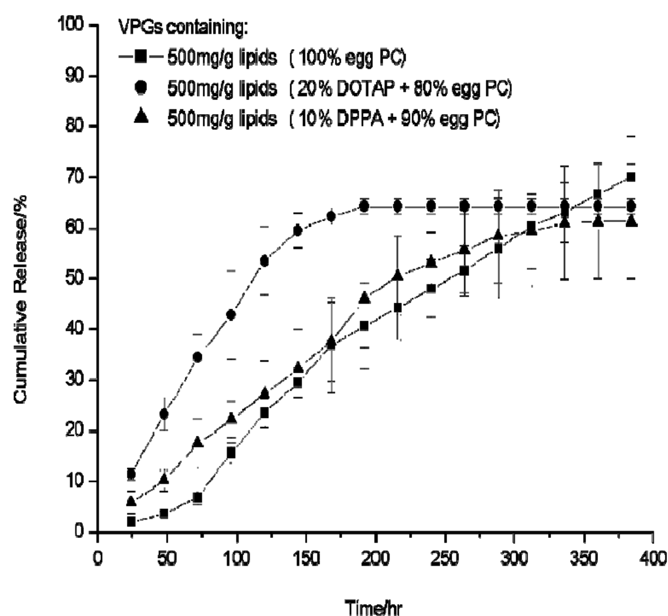


Figure V-16. Influence of lipid charge on the in-vitro release of EPO. The VPGs consist either of pure egg PC (LIPOID E80); a mixture of egg PC (LIPOID E80) and DPPA in the ration 9:1 or a mixture of egg PC (LIPOID E80) to DOTAP in the ratio 8 to 2. The lipid concentration in all formulations was 500 mg/ml (mean± standard deviation, n = 3 each).

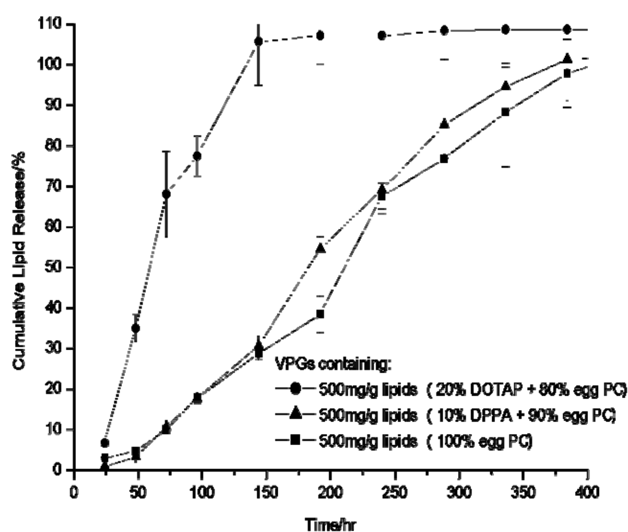


Figure V-17. Influence of lipid charge on the release of lipid. The VPGs consist either of pure egg PC; a mixture of egg PC and DPPA in the ration 9:1 or a mixture of egg PC to DOTAP in the ratio 8 to 2. The lipid concentration in all formulations was 500 mg/ml and the concentration of EPO was 3.8mg/g. (mean± standard deviation, n = 3 each).

However, in contrast the gel rigidity and viscosity measured (Chapter V.2) did not show pronounced differences between VPGs containing positively charged lipids and uncharged/negatively charged gels. Hence it was assumed that the electrostatic interaction between the positively charged lipid vesicles and the release medium (PBS buffer pH 7.4) facilitates the erosion as well as the EPO release from the system.

5.3. RELEASE MECHANISM

In general drug release from VPGs can be explained by two mechanisms: drug diffusion out of the lipid matrix and/or erosion of the matrix [11, 51].

As shown in Figure V-18 (taking VPGs containing 550mg/g lipids and 3.8mg/g EPO for example), the correlation between the lipid release (erosion of the phospholipid matrix) and the release of EPO was found constantly although the absolute values differed in various VPG formulations. Clearly, erosion is the dominant mechanism controlling EPO release. This is in agreement with former observations which showed that mainly matrix erosion governed the delivery of small molecular weight drugs [12, 50, 138, 155] and peptides [51] from VPGs. Moreover, it was found that the lipid charge was of dominant importance besides a strong influence of the lipid concentration on the matrix erosion.

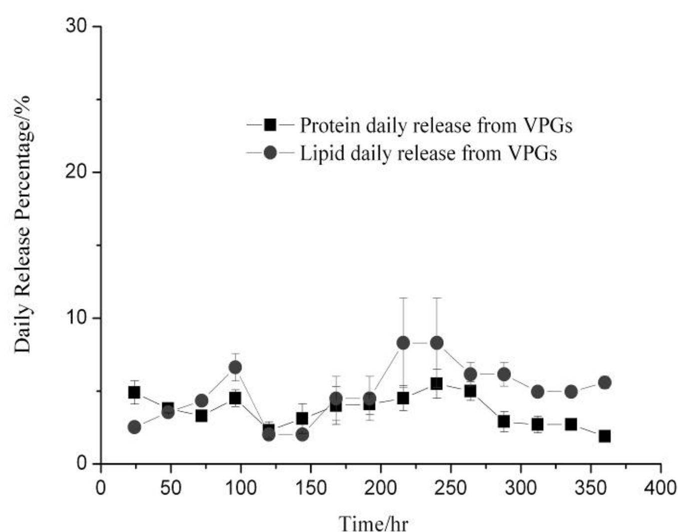


Figure V-18. Daily release percentage of EPO and lipid (erosion of phospholipid matrix) from VPGs. The y axis is the daily released percentage of the total protein/lipid content within VPGs. (VPGs contained 550 mg/g egg PC and 3.8 mg/g EPO. (Mean ± SD, n = 3 each)

5.4. INTEGRITY OF THE RELEASED PROTEIN FROM VPGS

EPO was liberated from VPGs in a continuous manner over days, delivering at least 60% of the incorporated proteins. The remaining protein was not detectable in the release medium despite the fact that the matrices were completely eroded after 3 weeks of incubation.

Consequently, the stability of proteins released from VPGs (Taking VPGs consisting of 400mg/g lipids and 3.8mg/g EPO for example) was examined. The proteins were extracted by applying chloroform (as described previously) from release fractions. SDS-PAGE was performed with subsequent silver staining.

As a result, the presence of dimer specimen was evident in all EPO samples (Figure V-19). However, the release process of EPO from VPGs did not feature the creation of this dimer fraction detected in all release fractions since the dimer was already present in the EPO bulk materials (referring to the SE-HPLC of EPO bulk, Figure V-11a).

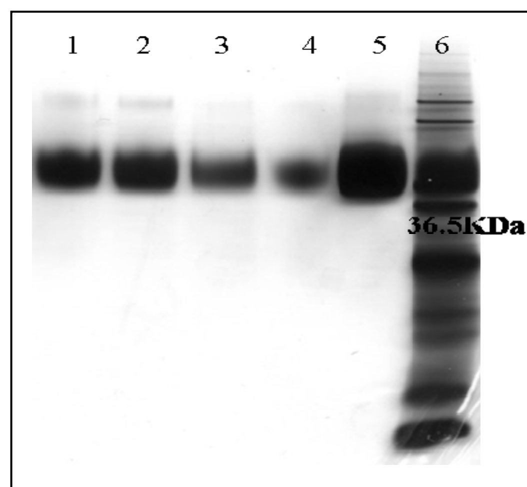


Figure V-19. EPO stability released from VPGs consisted of 400mg/g lipids and 3.8mg/g EPO. Lane1, EPO released after 216hrs; lane 2, EPO released after 168hrs; lane 3, EPO released after 96hrs; lane 4, EPO released after 48hrs; lane 5, EPO standard solution; lane 6, molecular weight maker.

5.5. PARTICLE SIZE DISTRIBUTION

The most important morphological characteristic of liposomes and VPGs is the vesicle size and distribution. The vesicular morphology has been confirmed by cryo electron microscopy of dispersed samples [152]. The other standard method for determination of the size distribution of liposomes is PCS.

In this work, the vesicle size of the release fractions was analyzed with PCS. The results were represented as intensity-weighted averages. As shown in Table V-10, the mean particle sizes of all the EPO VPG formulations were in the range up to ca. 270nm.

Table V-10. Mean particle size of release fractions from VPGs based on various contents of lipids.

EPO VPGs	Based on 400mg/g lipids	Based on 450mg/g lipids	Based on 500mg/g lipids	Based on 550mg/g lipids
Particle size of release fraction	172nm-205nm	175nm-239nm	156nm-261nm	188nm-271nm

In previous work, Grohganz [13] has determined the particle size of liposome dispersions from Cetrorelix loaded VPGs which were formulated by high pressure homogenizer. In his work, all the formulations showed a particle size of around 30 - 50 nm for more than 90% of all the particles. The relatively larger vesicles in our case can probably be ascribed to the different preparation technique (In our work, formulation of VPGs was performed on DAC) as already observed in the work of Massing [147]. However, the EPO loaded VPGs are suitable for application as a depot for in situ application.

6. ADSORPTION STUDY OF EPO

As shown in Figure V-14 and Figure V-16, protein release levelled off before reaching 100% of the total drug load. For PLGA based protein depot systems incomplete protein release was often correlated with protein denaturation and/or aggregation occurring during system preparation or within the release period or during storage [55, 208]. However, as explained above protein aggregation due to the manufacturing can be excluded. Protein loss might occur due to adsorption of EPO on exposed surfaces since as amphiphilic molecules proteins tend to adsorb on interfaces, In order to evaluate adsorption in the release setup EPO solution (10µg/ml) alone or spiked with highly diluted VPG dispersions (with a lipid content of 1.5mg/ml) was continuously pumped with a flow rate of 1ml/hr through the release system. As it can be seen in Figure V-20 already after 12 hours the protein concentration decreased by 10µg/ml to 82% for the unspiked and to 93% for the phospholipid spiked EPO solution. For the VPGs consisting of 300mg/g lipids and 3.8mg/g EPO, 75% of the protein was recovered after the total erosion of the matrices. Since EPO was expected to release from VPGs both in free form and in liposomally entrapped

form, the protein loss caused by unspecific adsorption was in the range from 7% to 18%. Accordingly, the incomplete protein release was mainly caused by unspecific adsorption onto the flow-through release system surfaces.

Typical means to avoid such adsorption, e.g. addition of surfactants were not applied because they would have affected erosion and release heavily by dispersion of the VPGs.

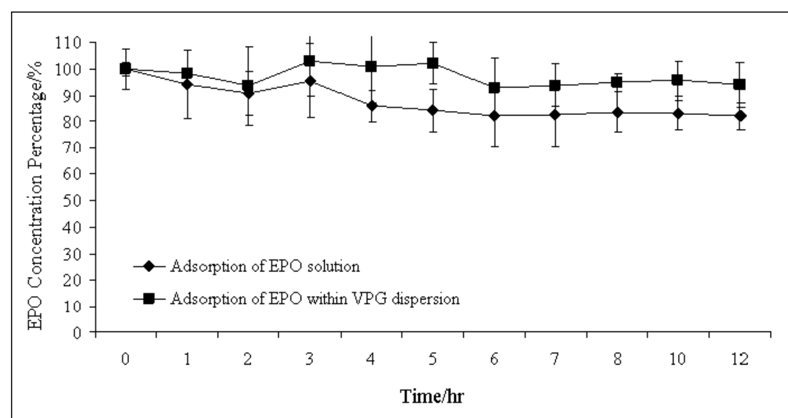


Figure V-20. Adsorption of EPO on the in-vitro release system. The adsorption behaviour of EPO was studied in the presence and in the absence of diluted VPG dispersions. The flow rate was fixed at 1ml/hr and the cells were incubated at 37°C.

7. SUMMARY AND CONCLUSION

In the first part of this section manufacturing strategy based on the dual asymmetric centrifugation was developed to produce EPO loaded VPGs. The temperature of the preparation process could be controlled with multiple centrifugations and cooling in-between.

The obtained VPGs all exhibited rubbery consistency with homogenous morphology under visual and microscopic inspection. On the other hand, increasing the lipid content in the VPG-formulations resulted in the increased gel rigidity and viscosity.

SDS-PAGE was performed to analyze the stability of EPO entrapped in VPGs. Extraction agents including organic solvents and surfactants were applied to eliminate the influence of the lipids. No detrimental effect of the extraction methods was found by the SDS-PAGE. Applying chloroform to extract lipids and subsequently recover EPO from the aqueous phase was found the most suitable extraction method which featured highest protein recovery. Moreover, denatured products of the protein could also be recovered from the lipid containing samples and detected by the SDS-

PAGE. Hence it could be stated the analysis method was established and well validated. SDS-PAGE analysis of EPO extracted from the VPGs matrix revealed no aggregates or fragments, and it was concluded that the formulation procedure caused no detrimental effect of EPO.

As the protein stability was not affected by the formulation process the prerequisite for a successful sustained release system based on lipids was fulfilled and further investigations on the in-vitro release studies were encouraged. However, a major obstacle was found in the protein quantification from the release fractions since the presence of lipids had strong interference on the routine analysis methods (e.g. HPLC, photometry). Therefore it was essential to develop an extraction method that allowed the quantitative recovery of the protein from the liposome fraction without having detrimental effects on the protein integrity. As for quantification, RP-HPLC was found to have more advantages in our case than SE-HPLC in robustness and flexibility in choosing the extraction agents. Among the organic solvents and surfactants tried out, applying chloroform for extraction of the lipids and subsequent recovering of EPO from the water phase was found most suitable. Calibration of extracted EPO from lipids was established with acceptable linearity. In the case of lipids containing egg PC or negatively charged lipid blends, good correlations were achieved between the detected AUCs of extracted and untreated lipid-free EPO solutions. However, due to the electrostatic interaction between the positively charged lipid vesicles and the negatively charged protein, the recovery of EPO from cationic lipid dispersions was dramatically reduced. Hence, the extraction protocol was modified by addition of at least 200 mM NaCl to reduce the interaction and consequently resulted in a suitable response curve similar to that of uncharged lipids. In addition, the influence of the centrifugation conditions on the protein recovery was evaluated to achieve an optimised process. Importantly for the release tests, variations of the lipid amount as well as variations in the protein amount had little effect on the EPO recovery. It was also possible to recover oxidized protein products from lipid containing samples with a comparable recovery. With the developed quantification method of EPO in lipid containing samples, it was possible for the further investigations on the in vitro release of VPGs.

The release profile could be tailored by varying the lipid content within VPGs formulations. Irrespective of the investigated formulation all VPGs delivered the

protein over prolonged periods of time at close to linear kinetics without any burst effect. For instance formulations based on 300mg/g lipid delivered 83% EPO after 280 hours while gels based on 550mg/g lipid liberated 64% within 400 hours. Beside the lipid content, the lipid charge also had a strong influence on the release behavior. Incorporating 20% of positively charged lipids (DOTAP) into VPGs resulted in a strongly accelerated release rate while adding the 10% of negatively charged lipids (DPPA) delivered the incorporated protein in a comparable manner to the uncharged formulation. It was assumed that the erosion of the VPG matrix was facilitated due to the ionic interaction between the lipid vesicle and the buffer solution in the case of the addition of positively charged lipids. The desired release kinetics can easily be achieved by adjusting the lipid content or the lipid charge. From the parallelism of release and erosion kinetics found for all formulations it was concluded that erosion rather than diffusion was the dominant release controlling mechanism for these macromolecule-loaded VPGs.

Importantly, SDS-PAGE analysis of EPO extracted from VPGs' release fractions revealed no further aggregates or fragments beside the denatured products that already existed in the protein bulk solutions. Hence the in vitro process featured no detrimental effect on the protein incorporated within VPGs detected by SDS-PAGE.

The vesicle size of the liposomes released from VPGs was majorly in the range up to 300nm .This result encouraged the expectation that a large number of protein drugs can be transferred into stable liposomes when using VPGs as a depot.

In the final section of this chapter, the protein adsorption onto the release system was investigated since incomplete release was observed for the in vitro release tests. As a result, the protein got 7% lost after 12 hours in presence of lipids while much more protein loss (18%) was observed without liposomal entrapment. Since the protein was released from VPGs both in free form and in liposome form, the adsorption of EPO in the release fractions was in the range from 7% to 18%. This unspecific adsorption to the release system mainly contributed to the incomplete release of EPO from the matrices and should be corrected from the results to reveal the real situation of in vivo application of the VPGs.

Thus it was assumed the unspecific adsorption led to the incomplete protein release from VPGs. However, surfactant was not used since it might affect erosion and release by dispersion of the VPGs.

As conclusion, a sustained release of EPO from VPGs over several hundred hours was observed depending on the formulations. The release of EPO from VPGs is dependant on the erosion of the lipid matrix. Varying the lipid content and composition can modify the erosion and the EPO release profile. Moreover, the protein released from VPGs is expected both in free form and in liposomally entrapped form. These two parts could be differentiated by using asymmetric flow Field Flow Fractionation (AFFF) technique in future work.

CHAPTER VI: VPGS FOR GROWTH FACTOR DELIVERY

Growth factors contribute to tissue regeneration at various stages of cell proliferation and differentiation [210-211]. Although many studies using growth factors have been carried out, limited success has been achieved due to their very short half-life time in vivo to retain the biological activity. Thus, many different controlled release devices have been optimized for controlled delivery of the growth factors. Choi [212] has reported a sustained release of G-CSF over a week in vitro by using PLGA based nanoparticles. Hydrogels based on polyethylene glycol or other copolymers have been investigated for delivering insulin-like growth factor (IGF-1) [213] and vascular endothelial growth factor (VEGF) [214]. A combination of PLGA microspheres embedded in a PLGA matrix has been used for rapid delivery of VEGF from the PLGA matrix followed by a sustained release of platelet derived growth factor (PDGF) from the embedded microspheres [215]. This combination delivery of multiple growth factors has shown improved growth of stable blood vessels around tissue engineering constructs. Gelatin based hydrogels were used for the immobilization and controlled release of human recombinant basic fibroblast growth factor (bFGF) and G-CSF. The co-delivery of the bFGF and G-CSF highly increased endothelial branch point formation than the delivery of either growth factor alone [216].

The aim of this chapter is to develop the VPGs with loading of growth factors. Various VPG-formulations of granulocyte colony-simulating factor (G-CSF) were prepared and characterized with the established methods. In vitro release behavior was evaluated to address the potential of the system for the delivery the growth factors. In vivo experiments would be carried out using rats as animal model. VPGs prepared with various doses of G-CSF, bFGF and a combination of the two growth factors would be applied in the rupture of the tendon to offer a sustained release of proteins. The therapeutic effect for the implantation of VPGs would be evaluated. In order to study the pharmacokinetics, ELISA was performed to develop a quantification method of G-CSF in the serum.

1. PREPARATION OF G-CSF LOADED VPGS

The G-CSF stock solution used here has a concentration of 4.0mg/ml in 10mM acetate buffer pH 3.9 containing 0.004% Tween and 5% sorbitol. Egg phosphatidylcholine (E80) were supplied from LIPOID. G-CSF VPGs were

formulated in DAC following the procedure described in the method section. In brief, the protein solution was added to the lipids. The mixture was homogenized in DAC by multiple runs of 1.5-minutes (to a total mixing time of 45 minutes) at a process speed of 3500 rpm. The samples were cooled at 2-8°C at interrupts (after 6-10 runs) to control the system temperature below 40°C. Several formulations were prepared to evaluate the influence of the lipid and protein content on the release behaviour. The lipid content was varied between 400mg/g and 550mg/g. The concentrations of G-CSF were varied from 2.2mg/ml to 8.4mg/ml.

2. TEXTURE ANALYSIS AND RHEOLOGY TEST OF G-CSF VPGS

The Texture analyser (TA.XT plus, Stable Micro Systems, UK) recorded the forces (Figure VI-1a) which occured when the probe (4mm in diameter) was pushed with a speed of 0.5mm/s into the tested VPGs. The gel strength of VPGs was represented as the maximal force value. The rheology behaviour of MAB VPGs was studied by a rotational viscometer (Physica MCR 100, Anton Paar) at 25°C. The shear viscosity was provided versus shear rate ranging from 10^{-1} and 100^{-1} (up and down curve, Figure VI-1b).

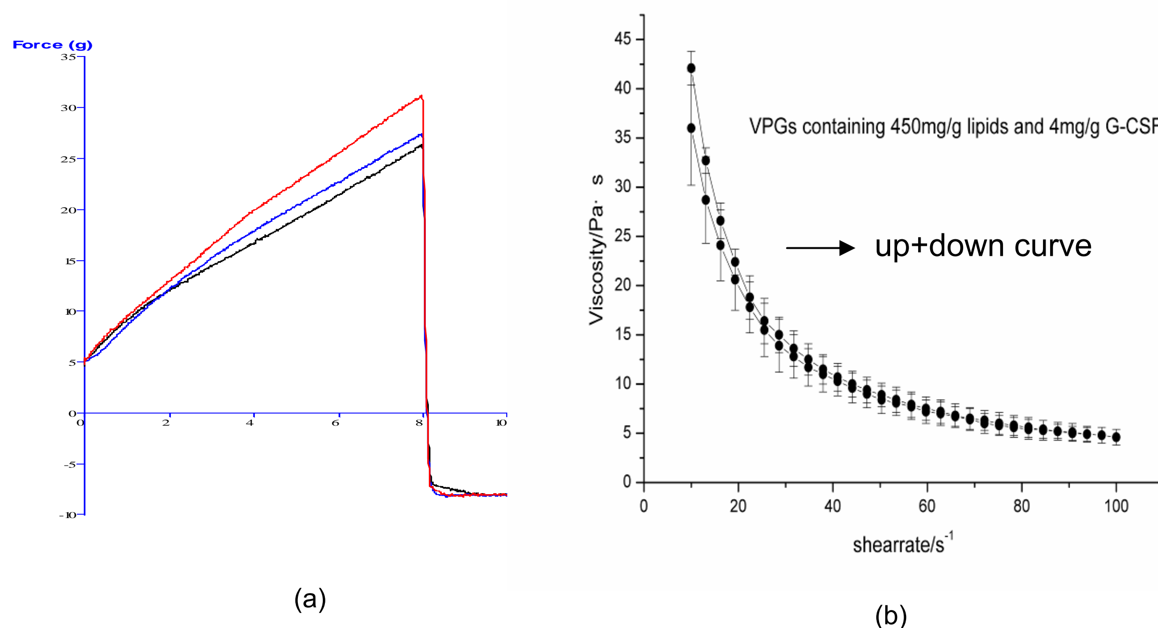
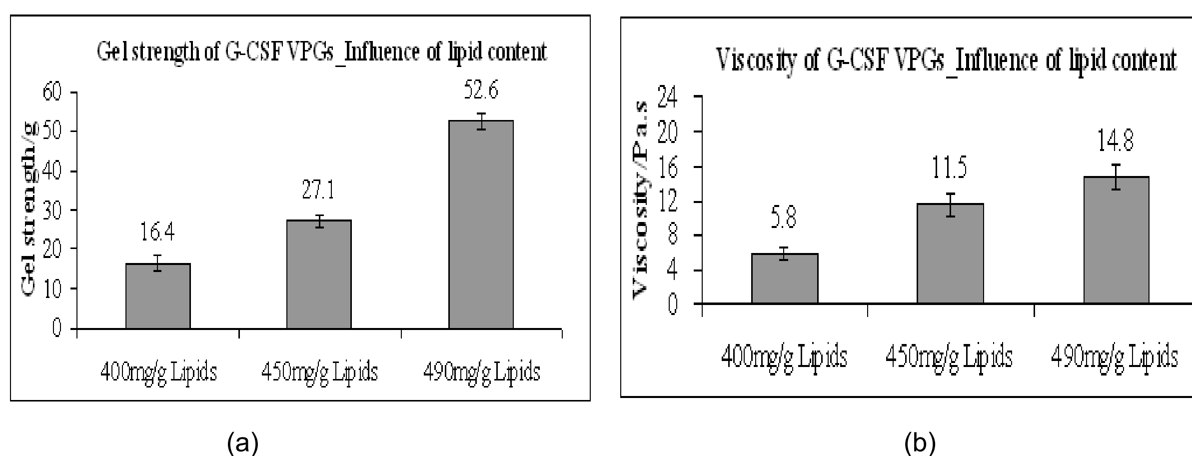


Figure VI-1. (a) Texture analysis of G-CSF loaded VPGs: recorded force trace when the probe (4mm in diameter) was pushed with a speed of 0.5mm/s into the tested VPGs. VPGs were based on 450mg/g lipids and 2.2mg/g G-CSF. (b) Rheological behaviour of VPGs. VPGs were based on 450mg/g lipids and 4mg/g G-CSF.

The influence of the lipid content in VPG-formulations on both the gel strength and viscosity was investigated. VPGs were prepared by various lipid contents ranging from 400mg/g to 490mg/g. A constant G-CSF concentration of 4mg/g was used in all the formulations. Increased gel strength was found by incorporating more lipids within VPGs (Figure VI-2a). The maximal force of VPGs based on 400mg/g lipids was 16.4g, while the value of VPGs consisted of 490mg/g lipids was 52.6g. As for the rheological behaviour of VPGs, the viscosity was compared at the same shear rate of $37.9s^{-1}$. As shown in Figure VI-2b, increasing the lipid content also resulted in increased viscosity.



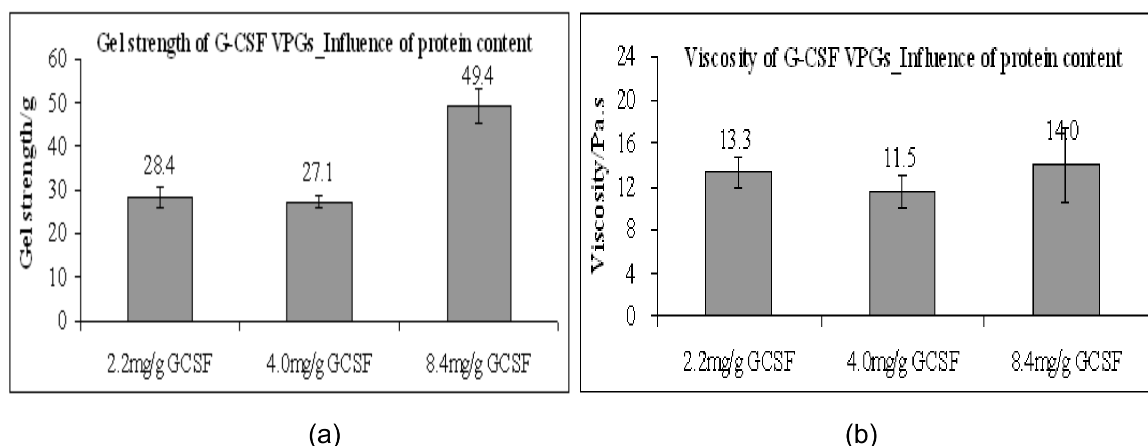
(a) (b)

Figure VI-2. (a) Gel strength (mean±SD, n=3) of G-CSF loaded VPGs. VPGs were prepared by various contents of lipids (as indicated in the figure) and a constant G-CSF concentration of 4mg/g. (B) Viscosity (mean±SD, n=3) of G-CSF loaded VPGs. VPGs were prepared by various contents of lipids (as indicated in the figure) and a constant G-CSF concentration of 4mg/g.

The influence of the G-CSF content within VPG-formulations on both the gel strength and viscosity was also investigated. VPGs were prepared by varying the protein contents from 2.2mg/g to 8.4mg/g. A constant lipid concentration of 450mg/g was used in all the formulations. As shown in Figure VI-3a, VPGs based on 2.2mg/g G-CSF had a gel strength value of 28.4g while VPGs containing 4.0mg/g G-CSF got a similar value of 27.1g. When the lipid content was further increased to 8.4mg/g, the gel strength was much higher (49.4g). However, the viscosity got little influenced by changing the protein content in this concentration range taken the standard deviation into consideration (Figure VI-3b). In accordance with the results of texture analysis, the viscosity (13.3 Pa.s) of VPGs based on 4.0mg/g G-CSF was slightly lower than that of VPGs based on 2.2mg/g.

Comparing G-CSF VPGs with EPO VPGs, the gel strength and viscosity of G-CSF VPGs were lower than those of EPO VPGs which were based on the same content of lipids (EPO was formulated with 20mM PBS buffer while G-CSF was formulated

with 10mM acetate buffer). Further experiments were performed and it was found that the buffer ingredients in the protein solutions have strong influence on the gel strength and viscosity. Therefore the difference in the gel texture was attributed to be the different buffers used in the different protein samples.



(a) (b)

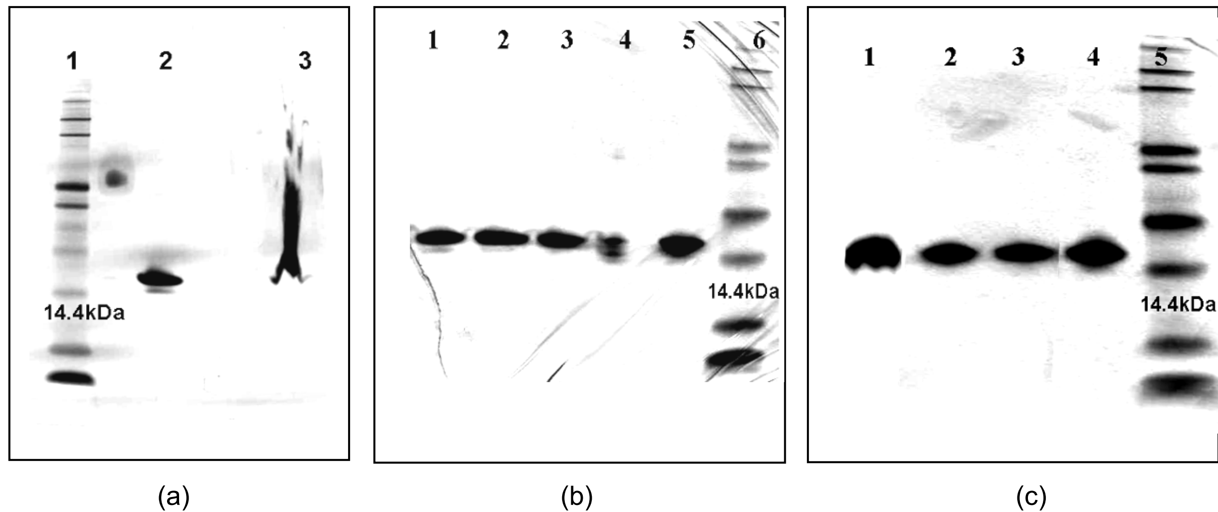
Figure VI-3. (a) Gel strength (mean±SD, n=3) of G-CSF loaded VPGs. VPGs were prepared by various contents of G-CSF (as indicated in the figure) and a constant lipid concentration of 450mg/g. (b) Viscosity (mean±SD, n=3) of G-CSF loaded VPGs. VPGs were prepared by various contents of G-CSF (as indicated in the figure) and a constant lipid concentration of 450mg/g.

3. EVALUATION OF G-CSF STABILITY BY SDS-PAGE

The integrity of G-CSF was analyzed by non-reducing SDS-PAGE with subsequent silver staining. In brief: the electrophoresis was performed on the Bis-Tris Pre-Cast Gel (Invitrogen, Groningen, Netherlands) in the MES running buffer (Invitrogen).

Without applying an extraction process, the analysis of G-CSF VPGs (redispersed in PBS buffer pH 7.4 resulting in a protein concentration of 100ug/ml) was strongly interfered by the presence of lipids (Figure VI-4a). Hence organic solvents and surfactants including chloroform, dichloromethane, ethanol and Triton X-100, were tried out to dissolve the lipids. As surface active molecules, proteins tend to adsorb at water/organic solvent interfaces. Since this adsorption step can induce protein instability [55, 217], the influence of the extraction agents on the protein stability was evaluated as the start point. It was found that adding ethanol to the G-CSF in absence of any lipids resulted in low protein recovery (as indicated by the band intensity). Beside the acceptable recoveries, no aggregation or fragmentation fraction was observed when other extraction agents were applied to the G-CSF without lipids (Figure. VI-4b). Then these extraction agents except ethanol were applied for the extraction of G-CSF from VPGs. As shown in Figure VI-4c, the dissolution of the

lipids by adding Triton X-100 resulted in the highest recovery of G-CSF without any detection of degradation products. Though the methods using chloroform and dichloromethane revealed lower extraction efficiencies, none of these methods featured aggregation or fragmentation of G-CSF. Accordingly, the formulation process to entrap G-CSF within VPGs suggested no detrimental effect on the protein stability.



(a) (b) (c)

Figure VI-4. Influence of the formulation process on the GCSF stability, by SDS-PAGE, silver staining.

(a): lane 1, molecular weight marker; lane 2, G-CSF standard; lane 3, G-CSF VPGs' dispersions without applying extraction process.

(b): lane 1, G-CSF solution (in absence of lipids) with adding Triton X-100; lane 2, G-CSF solution (in absence of lipids) with adding dichloromethane; lane 3, G-CSF solution (in absence of lipids) with adding chloroform; lane 4, G-CSF solution (in absence of lipids) with adding ethanol; lane 5, G-CSF standard; lane 6, molecular weight marker.

(c): lane 1 G-CSF VPGs, the protein was extracted by applying Triton X-100; lane 2, G-CSF VPGs, the protein was extracted by applying dichloromethane; lane 3, G-CSF VPGs, the protein was extracted by applying chloroform; lane 4, G-CSF standard; lane 5, molecular weight marker.

4. Characterisation of the secondary protein structure within VPGs

FTIR-spectroscopy has been suggested to analyze the secondary structure of proteins embedded within controlled release devices [218-220]. However, vibration of the matrix materials in the spectrum was observed, which might have interference on the spectra of the embedded proteins [219]. Van de Weert [219] has tried out to correct the spectra of lysozyme embedded within PLGA microspheres by the spectra of the blank PLGA microspheres. However, it was found that the background from the blank could not be completely removed because of the baseline slopes and distorted peak shapes especially in the carbonyl stretch vibration of PLGA. Moreover,

the different adsorption characteristics of protein–loaded microspheres were also suggested to restrict the subtraction of the spectrum of blank PLGA microspheres [219]. In the work of Herrmann [100], the same problem was observed for the IFN-α embedded in a lipid extrudate. The variations of the subtraction factor either involved an over-subtraction or an under-subtraction of the lipid contributions, leading to negative or positive features between 1700 and 1800 cm^{-1}.

In this context, FTIR with the ATR-technique was used for the analysis of G-CSF either formulated in the buffer (10mM acetate buffer pH 3.9) or embedded within VPGs.

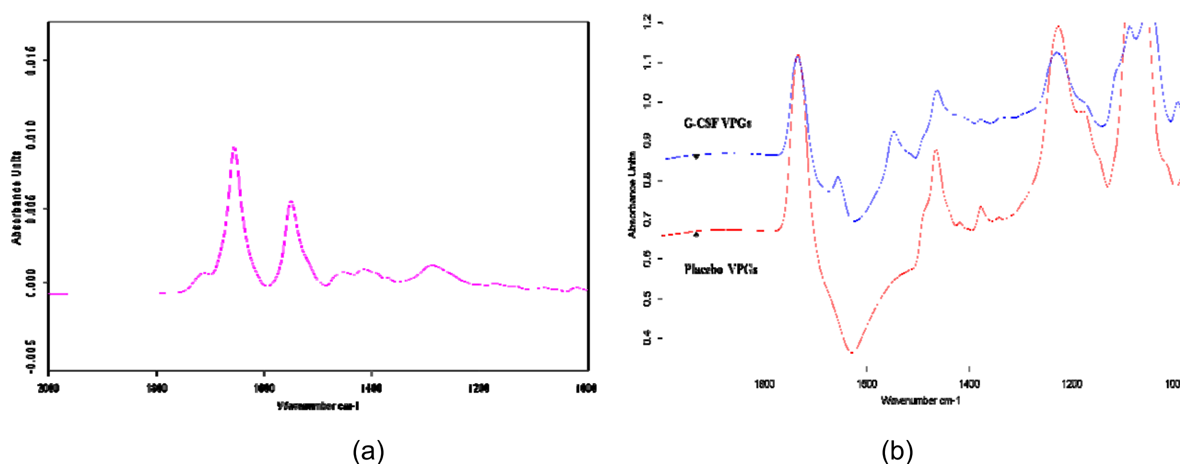


Figure VI-5: ATR-spectrum, (a) G-CSF in the buffer solution; (b) G-CSF VPGs (without subtraction) based on 450mg/g lipids and 4mg/g G-CSF and placebo VPGs based on 450mg/g lipds.

As illustrated in Figure VI-5a, the spectra recorded by the ATR-technique revealed a protein signal in the amid I/amid II region for the G-CSF in buffer solution.

The potential of FTIR to characterize the secondary structure of proteins embedded within VPGs was investigated subsequently. G-CSF VPGs were freshly prepared with 450mg/g lipids and 4mg/g G-CSF. As shown in Figure VI-5b, the spectrum of G-CSF VPGs was influenced by the background from placebo VPGs. Yang [220] suggested a flat baseline in the region at 1730 - 1710 cm-1as criterion for successful background subtraction. However, it was not possible to fulfil this request with the G-CSF loaded VPGs studied here.

In addition, several extraction methods were applied to eliminate the influence of the matrix background. However, lipids could not be removed clearly, and the left lipids still got strong interference. Hence we have no access to use FTIR to examine the protein stability in the study.

5. QUANTIFICATION METHOD FOR RELEASED G-CSF

A quantification and extraction method was needed for MAB analysis from lipid containing samples. Since the released amount of VPGs at each time interval was unknown, the quantification method should feature a high robustness against variations of the lipids as well as against variations in the protein amount which make this method suitable for protein quantification during in-vitro release studies. The fluorescamine assay and reverse phase HPLC method were assessed in the following.

5.1. THE FLUORESCAMINE ASSAY

Anderson [221] has used a fluorescamine assay to detect microgram quantities of GM-CSF in presence of dimyristoyl phosphatidyl choline (DMPC). Accordingly, the assay was performed for the quantification of G-CSF from the liposome fractions. A modified process using the 96 well-microplate as described by Lorenzen [194] was performed. Briefly, 150µl of the protein containing samples (in PBS buffer pH 7.4) and standards were pipetted into microplate wells (Nunc MaxiSorp™, Roskilde, Denmark). Fluorescamine was dissolved in acetone resulting in a concentration of 3mg/ml. The microplate was placed on a microplate shaker and 50µl of the fluorescamine solution was added to each well. After shaking for a minute, the fluorescence was determined by using an excitation wavelength of 400nm (bandwidth: 20nm), and an emission wavelength of 460nm (bandwidth: 20nm).

Placebo VPGs were dispersed with PBS buffer pH 7.4 resulting in lipid concentrations from 0.5mg/ml to 5mg/ml. To simulate the conditions expected during in-vitro release G-CSF solutions (10µg/ml) were spiked with diluted VPG dispersions and the recovery of G-CSF was determined and compared to the results of lipid-free G-CSF solutions by the assay. As shown in Table VI-1. The results of the assay were strongly influenced by the presence of various contents of lipids. Thus it could be stated the assay appeared not suitable in our case.

Table VI-1. Recovery of G-CSF from liposome containing samples, by the fluorescamine assay, n=8

Lipid concentration	0.5mg/g lipids	1.5mg/g lipids	5.0mg/g lipids
G-CSF recovery	142±13%	159±24%	243±37%

5.2. RP-HPLC METHOD

From the experiences in developing the quantification method of EPO, reverse phase HPLC appeared reasonable for G-CSF quantification in the samples containing liposomes. The method was developed with a ProSwift™ RP-2H column (4.6*50mm, Dionex). Gradient elution was performed as shown in Table VI-2. The eluent A consisted of 0.12% TFA in destilled water, and the eluent B consisted of 90% CH_3CN (in destilled water) and 0.1% TFA. The UV detector was operated at 215nm. The injection volume was 100µl.

Table VI-2. Gradient elution for RP-HPLC of G-CSF

Time/min	0	5	10	25	30	35	40	45
Eluent A%	100	80	60	60	40	20	0	100
Eluent B%	0	20	40	40	60	80	100	0
Flow rate	1.5ml/min							

G-CSF had a retention time around 28minutes (Figure VI-6). The calibration curve was generated with the protein concentrations ranging from 2.5µg/ml to 20.0µg/ml for the G-CSF solutions in absence of lipids (Figure VI-7).

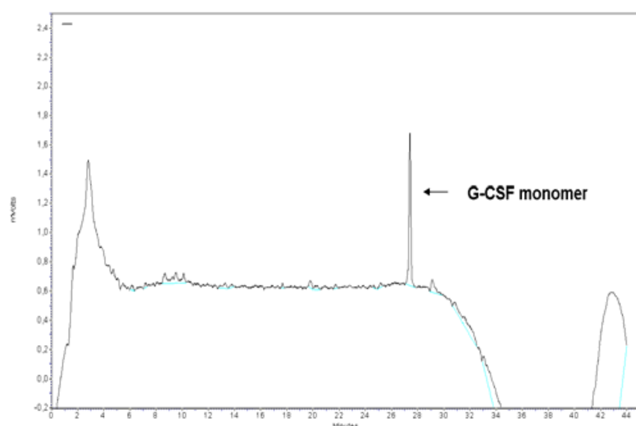


Figure VI-6. RP-HPLC chromatogram of G-CSF standard, 5µg/ml

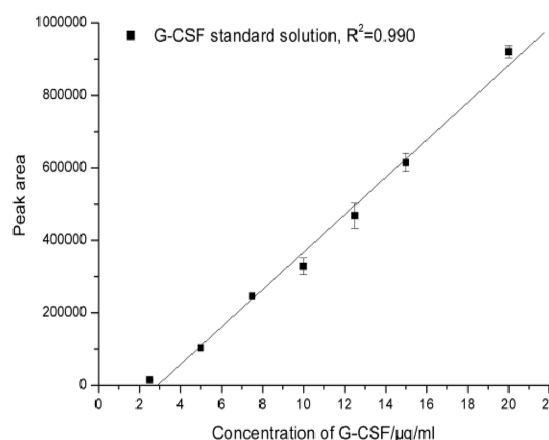


Figure VI-7. Calibration of G-CSF standard, concentrations of G-CSF ranged from 2.5µg/ml to 20.0µg/ml, R2=0.990

5.3. EXTRACTION METHOD

To determine the concentration of G-CSF in the release fractions, an extraction method is necessary for the dissolution of the lipids and extraction of the protein. The extraction method should allow the quantitative recovery of the protein from the

liposome fractions without having detrimental effects on the protein integrity. To simulate the conditions expected during in-vitro release G-CSF solutions were spiked with diluted VPG dispersions and the recovery of G-CSF was determined and compared to the HPLC-response of lipid-free G-CSF solutions after applying a detergent or an organic solvent. For the initial screening of the extraction methods, the samples were prepared with a G-CSF concentration of 10µg/ml and a lipid content of 1.5mg/ml.

5.3.1. METHOD DEVELOPMENT

In Chapter V it was shown that applying chloroform for the dissolution of lipids resulted in good recovery of EPO. Thus as a start point, chloroform (0.3ml per ml sample) was applied for extraction of G-CSF from lipids containing samples. The protein was recovered from the supernatant and subsequently quantified by the aforementioned RP-HPLC method. As shown in Figure VI-8a, a low protein recovery of 22.5±2.0% was observed for G-CSF extracted by applying chloroform. Thus other extraction methods had to be assessed.

In previous work, Grohganz [204] used Triton X-100 for the dissolution of liposomes and extraction of the decapeptide cetrorelix from VPGs. Accordingly, 5% Triton X-100 was applied for G-CSF extraction. As shown in Figure VI-8b, Triton X-100 has generated multiple peaks which could not be totally separated from the protein peak. This phenomenon was already observed in the application of Triton X-100 for EPO extraction. In addition, a fluorescence detector (Excitation wavelength: 280nm, Emission wavelength: 340nm) was also used aimed to eliminate the influence of the Triton X-100. However, similar results were obtained which made the extraction method with applying Triton X-100 not suitable in this case.

Methanol has been used to dissolve lipids in the G-CSF liposome samples prior to HPLC analysis [222]. Hence, this method was also assessed in our work. 1ml methanol was applied to extract G-CSF from the lipid (1.5mg/ml) containing samples. However, our challenge here is the variation of the lipid content as well as the variation of the protein content in the released samples.

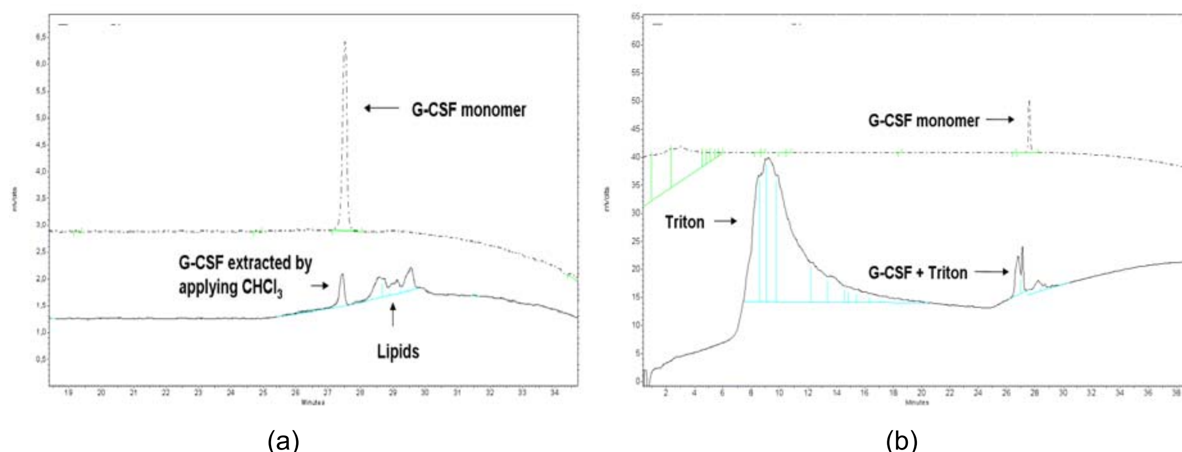


Figure VI-8. RP-HPLC chromatograms of G-CSF, (a) extracted G-CSF by applying chloroform, G-CSF standard was shown as reference, (b) G-CSF by applying Triton X-100 for dissolving the lipids, G-CSF standard was shown as reference.

Unfortunately, it was observed that the recovery of G-CSF was influenced by the original protein content in the samples when methanol was applied for lipid dissolution (Table VI-3). At lower protein concentrations the protein recoveries were implausibly high, which was probably caused by the baseline noise. In comparison about 48% was recovered from 10µg/ml G-CSF. However, the dilution effect of methanol has to be taken into account since methanol is miscible with the buffer solution. Regardless of this point, the method using methanol for extraction appeared not suitable for the quantification of released G-CSF from VPGs.

Table VI-3. Recovery of G-CSF from liposome containing samples, methanol was added to dissolve the lipids, n=3

Concentration G-CSF	2.5µg/ml	5.0µg/ml	7.5µg/ml	10µg/ml
G-CSF recovery±SD%	336.7±40.4%	95.9±-9.0%	58.9±1.9%	48.2±8.6%

When chloroform was added to the G-CSF samples, the recovery of G-CSF was only about 22.5% for the lipids (1.5mg/ml) containing samples, whereas it was interestingly found about 100% of the protein was recovered in absence of lipids. Our hypothesis was that the low recovery of G-CSF could be caused by the interaction between the protein and the lipids. Due to the interaction a part of the proteins were transported to the organic phase with the lipids, which consequently resulted in low protein content recovered from the aqueous phase. Some trials were made aimed to reduce the interaction: The pH of the solution was adjusted to change the protein charge (G-CSF has a pI of 6.1 [223], The zeta potential in various buffer conditions was shown in Table VI-4.). Chloroform was added for lipid extraction and the

recovered protein was quantified by the developed RP-HPLC method. As shown in Table VI-4, the recovery of the G-CSF was even worse at either a pH of 3.0 or pH 10.0. Thus varying the charge of the protein failed to improve the extraction efficiency of the method.

Table VI-4. G-CSF recovery: influence of the protein charge, the protein was extracted by applying chloroform.

G-CSF Sample	G-CSF in liposome containing samples, pH 7.4.	G-CSF in liposome containing samples, pH 3.0.	G-CSF in liposome containing samples, pH 10.0.
Concentration	10 µg/ml		
Zeta potential ζ/mV	-4.4±0.3	4.4±0.3	-20.8±1.0
Recovery±SD%	22.5±2.0%	10.3±5.8%	7.6±12.5%

However, low protein recovery by applying chloroform might also be induced by the protein instability occurred at the interface [209, 224] since the presence of lipids might create an improved interface between the protein and the organic solvent. Consequently some trials were made based on the report that adding a high amount of sucrose could improve the stability of the protein [225]. Accordingly, sucrose was added to the samples, and the G-CSF was extracted by applying chloroform and quantified by RP-HPLC. As a result, the addition of sucrose could not efficiently improve the protein recovery (Table VI-5) whereas resulted in high viscosity of the samples which might harm the HPLC column.

Table VI-5. G-CSF recovery: the addition of sucrose on the recovery of G-CSF. The protein was extracted by applying chloroform and subsequently quantified by RP-HPLC.

Concentration of sucrose	0	50mM	250mM	1M	2M
Concentration of G-CSF	10µg/ml				
Recovery±SD%	22.5±2.0%	14.7±5.8%	17.5±2.2%	34.3±2.0%	28.0±5.0%

5.3.2. VALIDATION OF THE FINAL EXTRACTION METHOD WITH N,N-DIMETHYLFORMAMID

N,N-Dimethylformamid (DMF) is a water-miscible organic solvent which has been used in the fluorescamine assay for protein-lipid isolation process. The function of the organic solvent was reported to suppress the influence of the residual lipids allowing direct comparison of samples with and without lipids [226]. Accordingly, 0.4ml DMF was added to 1ml G-CSF solutions (in PBS buffer) in presence of 1.5mg/ml lipids. After centrifugation (3000rpm for 30mins, at 15°C), the lipid was precipitated and the

protein was recovered from the solution. The protein was quantified by the developed RP-HPLC method.

As a result of the RP-HPLC analysis, the extraction by applying DMF at a pH environment of 7.4 featured the creation of oxidized G-CSF species whereas only monomeric protein with good recovery was observed at a pH of 3.5 (Figure VI-9). However, it was not surprising to find that the extraction effect was better at a low pH environment when G-CSF was exposed to organic solvent as CHI [223] had found out that G-CSF was more stable in a buffer solution of pH 3.5 rather than of pH 7.4.

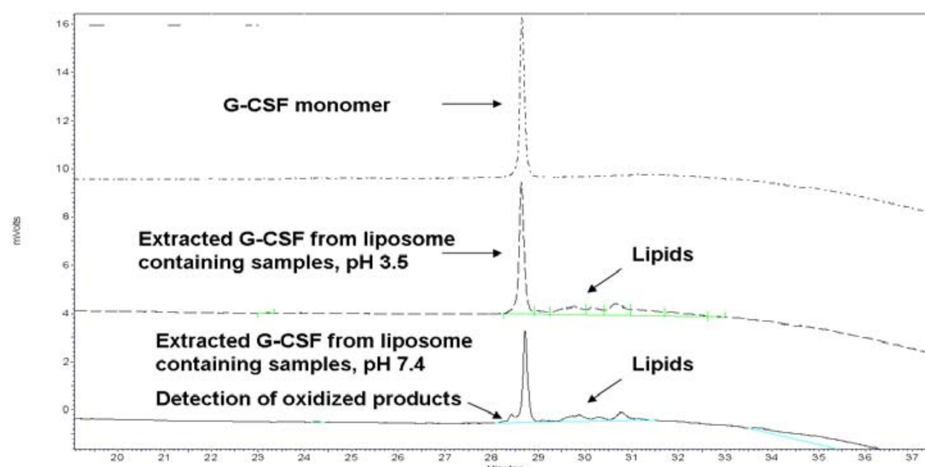


Figure VI-9. RP-HPLC of G-CSF, the protein was extracted from liposome containing samples (lipid concentration: 1.5mg/ml) by applying DMF. (a), G-CSF standard, 10µg/ml; (b), G-CSF extracted from liposome containing samples (PBS buffer pH 3.5); (c), G-CSF extracted from lipid containing samples (PBS buffer pH 7.4)

The extraction method with DMF was further evaluated at low pH. The recovery of G-CSF extracted by applying DMF from the lipid containing samples was calculated to be 91.4% at a pH of 3.5. Further investigations showed that in the pH range from 3.0 to 3.8, the protein recovery maintained relatively stable above 90% (Table VI-6). For the consideration of the in vitro release tests which were performed with PBS buffer pH 7.4, the release fractions needed a pH adjustment to 3.5 prior to the addition of DMF for extraction.

Table VI-6. Recovery of G-CSF: influence of the pH values of the PBS solution, the pH ranged from 3.0 to 3.8. The lipid content was 1.5mg/ml and the protein content was 10µg/ml in all the samples; DMF was used for protein extraction.

pH of the sample	3.0	3.2	3.5	3.8
G-CSF recovery±SD%	98.8±1.4%	96.1±-3.5%	91.4±1.9%	92.5±5.6%

Further investigations were carried out to see whether the extraction efficiency of G-CSF by using DMF was influenced by the lipid content within the samples. The lipid contents were varied between 0.5mg/ml and 5mg/ml (This concentration range was assumed to be around the lipid concentration in the release fractions).The protein concentration in all the samples was 10µg/ml. It was found that a lower protein recovery of 68% was obtained for the samples containing the lowest lipid content (0.5mg/ml). In comparison, there was a plateau of the extraction efficiency at a level of more than 90% for all the other samples containing a lipid content of more than 1.5mg/ml (Figure VI-10). This phenomenon can be explained in a way that the presence of sufficient amount of lipids can reduce the exposure of G-CSF to the organic solvent and consequently reduce the protein loss due to the addition of DMF. In this case, an extra amount of 1.5mg lipid (per ml sample) should be necessarily added to the release samples to achieve a high and repeatable protein recovery for the quantification aim.

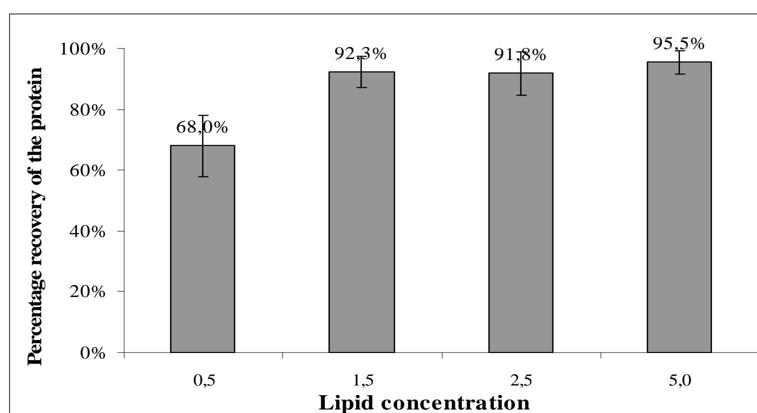


Figure VI-10. Recovery of the G-CSF: influence of the lipid content within the samples. The lipid content was varied from 0.5mg/ml to 5mg/ml; the protein content was 10µg/ml in all the samples; DMF was used for protein extraction.

In summary, the quantification method for G-CSF in the release fractions was fixed as followings: (1) 1.5mg/ml lipid (from VPG-disperions) is added to the release fractions. (2) The pH of the samples is adjusted to 3.5. (3) 0.4ml/ml DMF is added and the lipids are precipitated after centrifugation (3000rpm for 30mins, at 15°C). (4) G-CSF is recovered from the aqueous solution and subsequently quantified by the RP- HPLC.

Figure VI-11 shows the AUCs of G-CSF with this method in comparison to the calibration curve in the absence of any lipids (The calibrations were established with protein concentrations ranging from 2.5µg/ml to 20µg/ml). The extraction method by

applying DMF was used as standard method for G-CSF quantification for the in-vitro release studies.

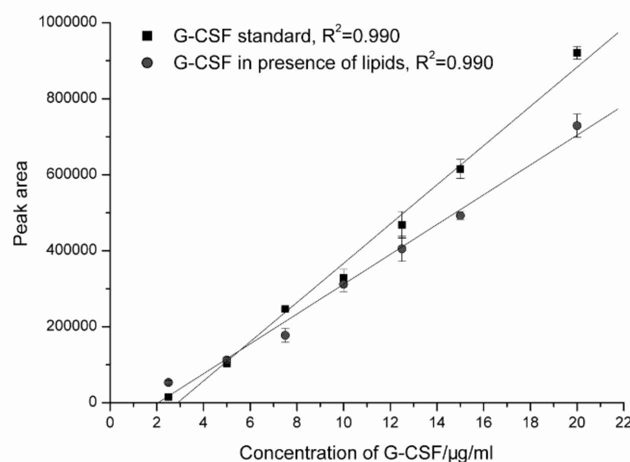


Figure VI-11. Response curve of G-CSF standard in comparison to the protein samples recovered after extraction from liposome containing samples. The lipids were precipitated by adding DMF.

6. In vitro release of G-CSF from VPGs

In vitro release tests were conducted within flow through cells at 37°C as described in the method section. PBS buffer pH 7.4 (20mM) was used as acceptor medium. The released G-CSF from VPGs was extracted by applying DMF and quantified by RP-HPLC. The release data are presented as the average of triplicates.

6.1. Influence of the lipid content

The influence of the lipid content on the release behavior of G-CSF VPGs was first investigated. VPGs were prepared with various lipid (egg PC, LIPOID E80) contents ranging from 400mg/g to 490mg/g (Table IV-7). The concentration of G-CSF was 4mg/g in all these formulations. The pH of release fractions were adjusted to 3.5 by adding hydrogen chloride for analysis if not otherwise stated.

Table IV-7. Formulations of G-CSF VPGs based on various contents of lipids.

	Lipid Concentration	G-CSF Concentration
G-CSF VPGs_1	400mg/g Egg PC (Lipoid E80)	
G-CSF VPGs_2	450mg/g Egg PC (Lipoid E80)	4mg/g
G-CSF VPGs_3	490mg/g Egg PC (Lipoid E80)	

As it can seen in Figure IV-12, G-CSF VPGs based on 400mg/g lipids delivered 63% of the total entrapped proteins over 408hrs while VPGs containing 450mg/g lipids

released 43% and VPGs with 490mg/g lipids released 35% within the same period. Increasing the lipid content from 400mg/g to 450 mg/g decreased the release rate of G-CSF from the VPGs. In comparison, the release behavior of the systems was little influenced when the lipid content was increased from 450mg/g to 490 mg/g. Moreover, it was observed that G-CSF was released from all formulations in a close to linear manner without initial burst.

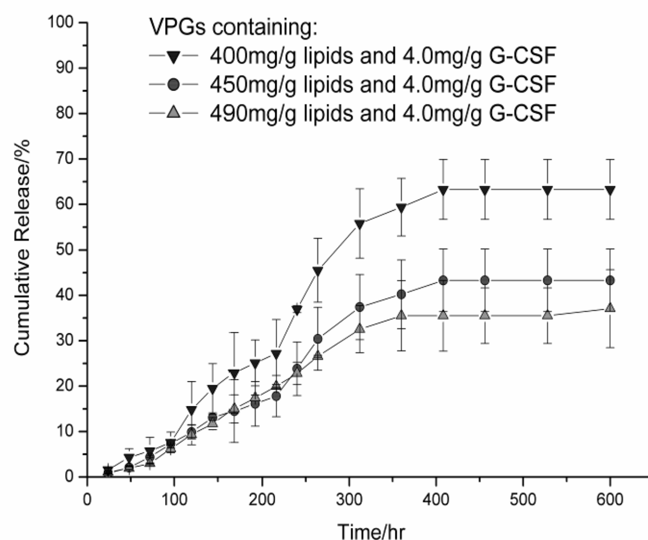


Figure VI-12. Cumulative release of G-CSF from VPGs based on various concentrations of lipids (egg PC, LIPOID E80) (Mean± standard deviation, n = 3 each).

6.2. INFLUENCE OF THE G-CSF CONTENT

In Grohganz's work, the release behaviour of the decapeptide cetrorelix from VPGs was found also dependant on the content of the peptide entrapped within VPGs [51].

Accordingly, the influence of the G-CSF content on the release behaviour of the system was investigated. VPGs were prepared with various concentrations of G-CSF as shown in Table VI-8. The lipid content in all these VPG-formulations was 450mg/g (egg PC, LIPOID E80).

Table VI-8. Formulations of G-CSF VPGs based on various contents of proteins

	Lipid Concentration	G-CSF Concentration (in the final formulations)
G-CSF VPGs_2	450mg/g Egg PC (Lipoid E80)	4.0mg/ml
G-CSF VPGs_4		8.4mg/ml

In comparison to VPGs based on 4.0mg/g G-CSF which delivered 43% of the incorporated proteins, the formulations containing 8.4mg/g G-CSF showed a far slower release from which about 11% of the total entrapped G-CSF was released over 600hrs (Figure VI-13). This finding was in agreement with the work on the decapeptide cetrorelix that increasing the content of the peptide within VPGs could result in reduced release rate. This behaviour may also be described as drug–drug and drug–lipid interactions, which in turn increase the overall stiffness of the VPGs[51] (as also indicated by the texture analysis of G-CSF VPGs in Figure VI-3a), thus slowing down the erosion of the lipid matrix and leading to a slower release of G-CSF.

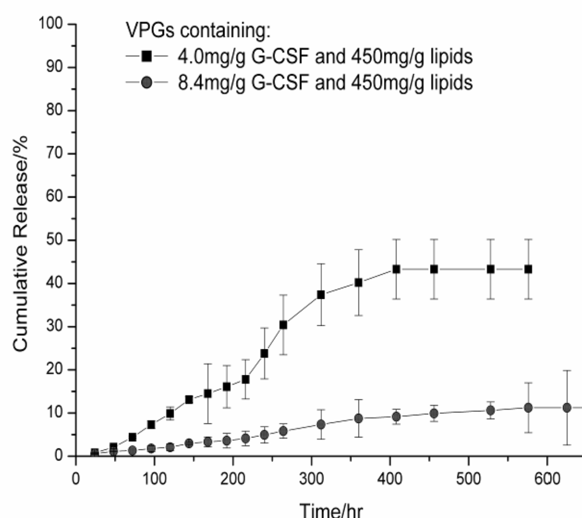


Figure VI-13. Cumulative release of G-CSF from VPGs based on various concentrations of G-CSF (Mean± standard deviation, n = 3 each). The lipid concentration in the formulations was 450mg/g.

6.3. DAILY RELEASE AMOUNT

The releases from various VPG-formulations were calculated and are summarized in Table VI-9. It was confirmed the VPG system showed no initial burst for the G-CSF release. From 450mg/g to 490 mg/g lipid the system was rather robust and the lipid concentration did not have much effect on the release kinetics. However, by increasing the lipid content from 400mg/g to 450mg/g the initial protein release and daily release amount were both reduced while increasing the content of G-CSF from 4.0mg/g to 8.4mg/g also decreased the release rate. For the effect of the protein content on the release behavior, our hypothesis was made that G-CSF plays a role as glue in-between the liposome vesicles within the matrix and consequently

increasing the protein content results in delayed erosion due to the enhanced structure which was also indicated in the gel strength as shown in Figure VI-3a. In summary, it can be stated that desired release profiles of G-CSF loaded VPGs could be achieved by varying either the lipid content or the protein content within VPGs. Moreover, the obtained results have revealed the possibility for the development of formulations for sustained delivery of G-CSF through local application.

Table VI-9. Daily release of G-CSF from 1g VPGs based on various compositions.

Formulation	G-CSF VPGs_1	G-CSF VPGs_2	G-CSF VPGs_3	G-CSF VPGs_4
Lipid content	400mg/g	450mg/g	490mg/g	450mg/g
G-CSF content	4.0mg/g	4.0mg/g	4.0mg/g	8.4mg/g
Initial release (Day 1)	64µg	32µg	43µg	49µg
Daily release amount	56µg-388µg	32µg-204µg	32µg-150µg	25µg- 76µg
Average release amount per day	149µg	88µg	89µg	43µg
Daily release percentage of the incorporated protein/%	3.7%	2.2%	2.2%	0.5%

6.4. INVESTIGATIONS ON THE IMCOMPLETE PROTEIN RELEASE

For all the G-CSF loaded VPG-formulations investigated above, the protein release leveled off before reaching 100% of the incorporated amount.

However, RP -HPLC did not indicate the existence of denatured protein materials in the release media (Figure VI-14).

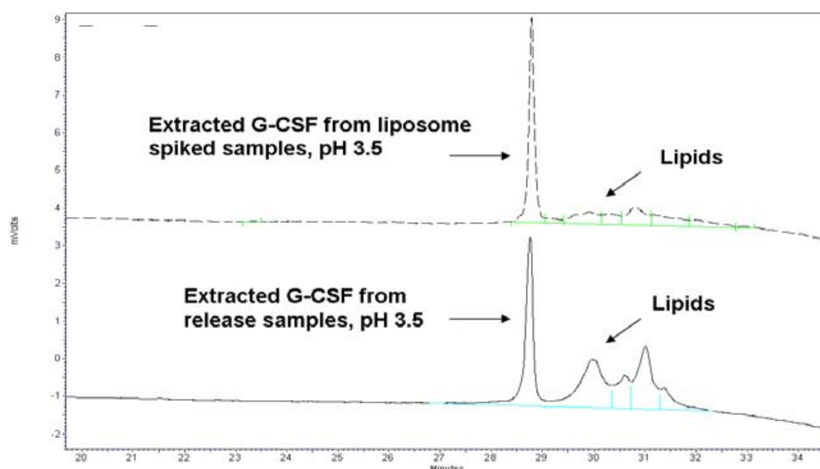


Figure VI-14. Chromatograms of GCSF. (1) G-CSF, DMF applied to dissolve lipids (2) G-CSF from release fractions, DMF applied to dissolve lipids.

Moreover, the stability of G-CSF (15µg/ml, in simulation to the concentration of G-CSF in the release fractions) at 37°C within buffer solutions was also investigated. It was found that G-CSF has a massive loss (63%) after 8 days incubation under a condition of 20mM PBS buffer pH 7.4 while 90% of G-CSF has left in a PBS buffer pH of 3.5 (Figure VI-15). No peak for the oxidized products was observed by the RP-HPLC.

Accordingly, the G-CSF bulk solutions used for VPGs' preparation were formulated with the low pH (3.5) and the presence of 5% sorbit. In the situation of in vitro release tests, PBS buffer pH 7.4 was used as acceptor medium. As G-CSF was theoretically entrapped within as well as in-between the lipid vesicles, it was easy to image that the part of G-CSF entrapped in-between the vesicles would be exposed to the massive volume of PBS release buffer pH 7.4 (which did not compromise the protein stability) along with the erosion process. Thus concerning the stability issue of G-CSF itself under the physical conditions, it was not surprising to find the incomplete release of G-CSF from the VPGs.

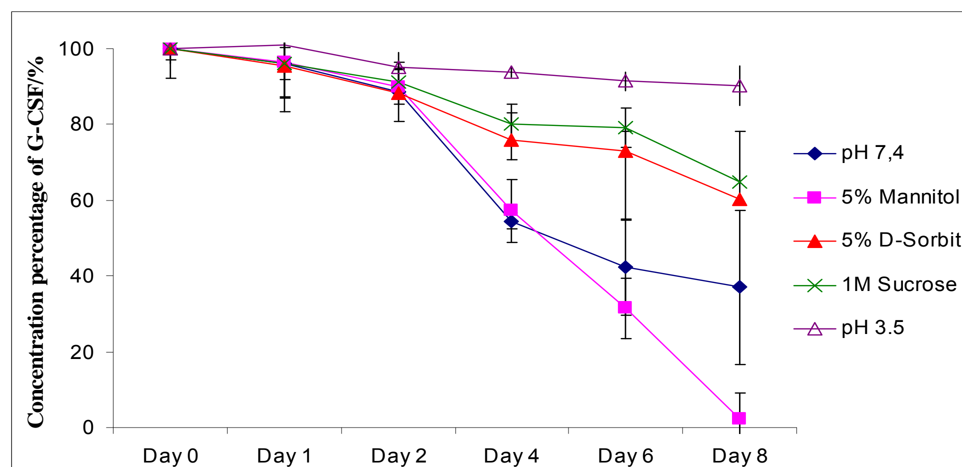


Figure VI-15. G-CSF stability in various buffer conditions, at 37°C, 15µg/ml.

Since the release tests were conducted with PBS buffer pH 7.4 at 37°C, it was not surprising to find incomplete release of the G-CSF from VPGs concerning the protein stability under the release conditions.

As it can be also seen from Figure 16, the addition of 5% sorbitol or 1M sucrose appeared to improve the stability of G-CSF at pH 7.4. Based on these results and the previous report that the stability of G-CSF could be improved at pH 7.4 in presence of

sucrose [223], G-CSF VPGs were prepared by incorporating sucrose (the formulation is shown in Table VI-10) and the release tests were performed subsequently.

Table VI-10. Addition of sucrose into the VPGs,

	Lipid Content	G-CSF Content (in the final formulations)	Sucrose Content
G-CSF VPGs_3	490mg/g Egg PC (Lipoid E80)	4.0mg/ml	-
G-CSF VPGs_5			0.16g

As it can be seen the VPGs containing sucrose delivered the incorporated G-CSF in a comparable manner to the VPGs without sucrose. Namely the addition of sucrose could not significantly improve the degree of G-CSF release from the systems (Figure VI-16). It was assumed that the mass of sucrose entrapped within VPGs was insufficient to function considering the large volume of the release buffer in the entire release tests.

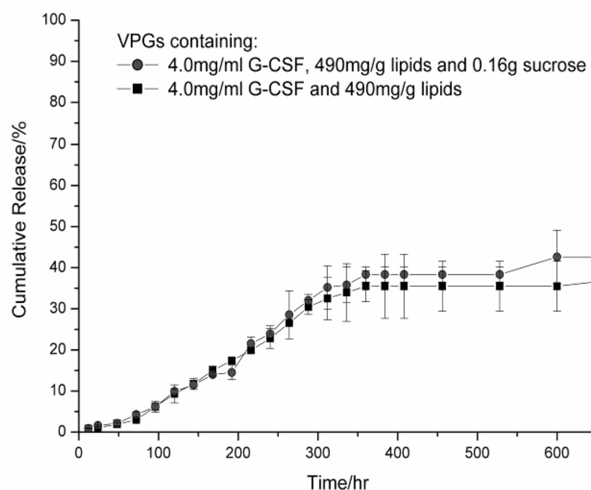


Figure VI-16. Cumulative release of G-CSF from VPGs with and without sucrose (Mean ± standard deviation, n = 3 each). The lipid concentration in the formulations was 490mg/g, and the protein concentration was 4.0mg/g.

7. IN VIVO EXPERIMENTS

7.1. PROVIDING BACK GROUNDS

Ruptures of the rotator cuff, in the supraspinatus muscles in particular, are among the most common degenerative injuries. They mainly occur in older age as degenerative diseases and are associated with pain and movement restrictions.

Untreated ruptures are deemed as a form of pre-arthrosis and therefore require an optimal treatment to prevent long-term damage to the shoulder osteoarthritis [227-228].

Growth factors represent one of the most important roles involved in tendon healing [229]. Chan [229-230] has demonstrated that the basic fibroblast growth factor (bFGF) is active in simulation of cellular proliferation and collagen III expression. G-CSF is shown to be capable in mobilizing cells [231-232]. In vivo use of the growth factors has shown some promise in recent years. Recent studies have suggested that by using two or more growth factors in combination may in fact promote mature vessel formation. [215-216]. However, a major challenge of the use of growth factors in vivo is the delivery of the therapeutic molecules to the target cells in a specific and sustained manner [210, 216, 233].

To achieve continuous therapeutic effect, various carriers have been investigated for the delivery of growth factors. These include gelatine hydrogels [234], collagen [235], and chitosan-based fleeces [236]. Pieper [237] has reported the loading of bFGF to cross-linked collagenous matrices with covalently attached heparin sulfate for subcutaneous implantation. For this system in vitro release was reported over 30 days. In vivo evaluation in rats has revealed an intense and prolonged tissue response and considerably promoted generation of new tissue. Application of the FGF entrapped chitosan hydrogel was found to significantly induce wound contraction and accelerate wound closure in mice [238]. No generally acknowledged dosing scheme has been established for the long-term application of the growth factors but the following data might be helpful for the estimation of therapeutical relevant doses: In the work of Pieper [237], loading 1260 µg/g matrix of bFGF resulted in an average release of 12ng/day during the in vitro release over 30 days. Ono [239] has reported that applying 1-10µg/day of bFGF both alone and in combination with collagen matrix was effective in wound contraction using a simulated in vivo delayed healing type model. Richa [240] has used 10 µg/kg/day of G-CSF for stem cell mobilization in humans. Single injections of 5µg/kg or 250 µg/kg of G-CSF were given to examine the pharmacological action of G-CSF in mice [241].

The biocompatibility and biodegradability of phospholipid based delivery systems have been widely demonstrated in previous works [242-246].

In this context, VPGs were loaded with growth factors including G-CSF and bFGF. Their implantation possesses a new and promising approach for the improved healing of the tendon and the angiogenic stimulation. Due to the specific characteristic of the VPG-system, a continuous release of the growth factors in situ with therapeutically relevant levels could be expected.

7.2. EXPERIMENTAL SET-UP

The in vivo evaluation of growth factor loaded VPGs will be performed using Sprague Dawley rats as the animal model (This work will be carried out by the Technical University Munich, Sport-orthopedics). In rats the structure of the shoulder girdle is similar to that of humans. Moreover, they have also proved as experimental animals in the study of the muscle-tendon pathology at the shoulder joint [247-248].

The tendon is separated from the shoulder muscle of the rats (Figure 17a). Growth factors (G-CSF and/or bFGF) are then applied in a continuous manner. This can be achieved either by infusion of the protein solutions using the osmotic pumps (model 2004, ALZET) or by the sustained release of proteins from VPGs which contain liposomal fractions. The therapeutic effect (e.g. histology) and pharmacokinetics of the protein will be evaluated.

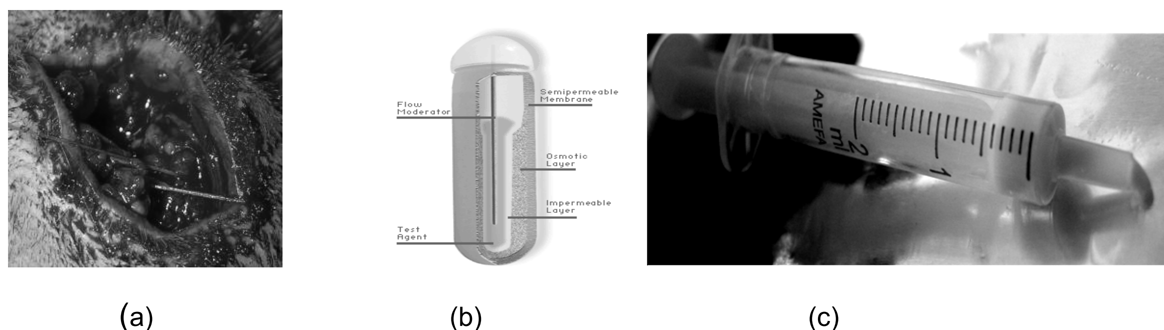


Figure VI-17. (a) Rupture of tendons in rats. (b) Cross section of osmotic pump, model 2004, ALZET. (c) Application of VPGs based on 450mg/g lipids and various concentrations of growth factors.

The ALZET pumps (Figure 17b) operate because of an osmotic pressure difference between a compartment within the pump, called the salt sleeve, and the tissue environment in which the pump is implanted. These mini osmotic pumps can deliver a variety of solutions including protein solutions at zero-order rates for periods up to 4 weeks [249]. The volume delivery rate of the pump was proved to be 0.28±0.01µl/hr by using methylene blue (at 590nm) for the pumps Model 2004 (lot.No.10172-07)

used in our study. Different dosing rates can be achieved by varying the concentration of agent in the solution or suspension used to fill the pump reservoir [249]. The filling volume was calculated from the net weight of the loading solutions, and in our case it was determined to be 251.8±6.0µl in average.

In our work, the desired dose infused daily from the pumps was 5µg/day for G-CSF while for bFGF was 0.5µg/day to maintain the therapeutic effect. As shown in Table VI-11, G-CSF was diluted from the bulk materials with the acetate buffer pH 3.9. bFGF lyophilized powder was obtained from PROSPEC. It was reconstituted with PBS buffer and 0.1% BSA [250]. A combination of the two proteins was also preparation in acetate buffer. The protein solutions were filled into the pumps and delivered for the animal experiment.

Table VI-11. Formulations of the growth factor solutions for the osmotic pumps.

	G-CSF	bFGF	G-CSF und bFGF
Concentration	834 µg/ml	83.4 µg/ml	G-CSF (834µg/ml) and bFGF (83.4µg/ml)
Dose pro 24h	5µg	0.5µg	5µg G-CSF and 0.5µg bFGF
Formulation	10 mM acetate buffer pH 3.9 0.004% Tween 5% sorbitol	10 mM PBS pH 7.4 0.1% BSA	10 mM acetate buffer pH 3.9 0.004% Tween 5% sorbitol

As described in the introduction section, VPGs can be easily prepared under sterile conditions. The lipid based system has good biocompatibility [242-246] and will dissolve after a defined period itself based on the observation of in-vitro release tests in our work. Furthermore, in vitro release tests have showed that VPGs loaded with G-CSF could deliver the protein with therapeutically relative dose over a defined period of up to 4 weeks.

Table VI-12. Formulations of VPGs, loaded with various contents of growth factors

Protein	G-CSF	
Buffer in protein solutions	10 mM acetate buffer pH 3.9 0.004% Tween 5% sorbitol	
Protein concentration per ml VPGs	200µg	2mg
Lipid content per ml VPGs	450mg/g	
Expected release amount pro 24h	1µg	10µg

Concerning the texture of the gels, VPGs based on 450mg/g lipids (egg PC, LIPOID E80) were chosen for the in vivo tests. The protein solutions (or buffer solutions) will be added to be sterile phospholipids in the sample containers (25ml, PP) under the

laminar flow. Aseptic production is achieved using the hermetically sealed sample vials. The prepared gels will be transferred into 1ml syringes (B.Braun Melsungen AG, Germany) under the aseptic conditions. 0.2ml of VPGs will be implanted to the rupture of the tendons in each of the rats (10 rats for each group). Placebo VPGs (based on 450mg/g lipids) will first be applied as control group and to evaluate the biocompatibility of the gels as well. The potentially therapeutically relevant formulations were planned as shown in Table VI-12. Various protein concentrations were entrapped within VPGs to achieve different doses. The serum samples will be taken from the rats after implantation of the gels at planned intervals, and the concentration of G-CSF will be quantified with the developed method of ELISA for the pharmacokinetics study.

7.3. ELISA OF HUMAN G-CSF

In order to investigate the pharmacokinetics of G-CSF from VPGs, the rat serum will be collected at predetermined intervals. An ELISA was developed as quantification method for G-CSF in serum.

As a start point, the cross-reactivity of the ELISA assay was evaluated. To simulate the conditions from the animal tests, G-CSF was spiked with placebo rat serum. Three ELISA kits have been assessed. As for the RayBio Human G-CSF ELISA, the placebo serum samples got a high OD background (2.477±0.746) which indicated cross-reactivity with the assay, and no correlation could be established for the serum spiked samples.

Another ELISA kit (Human G-CSF Instant ELISA, Bender) was also evaluated. The working procedure of the assay was simplified as the detection antibody and streptavidin-HRP (color reagent) beside the coating antibody were readily included in the assay plate so that the binding starts simply by adding the samples or destilled water. A correlation (R^2=0.971) could be established for the protein stock solutions (Figure VI-18). However, the serum samples appeared to have strong cross-reactivity with the assay as the placebo serum alone got strong background signals.

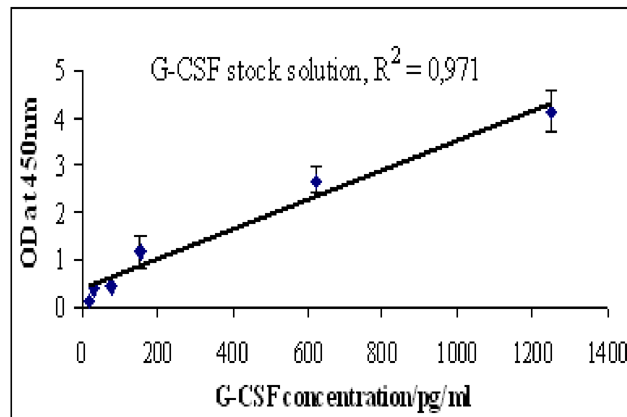


Figure VI-18. Response curve of Human G-CSF stock solution, by Instant Human G-CSF ELISA (Bender) n=3.

The Quantikine G-CSF Immunoassay (DCS50, R&D Systems) contains E. coli-expressed recombinant human G-CSF and antibodies raised against the protein. It has demonstrated accurate quantification of the recombinant human G-CSF without measurable cross-reactivity with mouse serum[251].

The optical density was determined at 490nm and subtracted from the readings at 650nm for correction. It was recommended in the manual that the log transformation of the data could result in better linearity. Correlation was established for the G-CSF stock solutions with concentrations ranging from 78pg/ml to 1250pg/ml (Figure VI-19).

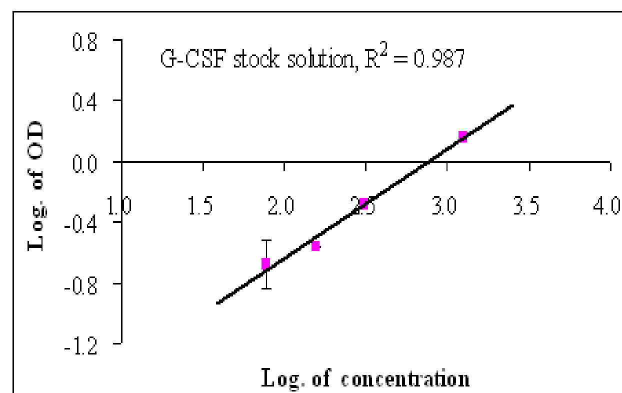


Figure VI-19. Response curve of Human G-CSF stock solution, Quantikine Human G-CSF ELISA, n=3

Importantly, placebo serum samples showed little background (Table VI-13). To evaluate the influence of the serum spiking, the G-CSF bulk was spiked with serum in two different levels resulting in the same protein concentration (625pg/ml). The result showed the recoveries of G-CSF from spiking were both around 78% (Table 12), which could be acceptable for the study. Moreover, the spiking ratio has shown little influence on the result which is promising for the quantification of released G-

CSF since the protein-serum ratio is uncertain in the serum samples from the animal experiments.

Table VI-13. ELISA of Human G-CSF spiked with serum samples: influence of the serum spiking.

Sample	Optical density	Recovery/%
Serum Blank	0.072±0.010	-
G-CSF stock, 625pg/ml	1.232±0.136	
G-CSF spiked with serum in a ratio of 1:1, 625pg/ml in the final solution	0.966±0.043	78.4%
G-CSF spiked with serum in a ratio of 1:4, 625pg/ml in the final solution	0.968±0.126	78.5%

The calibration of G-CSF spiked with serum samples (in a ratio of 1:1) was established as shown in Figure VI-20. Good correlation was established for the response curve of the stock materials and the serum spiked samples. Thus based on the obtained results, this Quantikine Human G-CSF ELISA was found suitable for the quantification of G-CSF in the rat serum. However, currently no pharmacokinetics results were obtained because the animal studies have been postponed due to the fact that the authorization by the clinical committee has been delayed.

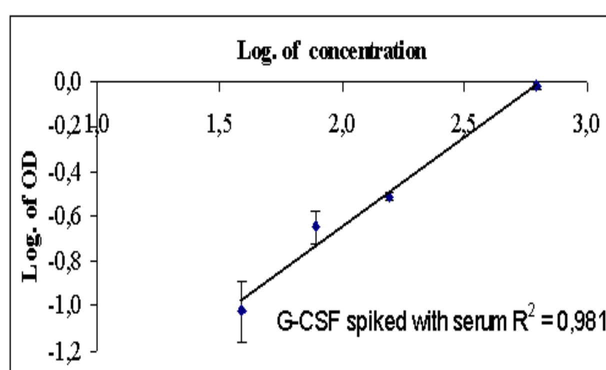


Figure VI-20. Response curve of Human G-CSF spiked with rat serum in a ratio of 1:1, Quantikine Human G-CSF ELISA, n=3

8. Conclusion

In this chapter, VPGs are used for the delivery of growth factors. Various formulations of G-CSF VPGs were prepared and characterized by the texture analysis and rheological tests. Suitable gel strength and viscosity for the aim of easy application and sustained release could be achieved by adjusting the lipid content and the protein content within the matrices.

No detrimental effect of G-CSF was found of the preparation process in dual asymmetric centrifuge analyzed by SDS-PAGE. FTIR was not assessable for the analysis of G-CSF within VPGs.

For the quantification of G-CSF from liposome containing samples, a reverse-phase HPLC method was developed. Several extraction methods were assessed according to the literatures and previous work. When chloroform was applied to the G-CSF in absence of any lipids, the protein was 100% recovered from the aqueous solution. However, it was found that the presence of lipids highly reduced the protein recovery from liposome containing samples when chloroform was applied for lipid extraction. Thus it was assumed that interaction existed between the lipids and the proteins. Accordingly, N,N-Dimethylformamid was used for the protein-lipid isolation. The lipids were precipated and G-CSF was recovered from the aqueous solution. The extraction method works under a buffer condition of pH 3.5 whereas there were oxidized products observed for G-CSF exposed to DMF at pH 7.4 which was associated with the fact that G-CSF has better stability in an acidic environment [225]. A correlation was established between the extracted protein and the standard. Thus, the method was applied as the standard for quantification of G-CSF in the release fractions.

Generally, the in-vitro release studies demonstrated the potential of VPGs for sustained delivery of G-CSF. It was observed that G-CSF was released from all formulations in a linear manner following the zero-order kinetics. Increasing the lipid content from 400mg/g to 450mg/g resulted in slower release rate. However, the VPG-system appeared robust when the lipid content was changed from 450mg/g to 490mg/g (For VPGs based on 400mg/g lipids and 4.0mg/g G-CSF 63% of the total entrapped protein was delivered over 408hrs whereas VPGs based on 450mg/g lipids delivered 43% and VPGs with 490mg/g lipids delivered 35% within the same period.). To investigate the influence of the loading amount of proteins, VPGs were also prepared with a higher protein concentration (8.4mg/g). As a result, higher protein loading resulted in a much slower release of 11% within the same period. Concerning the determined gel strength and rheology, the VPGs with loading of 8.4mg/g G-CSF had a higher gel strength and viscosity (see Figure VI-3) compared with the VPGs containing 4.0mg/g G-CSF. It was assumed that the delayed release

of G-CSF was attributed to the enhanced gel properties due to the interaction between the drug and drug as well as the interaction between the drug and lipids.

The incomplete protein release was attributed to the instability of G-CSF under the release conditions (PBS buffer pH 7.4, 37°C). More than 60% of G-CSF was lost after 8 days (216hrs) incubation at 37°C. Though the addition of sucrose or sorbitol was proved to improve the stability of G-CSF at pH 7.4, no obvious improvement in the protein recovery from the release of VPGs containing sucrose was observed. Comparing with the massive volume of the release buffer, the amount of sucrose within VPGs was too little to function. However, it was not possible to incorporate a large amount of sucrose into the matrices.

Concerning the in vivo tests, the experiment was set up based on the fact that the lipid based VPG-system possesses excellent biocompatibility [50, 123] and the growth factors were shown healing effect of wounds in recent research [229]. In collaboration with Sport-orthopedics of Technical University Munich, the experiment was performed using rats as animal model. The basic fibroblast growth factor (bFGF), granulocyte colony-simulating factor (G-CSF), and a combination of both will be applied for the rupture of tendons. VPGs with the loading of these growth factors were expected to release the entrapped protein in a sustained manner after implantation based on the results of the in vitro release. On the other hand, osmotic pumps were used to offer a stable infusion of the protein solutions over a period of 28 days for the matters of comparison. The healing effect (e.g. histology) and the pharmacokinetics of the protein released from VPGs will be evaluated. The Quantikine G-CSF Immunoassay was validated for the quantification of G-CSF in the serum samples. No cross-reactivity of the assay was observed with the rat serum. However, the experiment was still ongoing since the animal studies have been postponed due to the fact that the authorization by the clinical committee has been delayed. The related results would be included later in a publication.

CHAPTER VII: MAB LOADED VPGS

Our data so-far indicated that the advantageous use of VPGs can be extended to protein drugs. In Chapter V and Chapter VI, cytokines which possess a molecular weight below 40kDa were used as model proteins. However, considering the growing value of the global therapeutic monoclonal antibody (MAB) market an increasing demand for improved delivery of these large multidomain proteins arose. With more than 400 ongoing studies targeting a broad range of diseases MAB therapeutics will surely dominate the new approvals of biotech drugs [252].

Nevertheless, only limited work has been conducted in the field of controlled and/or local release of MAB. Today most approved MAB therapeutics are formulated for intraveneous infusion (i.v.) or repeated subcutaneous injection (s.c.) requiring large quantities of drug for systemic distribution [253-254]. Due to the relatively high costs of both the drug itself and the way of administration antibody therapy is comparatively expensive. In contrast, the direct local controlled release of antibodies at the intended site of action (e.g. in knee joints, at a tumour site, at the eyes, and other topical application sites) would allow a spatial control over antibody distribution and thus lower dosing requirements. In addition, the typical benefits of controlled release formulations such as improved patient compliance reduced systemic exposure would be associated with this way of administration.

As for proteins in general controlled delivery of antibodies requires sophisticated formulation strategies to meet the demands of instable and complex bio-macromolecular drugs. So far only polymers were exploited as carriers for controlled release devices lacking either biodegradation, maintenance of antibody stability or sufficient release kinetics [2, 4, 254].

Based on the investigations on VPGs for cytokine delivery these lipid-based formulations appear suitable to form a new delivery platform for monoclonal antibodies. Therefore, the main object of the study in this Chapter will be a careful evaluation of the benefits and boundaries of VPG-based controlled antibody delivery. To fulfil a broad range of needs for various indications an ideal delivery system should permit tailoring of antibody dose and release kinetics. Therefore, within the context of this study various formulation strategies will be investigated to modify the release kinetics.

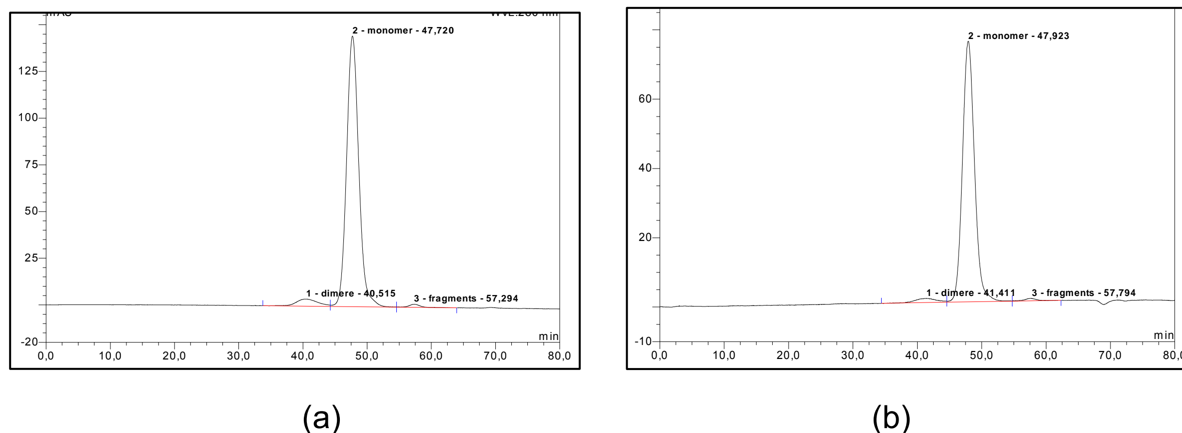
1. PREPARATION OF MAB LOADED VPGS

1.1. CONCENTRATING MAB BULK

A high protein concentration was necessary for the preparation of MAB VPGs considering the therapeutically relevant doses. MAB stock solution (17mg/ml) was concentrated by ultrafiltration with Vivaspin tubes (100KDa, 20ml). The concentration of MAB was determined with UV photometry at 280nm using the extinction coefficiency (1.499ml/mgXcm). SE-HPLC was performed to check the stability of the protein after the concentrating process. As shown in Figure. VII-1, the MAB monomer was maintained the same before and after the up-concentrating, namely no detrimental effect was induced by the ultrafiltration process. Besides, the percentage of protein dimer and fragments were slightly reduced in the concentrated protein samples (Table VII-1).

Table VII-1. SE-HPLC results of MAB bulk and concentrated samples. mean±SD, n=3

	Monomer%	Dimer/%	Fragments/%
MAB Bulk	94.63±0.15%	4.4±0.09%	0.97±0.09%
MAB concentrated	96.40±0.13%	2.75±0.05%	0.79±0.09%



(a) (b)

Figure VII-1. SE-HPLC of MAB. (a) bulk solution, unconcentrated, 1.55mg/ml; (b) concentrated MAB solution, 0.67mg/ml (100 times diluted).

1.2. FORMULATION OF MAB LOADED VPGS

In general, the preparation of MAB VPGs followed the procedure described in the method section. MAB was formulated with 50 mM phosphate buffer pH 6.4, containing 150mM sodium chloride and 0.1% BSA. The phospholipid used here was egg phosphatidylcholine (E80, LIPOID). Homogenization of the mixture was performed by means of the dual asymmetric centrifugation in a cylindric container of

25 ml total volume. Multiple runs of 1.5-minutes were added up to a total mixing time of 45 mins at a process speed of 3500 rpm. The samples were cooled at 2-8°C at interrupts to control the system temperature.

A number of different VPGs were produced in order to evaluate the influence of VPG composition on the release properties (Table VII-2).The content of phospholipid was varied between 350 and 450 mg/g. A variety of MAB concentrations was investigated ranging from 9.4 mg/g (in the final VPGs formulations) for basic research to 37.4 mg/g for therapeutically relevant levels.

Table VII-2. Formulation of MAB VPGs

(a) Influence of the lipid concentration

Formulation	MAB VPGs_1	MAB VPGs_2	MAB VPGs_3
Lipid content	350mg/g	400mg/g	450mg/g
MAB content	9.4mg/g		

(b) Influence of the protein concentration

Formulation	MAB VPGs_3	MAB VPGs_4	MAB VPGs_5
Lipid content	450mg/g		
MAB content	9.4mg/g	17.6mg/g	37.4mg/g

2. TEXTURE ANALYSIS AND RHEOLOGY

The texture analyser (TA.XT plus, Stable Micro Systems, UK) recorded the forces (Figure VII-2a) which occured when the probe (4mm in diameter) was pushed into the tested VPGs. The gel strength of VPGs was represented as the maximal force value according to the “Bloom test” method. The rheology behavior of MAB VPGs was studied by a rotational viscometer (Physica MCR 100, Anton Paar) at 25°C. The shear viscosity was provided versus shear rate ranging from 10^{-1} and 100^{-1} (Figure VII-2b).

As shown in Figure VII-3a, the gel strength increased with the lipid content when the content of MAB was kept constant (9.4mg/g) within VPGs. The maximal force reached a value of 64.2g for VPGs containing 500mg/g lipids, whereas VPGs containing 350mg/g had a value of 8.1g. Figure VII-3b showed the gel strength of VPGs containing 17.6mg/g MAB (with a value of 40.3g) was a little higher than the VPGs containing 9.4mg/g MAB (with a value of 36.7g). However, it was surprisingly found that the gel strength value of VPGs containing 37.4mg/g MAB was much lower than the other two formulations. Our explanation was that the presence of very high content of proteins may reduce the gel strength whereas the gel strength increases

with the protein concentration in a low concentration range (less than 17.6mg/g in the case of MAB).

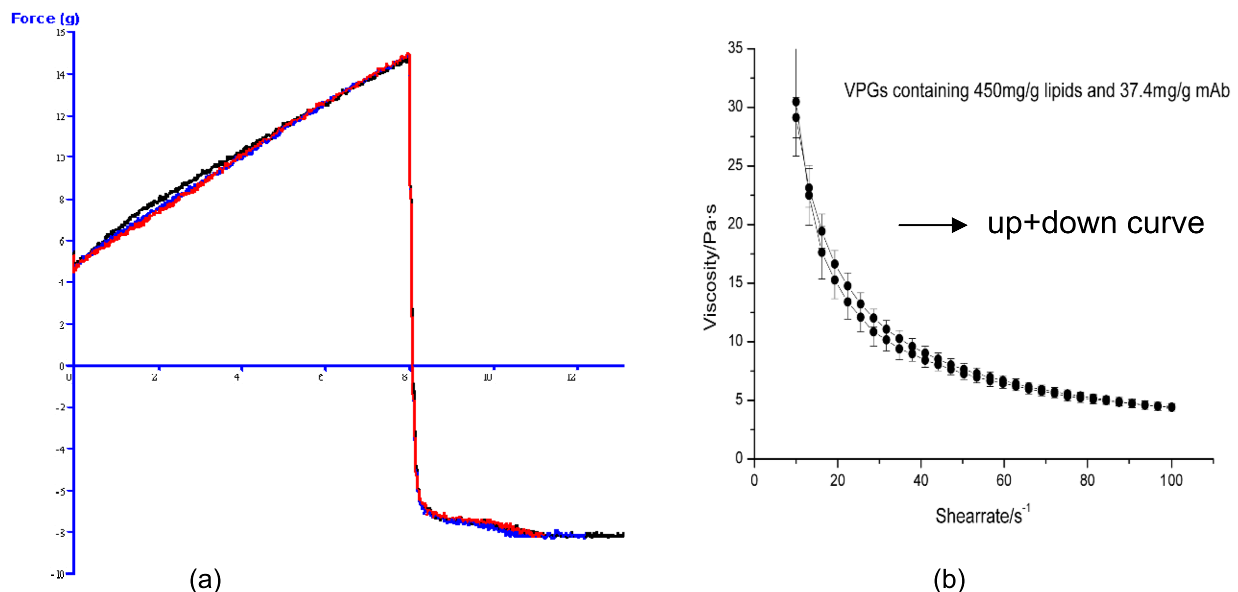
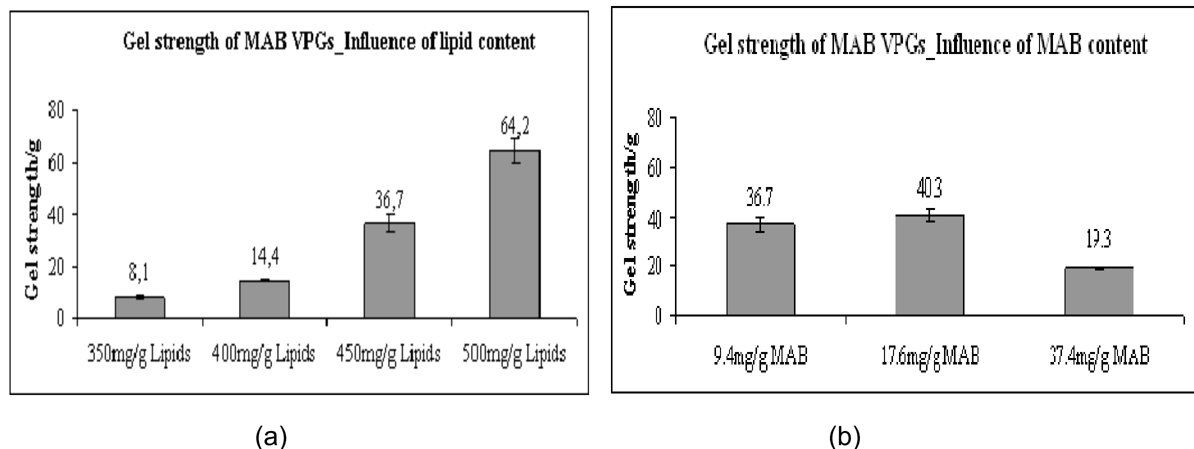


Figure VII-2. (a) Texture analysis of MAB loaded VPGs: recorded force trace when the probe (4mm in diameter) was pushed with a speed of 0.5mm/s into the tested VPGs. (VPGs containing 400mg/g lipids and 9.4mg/g MAB) (b) Rheological behaviour of VPGs. Gel strength (VPGs containing 450mg/g lipids and 37.4mg/g MAB).

As shown in Figure VII-3a, the gel strength increased with the lipid content when the content of MAB was kept constant (9.4mg/g) within VPGs. The maximal force reached a value of 64.2g for VPGs containing 500mg/g lipids, whereas VPGs containing 350mg/g had a value of 8.1g. Figure VII-3b showed the gel strength of VPGs containing 17.6mg/g MAB (with a value of 40.3g) was a little higher than the VPGs containing 9.4mg/g MAB (with a value of 36.7g). However, it was surprisingly found that the gel strength value of VPGs containing 37.4mg/g MAB was much lower than the other two formulations. It was assumed that the gel strength and viscosity could reach a peak value at an optimized drug-lipid ratio due to the drug-drug and drug-lipid interaction. Non-linear trend could be expected for the influence of the protein content on the gel strength and viscosity. In a low protein concentration range, the gel strength increases with increasing the protein content within the matrices whereas the gels got less stiff at a much higher protein content.

The viscosity of various VPG formulations was compared at the shear rate of 37.9s^{-1}. When the lipid content was increased from 350mg/g to 500mg/g, the viscosity of the gels increased from 0.6Pa.s to 21.6Pa.s (Figure VII-4a). Increasing the content of

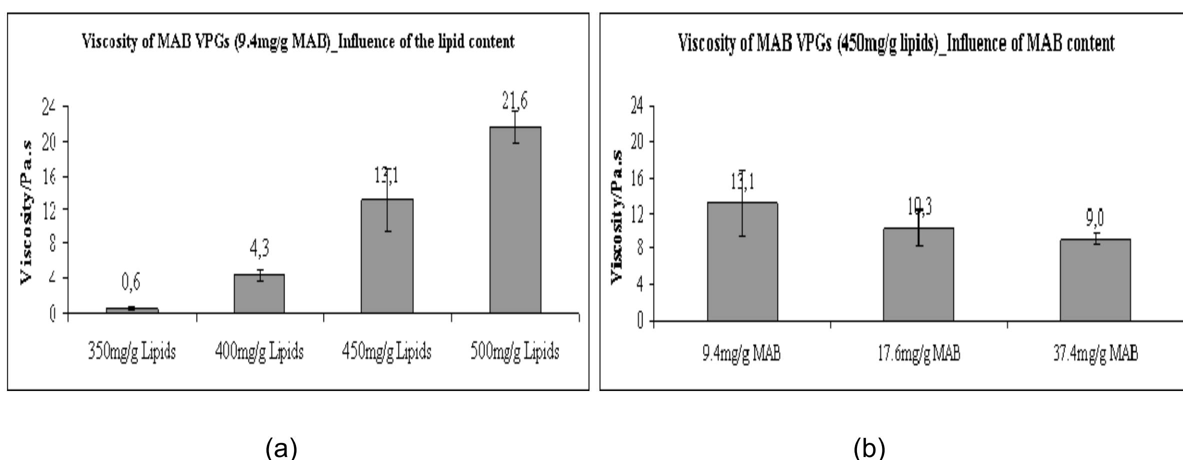
MAB resulted in decreased viscosity of VPGs (Figure VII-4b). However, the influence is not dramatic concerning the deviations.



(a) (b)

Figure VII-3. Gel strength (mean±SD, n=3) of mAb loaded VPGs. (a) Influence of the lipid content, VPGs were based on 9.4mg/g mAb IgG; (b) Influence of the protein content, VPGs were based on 450mg/g lipids.

As a conclusion, the gel strength and viscosity of VPGs both increased when the lipid content increased, whereas the gel strength and viscosity were of low values when there was a high content of MAB incorporated within VPGs.



(a) (b)

Figure VII-4. Viscosity of mAb VPGs (mean±SD, n=3). (a) influence of the lipid content, VPGs were based on 9.4mg/g mAb IgG; (b) Influence of the protein content, VPGs were based on 450mg/g lipids.

3. EVALUATION OF MAB STABILITY BY SDS-PAGE

SDS-PAGE was conducted to examine the stability of MAB entrapped into VPGs. Non-reducing SDS-PAGE analysis was performed with subsequent silver staining. 0.05g freshly prepared VPGs (500mg/g lipids) were accurately weighed and redispersed in 4ml PBS buffer (40mM, pH 7.4). The concentration of the MAB in the VPG-redispersions was around 100µg/ml. In order to eliminate (or minimize) the

influence of lipids on the results of SDS-PAGE, various agents including chloroform, 5% Triton X-100, and 5% SDS, have been tried out to dissolve the lipids and extract the antibody. 0.3ml/ml chloroform was added to dissolve the lipids. The MAB was recovered in the supernatant aqueous solution after centrifugation at 3000rpm for 30mins. Chloroform was also added to MAB standard solutions to investigate the influence of the organic solvent itself on the protein integrity. On the other hand, 5% Triton or 5% SDS was added to the lipid containing solutions. The surfactant containing samples were incubated at 37°C for 30mins respectively before running of the SDS-PAGE.

The results were shown in Figure VII-5. The MAB solutions with the addition of chloroform (lane 3/8) showed exactly the same bands as the standard solutions (100µg/ml, lane 2/7), which indicates that adding chloroform causes no instability of the antibody. When chloroform was used to extract the protein from VPGs, no difference was observed for the extracted MAB (lane 4/9) from the standard (lane 2/7). Moreover, judged on the stained density the extraction recovery of MAB by adding chloroform was high. In the case of adding Triton to dissolve liposomes, band shrifts (lane 5/10) were observed which might be probably induced by interaction between the proteins and Triton. By adding SDS, we observed more pronounced aggregations and fragments (lane 6/11).

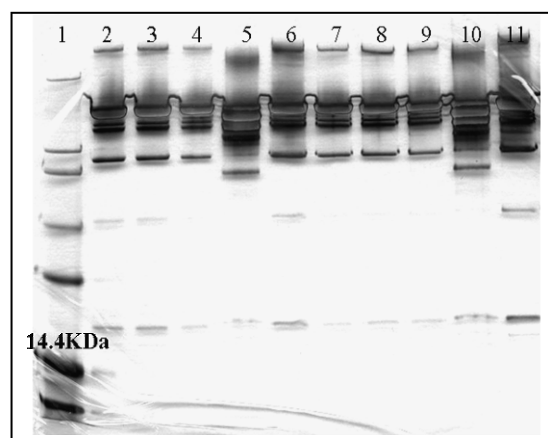


Figure VII-5. SDS-PAGE analysis of MAB integrity:

Lane 1, molecular weight marker
Lane 2/7, MAB standard
Lane 3/8, MAB with adding chloroform
Lane 4/9, MAB VPGs extracted by adding chloroform
Lane 5/10, MAB VPGs extracted by adding 5% Triton X-100

Hence it can be stated that: (1) Adding chloroform for extraction causes no instability of the MAB; (2) Aggregate and fragment fractions beside the monomer can also be extracted by adding chloroform; (3) The formulation process to entrap MAB into VPGs by dual asymmetric centrifugation has no influence on the protein stability. In summary, the SDS-PAGE results provide evidence that the formulation technology of VPGs is applicable for MABs, and adding chloroform is suitable to extract MAB for SDS-PAGE analysis.

4. Quantification method for MAB in presence of lipids

Quantification and extraction method were needed for MAB analysis from lipid containing samples. Since the released amount of VPGs at each time interval was unknown, the quantification method should feature a high robustness against variations of the lipids as well as against variations in the protein amount which make this method suitable for protein quantification during in-vitro release studies. CBQCA assay and micro BCA protein assay were tried out to quantify MAB instead of the HPLC method due to the detection limit and sensitivity of the SEC column.

4.1. CBQCA Assay

CBQCA Protein Quantitation Kit (Molecular Probe, Invitrogen) provides a rapid and highly sensitive method for the quantitation of proteins in solution. The kit utilizes the ATTO-TAG CBQCA reagent (3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde) originally developed as a chromatographic derivatization reagent for amines. This reagent has also proven extremely useful for quantitating amines in solution, including the accessible amines in proteins. Importantly, it was reported by using this assay lipoproteins and other lipid associated proteins could be assayed without any manipulation to extract lipids [255].

4.1.1. Calibration

A calibration curve for MAB without lipids was generated in the concentration range between 0.74 µg/ml and 44.40 µg/ml. BSA was assayed as reference. A series of lipid containing MAB samples were prepared with a lipid content of 5mg/ml. In order to minimize the influence of lipids in the samples on the protein analysis, 0.3 ml/ml chloroform was added to extract the lipids before the assay. Fluorescence

measurements were performed with 96-well (white) micro-plates (Nalge NUNC, Denmark) and FLUOstar Omega multifunctional micro-plate reader (BMG LABTECH, Germany). The excitation wavelength was set to 485nm, and the emission was measured at 580nm (The choice of excitation and emission wavelengths was limited by the device filters).

For each calibration, PBS buffer (40mM) pH 7.4 was assayed as blank and used for background correcting. As shown in Figure VII-6: There was good calibration line established for BSA as the reference standard (R^2 = 0.994). For MAB without lipids, similar response curve was obtained despite that the linearity was poor (R^2 = 0.963) which was probably caused by operating errors (Large deviations might occur in handling with 5µl and 10µl sample volumes).

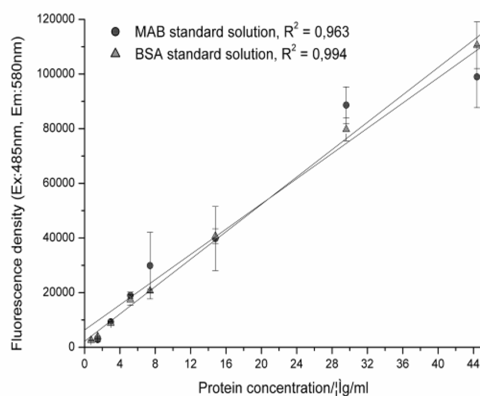


Figure VII-6. Calibrations of the proteins by CBQCA Assay. BSA was used as the reference standard. Lipid free MAB and MAB extracted from lipids were assayed to develop the method.

4.1.2. INFLUENCE OF THE LIPID CONTENT

As described previously in the method development of EPO and G-CSF quantification, the influence of the lipid content on the assay was investigated since the released amount of VPGs at each time interval was unknown. Various amounts of lipids were dispersed into PBS buffer resulting in concentrations ranging from 0.5mg/ml to 5.0mg/ml. The concentration of MAB was 7.4µg/ml in all the samples. The lipids were extracted by applying 0.3 ml/ml chloroform and the proteins were recovered from the aqueous supernatant.

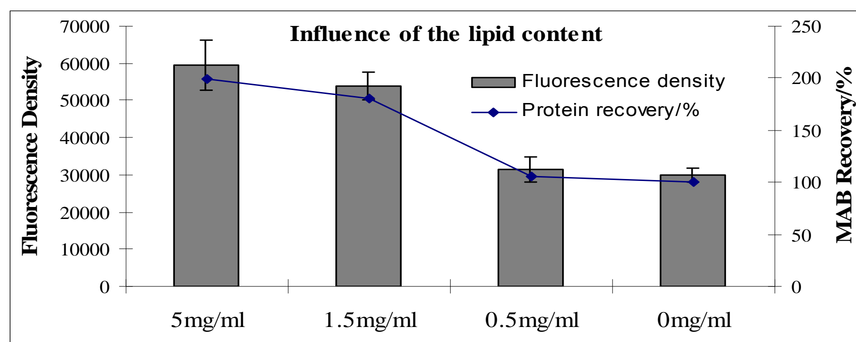


Figure VII-7. Influence of lipid content on the protein recovery. The lipid concentration within the samples was varied between 0mg/ml and 5mg/ml. The protein concentration was determined by the CBQCA assay. The results were represented by the fluorescence density as shown for the left axis, n=9. The protein recovery was calculated as compared to the original protein concentration and shown for the right axis.

Unfortunately, it was found that the lipid content had influence on the results of the assay (Figure VII-7). When the lipid content was more than 1.5mg/ml, the fluorescence density was much higher than expected. We have applied chloroform to remove a major part of the lipids. However, it was assumed there were a small part of lipids remained in the supernatant due to the interaction between the protein and lipids. The presence of lipids obviously disturbs the assay. However, this phenomenon was undesirable in our case since the lipid content was uncertain for the release samples and it might consequently led to bad repeatability in the MAB quantification from the release samples. Moreover, the CBQCA assay was hard to handle due to the small sample volumes (5µl-10µl) and the very toxic substances e.g. potassium cyanide used for reaction. Therefore, the CBQCA assay appeared not suitable for the need of quantification of MAB for the release tests.

4.2. MICRO BCA ASSAY

4.2.1. THE METHOD

The principle of the micro BCA assay is that colored products are formed when the working reagent (Cu^{2+}) is reduced by protein in an alkaline environment. This water soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations.

The procedure for microplate mode follows the instructions provided by the manufacture of the test reagents. Briefly, the working reagent is added to sample

solutions in the microplates. The mixture is incubated at 37°C for 2hrs. The optical density is measured at 562nm after the mixture is cooled down to room temperature.

Lipids are known to cause high background in the assay. Therefore, an extraction method is necessary to remove lipids and recover protein before the assay is applied. Some often used organic solvents and surfactants, including Triton, DMF, Tween have interference with the assay. Adding SDS can not efficiently eliminate the influence of the lipids [256-258]. Based on the experience from EPO extraction, chloroform has tried out as the start point.

4.2.2. CALIBRATION OF THE STANDARD

BSA was used as a standard reference to validate the assay. Calibration of MAB standard solutions was established with concentrations in the range from 0.5μg/ml to 20μg/ml (Figure VII-8).

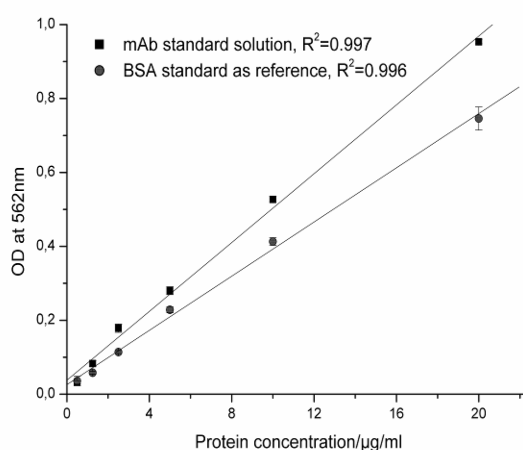


Figure VII-8. Calibration of MAB standard solutions, BSA was used as reference, n=6.

In general, the OD values of MAB were slightly higher than the reference BSA. Good linearity and repeatability were achieved for both proteins. Therefore this assay was applicable for quantification of MAB. Further investigations were carried out for in presence of lipids.

4.2.3. EXTRACTION METHOD WITH CHLOROFORM

4.2.3.1. CALIBRATION

A series of lipid containing samples was prepared with a constant lipid concentration of 1.5mg/ml and various MAB concentrations between 0 and 20µg/ml. 0.3 ml/ml chloroform was added to dissolve the lipids. The antibody was recovered in the upper aqueous solutions and quantified by the micro BCA assay. As shown in Figure VII-9, good linearity and acceptable repeatability were achieved for the extracted MAB. A good correlation was established for the lipid-free MAB and the extracted samples. The recovery of the extraction method by applying chloroform was calculated 72% by using the slopes of the two calibration curves.

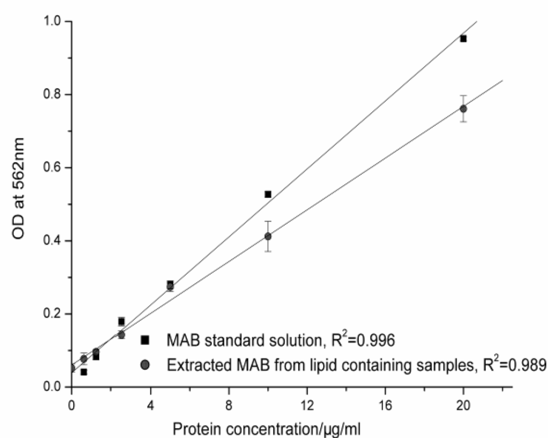


Figure VII-9. Calibration of extracted MAB samples, MAB standard solution was used as reference, linear fit, n=12.

4.2.3.2. INFLUENCE OF LIPID CONTENT ON THE MAB RECOVERY

The influence of the lipid content on the recovery of MAB was investigated by using the micro BCA assay in combination with the extraction method. Various amounts of lipids were dispersed into PBS buffer resulting in concentrations ranging from 0.5mg/ml to 5.0mg/ml. The concentration of MAB was 5µg/ml in all the samples. As shown in Figure VII-10, the recovery of MAB was stable (91.7%~97.9%) when the lipid content was between 0 and 1.5mg/ml. However, the recovery was much higher (138.9%) than expected when the lipid content was increased further to 5.0mg/ml. It was consequently assumed that a part of lipids might remain in the aqueous solution and cause additional optical density when the amount was high enough. Hence, further investigation was carried out aimed to eliminate the influence by adding more chloroform (applying 1ml or 3ml chloroform per ml sample) to dissolve the lipids.

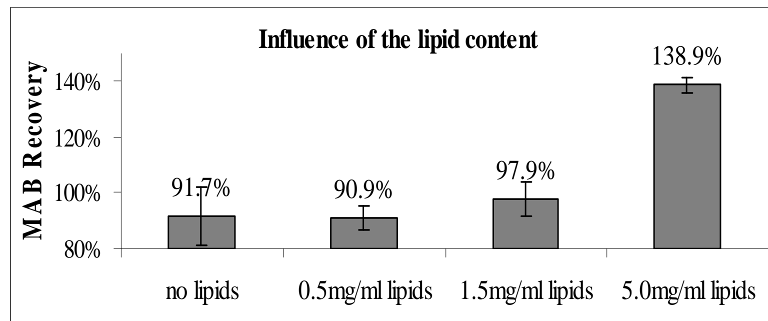


Figure VII-10. Influence of the lipid content on the MAB recovery, micro BCA assay, MAB concentration: 5µg/ml, 0.3ml/ml chloroform was added to dissolve the lipids.

4.2.3.3. Influence of chloroform amount

A series of MAB samples (MAB concentration: 5µg/ml) were prepared with lipid content varying from 0.5mg/ml to 5mg/ml. Various amounts of chloroform (0.3ml/ml, 1ml/ml, and 3ml/ml) were applied to dissolve the lipids within the samples. As shown in Figure VII-11, the amount of chloroform applied in the range between 0.3ml/ml and 3ml/ml got marginal effect on the assay results. Namely, adding more chloroform was not efficient to dissolve the rest part of lipids in the aqueous solution. Therefore, it was a must to find another method to remove the lipids which caused the interference. A protein precipitation method by using sodium deoxycholate was tried out for the next step.

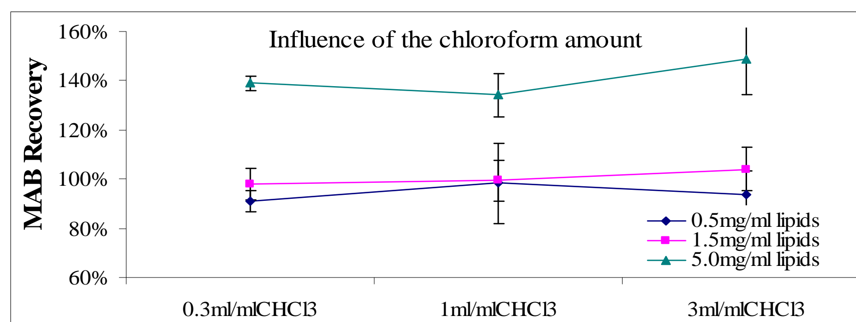


Figure VII-11. Influence of the chloroform content on the MAB recovery, micro BCA assay, MAB concentration: 5µg/ml.

4.2.4. Adding sodium deoxycholate

Sodium deoxycholate (DOC) is a kind of water soluble ionic detergent, commonly used for membrane protein and lipid isolation. In order to eliminate the influence of lipids, DOC was added before adding chloroform aimed to reduce the interaction between the protein and lipids. 0.01ml 1.5% sodium deoxycholate was added to 1ml

lipid containing MAB samples. After incubating at 37°C for 20mins, 1ml/ml chloroform was added to dissolve the lipids. The protein remained in the aqueous phase. Samples from the upper solution were assayed by the micro BCA assay.

As a start point, the influence of adding DOC and chloroform on the protein recovery in absence of lipids was first evaluated. 79% of the MAB were recovered from lipid extraction at the low concentration of 2.5µg/ml whereas 95% were recovered at the high concentration of 20µg/ml. This phenomenon was similar to the situation of applying chloroform alone for EPO extraction.

A series of samples were prepared with various lipid contents ranging from 0.5mg/ml to 5mg/ml. The MAB concentration was 5µg/ml in all the samples. DOC and chloroform were applied and the protein was recovered from the aqueous solution. The supernatant was assayed after centrifugation process.

For the samples containing 0.5mg/ml lipids protein recovery was 115.7% whereas the protein recovery was around 100% for the samples containing 1.5mg/ml or 5.0mg/ml lipids (Figure VII-12). However, compared with the other methods it could be stated the lipid content got less interference on the recovery of MAB extracted from lipids when DOC and chloroform were applied, which encouraged further investigations.

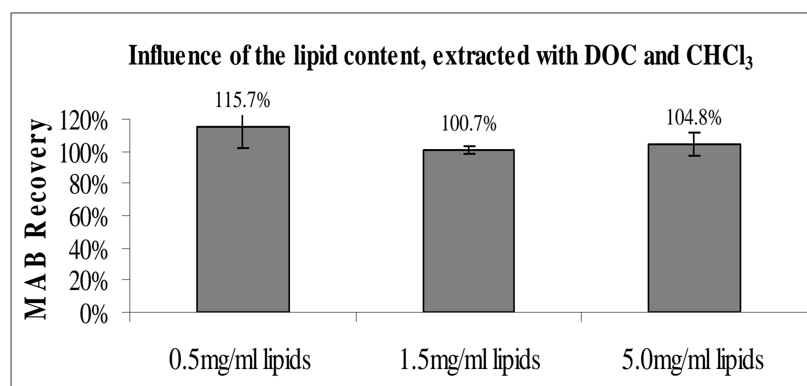


Figure VII-12. Influence of the lipid content on the protein recovery, DOC and chloroform were applied for extraction. n=12

MAB solutions of known concentrations (2.5 – 20µg/mL) in presence of 5.0mg/ml lipids were used to generate the calibration curve (Figure VII-13). The AUCs of extracted MAB by adding DOC and chloroform were in comparison to the calibration curve in the absence of any lipids. Obviously, a good correlation was achieved between the detected AUCs of extracted and untreated MAB solutions. Based on

these data and an acceptable linearity the extraction by adding DOC and chloroform appeared suitable for MAB quantification for the in-vitro release studies.

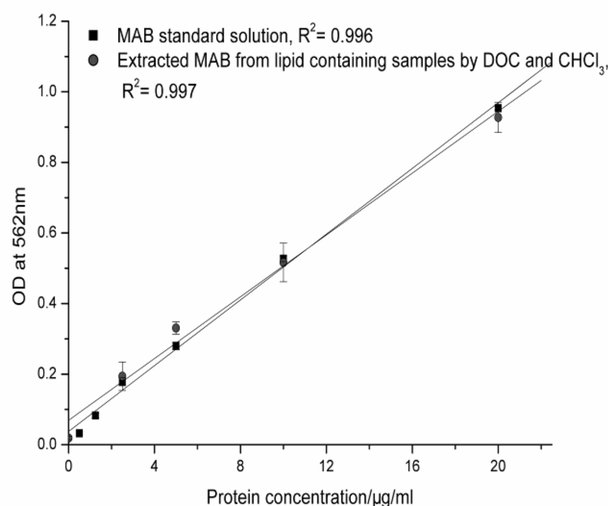


Figure VII-13. Calibration of MAB standard and extracted MAB from lipid containing samples, extraction was performed by adding DOC and chloroform, n=12.

4.2.5. SUMMARY OF THE QUANTIFICATION METHOD

The major criteria to develop the quantification method of the MAB for the in vitro release tests is the robustness against the variation of the lipid and protein content in the release fractions. For the CBQCA assay, the presence of lipids showed great interference on the fluorescence output. Different calibration curve of extracted MAB from lipid containing samples was observed from the lipid-free samples. Alternatively, a commonly used protein assay - micro BCA assay was used for the quantification of MAB. Calibration of the MAB standard was first established with good linearity and repeatability. Applying chloroform alone for MAB extraction could not eliminate the influence of the lipids when there was a large amount of lipids in the samples. It was assumed that there was interaction between the MAB and lipids. Therefore, DOC was used for the protein and lipid isolation before adding chloroform for extraction. As a result, the influence of the lipids was minimized. The calibration was established for the extracted MAB in simulation to the MAB standard samples in absence of lipids. In summary it could be stated that the micro BCA assay in combination with the developed extraction method using DOC and chloroform, suitable for the determination of MAB in the release fractions, was developed and validated for the

quantification of MAB in presence of liposomes taking the variation of lipid and protein amount into account.

5. STABILITY STUDY OF MAB AT 37°C

The influence of the manufacturing process on the integrity of MAB was studied by extracting the protein from VPGs and subsequent analysis by SDS-PAGE. As shown in Figure VII-5 similar bands were observed to the MAB standard. Encouraged by these results the question arose whether this excellent stability could also be maintained at elevated temperature during in-vitro release. To simulate the conditions during in-vitro release VPG formulations based on 450mg/g lipids and 9.4mg/g MAB were incubated at 37°C in Eppendorf tubes. At predetermined points of time the samples were taken out and the protein was extracted by applying DOC and chloroform (as described above). For matters of comparison MAB solutions with a concentration of 9.4mg/g in PBS buffer (40mM) was incubated at 37°C.

The recovery from the gel formulation was comparable with that observed with the liquid formulation (Figure VII-14). Both got little protein loss (less than 10%) after 14 days incubation at 37°C.

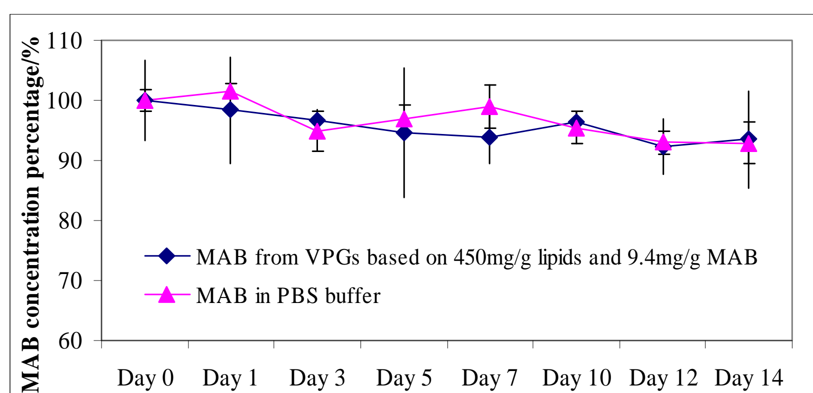
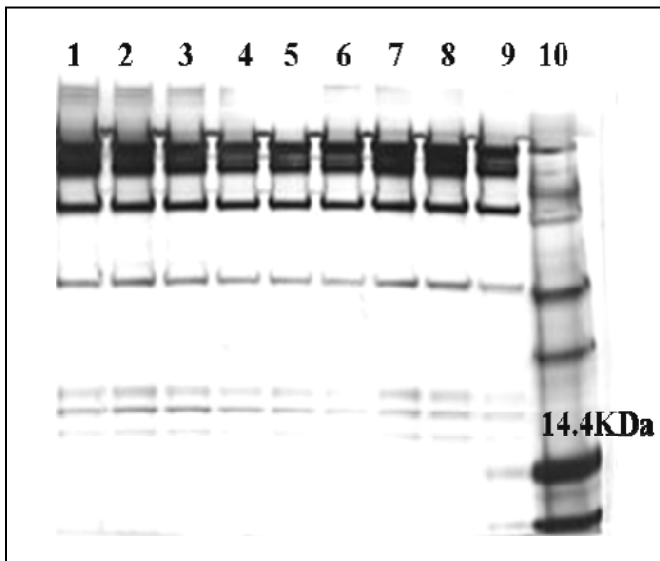


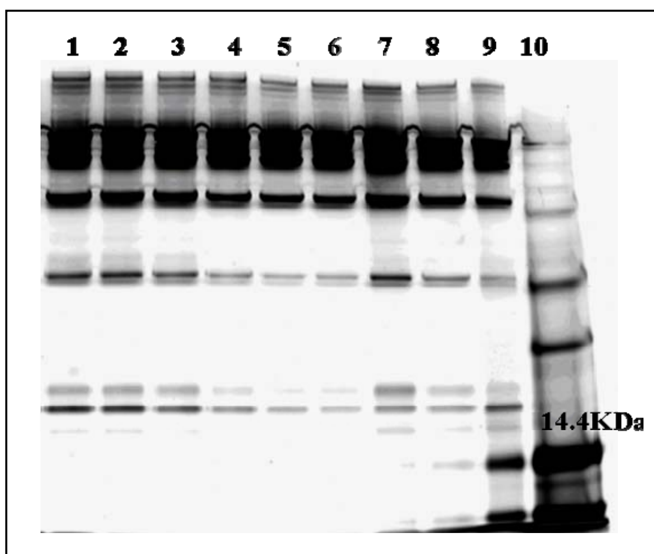
Figure VII-14. Stability of MAB with a VPG formulation based on 450mg/g lipids and 9.4mg/g MAB compared to the protein stability in PBS buffer. Both the liquid and the gel formulation were incubated at 37°C (mean± standard deviation, n = 3 each).

Beside the quantitative analysis of MAB during the incubation, SDS-PAGE was performed (as described previously see Chapter VII.3) to reveal the stability of the proteins at 37°C.



Lane 1, MAB solution after 14 days incubation
Lane 2, MAB solution after 12 days incubation
Lane 3, MAB solution after 10 days incubation
Lane 4, MAB solution after 8 days incubation
Lane 5, MAB solution after 6 days incubation
Lane 6, MAB solution after 4 days incubation
Lane 7, MAB solution after 2 days incubation
Lane 8, MAB solution after 1 days incubation
Lane 9, MAB standard solution
Lane 10, molecular weight maker

(a)



Lane 1, MAB VPGs after 14 days incubation
Lane 2, MAB VPGs after 12 days incubation
Lane 3, MAB VPGs after 10 days incubation
Lane 4, MAB VPGs after 8 days incubation
Lane 5, MAB VPGs after 6 days incubation
Lane 6, MAB VPGs after 4 days incubation
Lane 7, MAB VPGs after 2 days incubation
Lane 8, MAB VPGs after 1 days incubation
Lane 9, MAB standard solution
Lane 10, molecular weight maker

(b)

Figure VII-15. SDS-PAGE analysis of MAB stability incubated at 37°C: (a) solution of MAB in PBS buffer pH 7.4, 9.4mg/ml. (b) MAB VPGs based on 450mg/g lipids and 9.4mg/g. The protein was extracted by applying chloroform.

For both the MAB solutions (Figure VII-15a) and the MAB extracted from VPGs (Figure VII-15b), the band pattern observed from the incubated samples was the same as for the protein bulk materials. Therefore it could be stated the solution of MAB (PBS buffer pH 7.4) had good stability at 37°C. Also a good stability of MAB incorporated within the VPGs was revealed: compared to the protein standard no pronounced aggregation or degradation products showed up during the whole incubation period at 37°C.

6. ADSORPTION OF MAB TO THE RELEASE SYSTEM

It was observed for the EPO VPGs that protein release leveled off before reaching 100% of the total protein incorporated. It was found that incomplete release of EPO from VPGs was mainly caused by unspecific adsorption onto the flow-through release system surfaces. Therefore, the adsorption of MAB to the release system was first evaluated before performing the in vitro release tests of MAB VPGs. The MAB solution (in PBS buffer pH 7.4) with a concentration of 10µg/ml was pumped through the release set with a flow rate of 1ml/hr over a time period of 11hrs. In contrast to the results of EPO, no adsorption of MAB was observed to the release system (Figure VII-16), which made it possible to expect 100% release of the total MAB incorporated into VPGs.

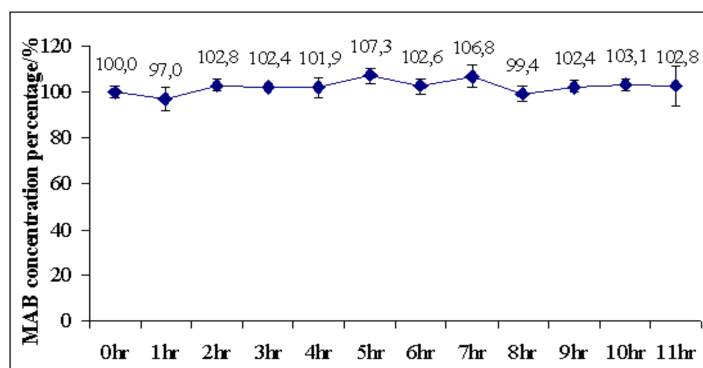


Figure VII-16. Adsorption of MAB onto the in-vitro release system. The solution of MAB with a concentration of 10µg/ml was pumped through the release system with a flow rate of 1ml/hr. The system was incubated at 37°C.

7. IN VITRO RELEASE BEHAVIOUR OF MAB (IGG) LOADED VPGS

7.1. INFLUENCE OF THE LIPID CONTENT

In vitro release tests of MAB VPGs were conducted at 37°C within flow through cells as described previously. PBS buffer (40mM) pH 7.4 was used as acceptor medium. The flow rate was set to 1ml/hr controlled by the syringe pump.

VPGs were prepared with various lipid contents (Table VII-1a). The lipid content ranged from 350mg/g to 450mg/g. The MAB content within VPGs was 9.4mg/g in these formulations. In general, the influence of the lipid content on the release behavior was not pronounced (Figure VII-17). VPGs based on 350mg/g delivered 89% of the total MAB loaded into VPGs over 600hrs (25 days), whereas VPGs

containing 400mg/g released 90% over 750hrs (over 31 days) and VPGs based on 450mg/g lipids released 100% over 1000hrs (over 41 days). Moreover, the initial release rate of VPGs based on 350mg/g lipids was much higher (ca. 16%). A close to zero-order release kinetics was observed for VPGs based on 400mg/g and 450mg/g lipids.

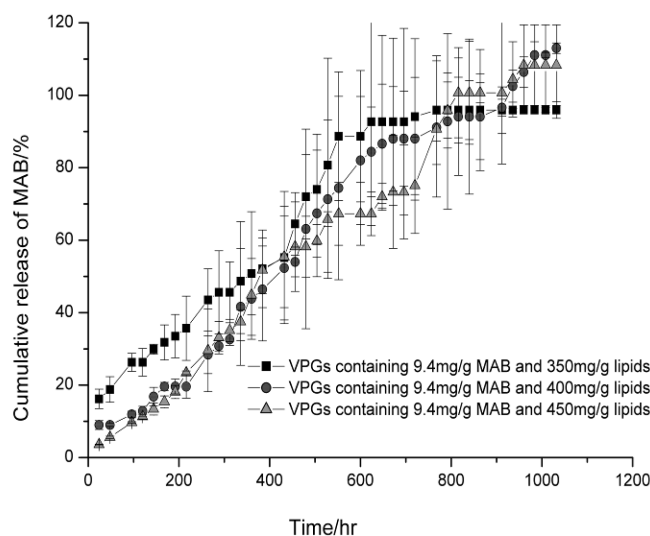


Figure VII-17. Cumulative release of MAB from VPGs based on various contents of lipids (Mean± standard deviation, n = 3 each). The protein concentration in all formulations was 9.4mg/g.

7.2. INFLUENCE OF THE MAB CONTENT

In order to investigate the influence of the MAB content in VPGs on the release profiles, VPGs were prepared with various MAB content ranging from 9.4mg/g to 37.4mg/g (Table VII-1b), while the lipid content was 450mg/g in all the formulations. For the three formulations, total release of entrapped MAB from VPGs was achieved over 1000hrs (over 41 days, Figure VII-18). In general, the MAB content within VPGs showed little influence on the release behavior. However, it was interestingly observed that there was a fast release phase for the VPGs based on 37.4mg/g MAB in the time period between 700hrs and 900hrs, which was probably caused by the accelerated erosion due to the lack of stiffness in the later phase of the release.

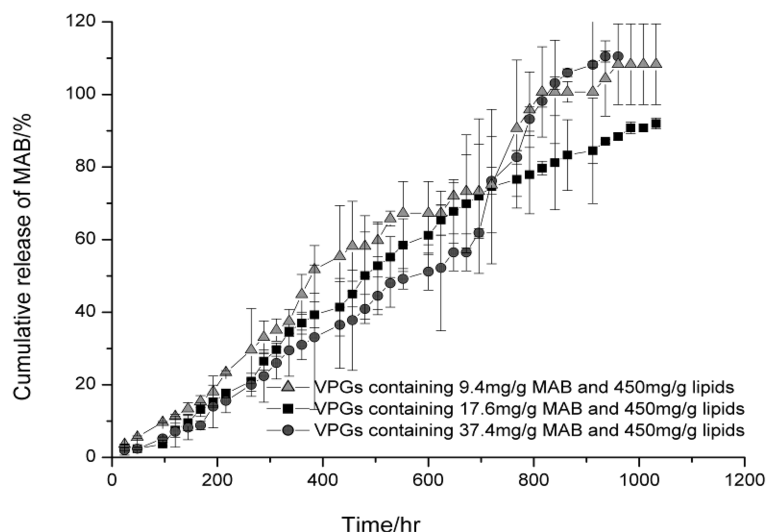


Figure VII-18. Cumulative release MAB from VPGs based on various contents of MAB (Mean± standard deviation, n = 3 each). The lipid concentration in all formulations was 450mg/g.

7.3. QUANTIFICATION OF PHOSPHATIDYLCHOLINE CONTENT

The LabAssay Phospholipid (Wako, Neuss, Germany) was used to quantify the phospholipid content in the release fractions based on the method described previously. The assay was performed by measuring the absorption at 600 nm. In Figure VII-19 it was shown that the release of lipids from various VPG formulations. Faster lipid erosion was observed for the VPGs based on 350mg/g lipids. Besides, the other formulations showed similar erosion profiles. Comparing the release profiles of MAB from VPGs (Figure VII-17 and Figure VII-18), it was found that the lipid erosion was in parallel with the protein release (Figure VII-20 showed the VPGs containing 37.4mg/g MAB and 450mg/g lipids for example). It could consequently be stated that erosion is the dominant mechanism controlling the release of MAB from VPGs, which is in agreement with former observations in the EPO release.

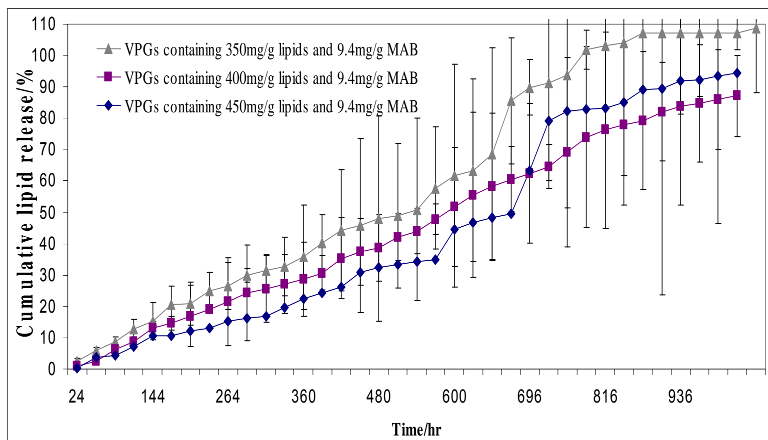


Figure VII-19a. Cumulative release amount of phospholipid from VPGs based on various lipid contents. (Mean± standard deviation, n = 3 each).

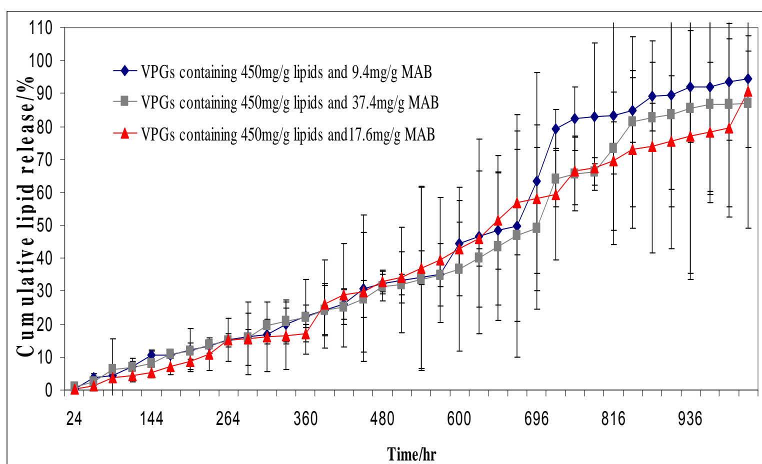


Figure VII-19b. Cumulative release amount of phospholipid from VPGs based on various MAB contents. (Mean± standard deviation, n = 3 each).

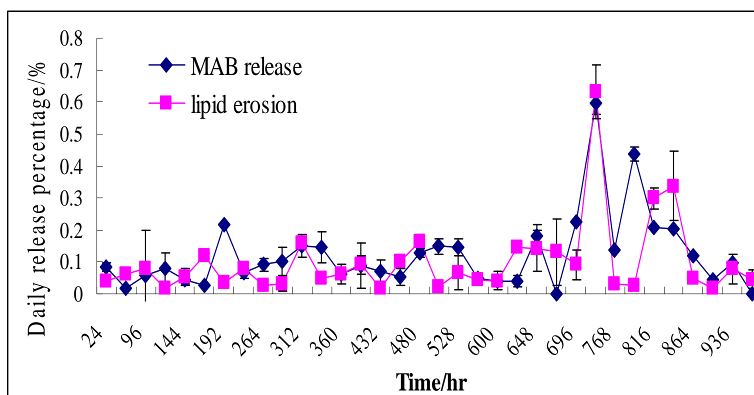
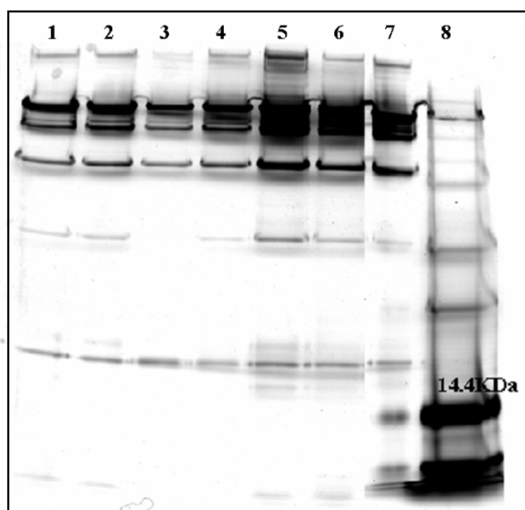


Figure VII-20. Daily releasae percentage of MAB and lipid from VPGs based on 450mg/g lipids and 37.4mg/g MAB. (Mean± standard deviation, n = 3 each).

7.4. SDS-PAGE OF THE RELEASED PROTEINS FROM VPGS

The quality of released MAB from VPGs (Taking VPGs based on 450mg/g lipids and 37.4mg/g EPO for example; only limited samples can be analyzed due to the low protein concentration in the release fractions.) was examined with SDS-PAGE. The proteins were extracted by adding DOC and chloroform subsequently (as described above) from release fractions. Non-reducing SDS-APGE were performed with silver staining. 50µl of extracted protein solutions were diluted into 100µl with Tris-buffer pH 6.8 containing 2 % SDS. Electrophoresis was performed in a 7% Tris-acetate/SDS running buffer (Invitrogen).

MAB recovered from release fractions of VPGs showed fraction bands similar to the MAB standard (Figure VII-21) despite that some fragment species in the standard were not detectable in the release samples (Some fragmentation fractions might be removed in the concentrating process). However, the release procedure of VPGs did not feature the creation of further aggregation or fragmentation species.



Lane 1/2, MAB release after 216hr
Lane 3/4, MAB release after 264hr
Lane 5/6, MAB release after 192hr
Lane 7, MAB standard solution
Lane 8, molecular weight marker

Figure VII-21. MAB stability from release samples of VPGs based on 450mg/g lipids and 37.4mg/g MAB. SDS-PAGE with silver staining.

8. SUMMARY AND DISCUSSION

In Chapter V and Chapter VI, EPO and G-CSF have been loaded into VPGs respectively. In vitro release tests showed the potential of VPGs for sustained delivery of these cytokines. Based on the previous work, the suitability of VPGs as the delivery system for the monoclonal antibody was evaluated. Various MAB VPG formulations were development which allowed an almost complete and sustained release of the entrapped antibody.

Initially, VPGs were prepared with various lipid and MAB contents. The obtained formulations were characterized by texture analyzer and rheometer. Increasing the lipid content could increase both of the gel strength and viscosity whereas incorporating more protein resulted in slightly decreased gel viscosity.

Again, the influence of the formulation process on the stability of the entrapped proteins was evaluated. Taken the experiences from previous work, chloroform and several surfactants were tried out for MAB extraction. The extracted MAB by applying chloroform showed the same bands with the standard samples. It was consequently concluded that MAB maintained stable within VPGs after formulation process in DAC, which fulfilled the prerequisite for a successful sustained release system.

For quantification of MAB in the release fractions from VPGs, CBQCA and micro BCA assay were tried out. However, the release samples could not be directly assayed since the lipids thereof got strong interference on the assay output. Moreover, the screening of extraction agents was limited for the both assays since some mostly used surfactants or organic (e.g. Triton X-100) were not compatible. Thus chloroform appeared the first choice for the MAB extraction in this case. Despite that the CBQCA assay was reported to work well in presence of lipids [255], it was found the results of the assay were influenced by the lipid content in the samples even though a major part of the lipids had been removed by applying chloroform. Besides, poor linearity and repeatability were observed for the CBQCA assay. As for the micro BCA assay, the protein recovery was also affected by the lipid content at a high concentration if adding chloroform alone for extraction. However, it was also found adding more chloroform could not efficiently change the extraction efficiency. Finally the extraction method was improved by using a surfactant – sodium deoxycholate before chloroform was added. The effect of the DOC (sodium deoxycholate) was assumed to reduce the interaction between the MAB and the lipids, and thus more lipids could be extracted to the organic phase by adding chloroform subsequently. The quantification method was established for the extracted MAB from lipid containing samples. A good correlation was generated for the results of the lipid containing and lipid-free samples. Based on the developed method, it was possible to quantify the MAB from VPGs or from VPGs' release fractions.

Protein denaturation and/or aggregation were found occurring within the release period or during storage for PLGA based protein depot systems, which consequently correlated with the incomplete protein release [55, 208]. Thus, the stability of MAB within VPGs was examined after incubation at 37°C to simulate the conditions during the in vitro release tests. As a result, the stability of MAB from VPGs was comparable to the MAB solutions, for both of which little protein was lost after 14 days incubation at 37°C. SDS-PAGE also confirmed that MAB maintained the stability both in solutions and in VPGs. It could be stated that the encapsulation of MAB within the lipid matrix did not feature the creation of protein instabilities under the conditions during in vitro release tests.

However, protein loss can also be induced by adsorption on exposed surfaces as amphiphilic molecule proteins tend to adsorb on interfaces. In the case of MAB, it was found that there was no protein lost after pumping the MAB solutions through the release set over a time period of 11hrs. Thus the unspecific adsorption of MAB could be excluded. Based on the promising results of the adsorption and stability study, it was expected 100% protein recovery could be achieved from the in vitro release tests.

Then the in vitro release tests of various MAB VPG formulations were carried out. In general, the lipid content as well as the protein content incorporated into VPGs had no dramatic influence on the release profiles. All the formulations showed sustained release of MAB from VPGs over at least 600hrs: VPGs based on 350mg/g delivered 89% of the total MAB within VPGs over 600hrs, whereas the other VPGs containing 400mg/g or 450mg/g lipids released 90% over 750hrs. A close to zero-order release kinetics was observed for all the formulations. Linear fit was made for the release curves. Judged on the slope, it was found increasing either the lipid content or the MAB content could slight decrease the release rate. Moreover, it was shown that the initial release and daily release amount of MAB from VPGs could be modified by varying the compositions of the VPGs. In addition, SDS-PAGE analysis revealed that the stability was maintained of MAB released from VPGs. Therefore it can be stated that the VPG formulations feature the flexibility for therapeutically applications besides the fulfilled protein stability issues.

In addition, the release of lipids was found in line with MAB release. Thus erosion was proved to be the dominant mechanism controlling the MAB release from the lipid matrix which was in agreement with the results of EPO VPGs.

In a summary, it can be concluded that the VPGs possess a great potential as an alternative approach to formulate depot delivery systems for MAB. The protein instability problems which always hampered the development of the other sustained release systems were solved by using VPGs. The MAB maintained the stability during system preparation, as well as within the release period. Moreover, the release profiles can be easily modified to achieve desired kinetics.

CHAPTER VIII: FINAL SUMMARY

Within the scope of the present thesis, it was aimed to evaluate the potential of vesicular phospholipid gels as local depot system for protein delivery. Focus was also necessarily put on the development of suitable quantification methods for various proteins in presence of lipids to meet the requirement of the in vitro release tests.

In Chapter I, the protein release systems are introduced in general. Background of the application of pharmaceutical proteins is first presented. Controlled release systems for protein delivery are reviewed into aspects of the polymeric systems and the lipid based systems. The success and major limit of the polymer based PLGA and hydrogel systems are stated. On the other hand, the increasing interest is revealed in the development of protein release devices based on lipidic materials. The principles of the lipid based systems and several promising features are presented. A close overview is focused on the novel lipid based systems: lipid implants, multivesicular liposomes, and vesicular phospholipid gels. The manufacturing technique and the related application are illustrated in details.

The objectives of the thesis are summarized in Chapter II. Chapter III lists the materials and methods that are employed in the present work, where three different protein drugs are used: erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), and monoclonal antibody immunoglobulin G (MAB IgG)

In Chapter IV, grounds are provided using FITC-Dextran as a macromolecular model for protein delivery from vesicular phospholipid gels. The high pressure homogenization and dual asymmetric centrifugation (DAC) technique were utilized for the production of VPGs. DAC appeared a suitable device for the lab-scale preparation of VPGs. The release system was set up with flow through cells for the in vitro release tests as described by Tardi[11]. Sustained release of FITC-Dextran from various VPG formulations was observed from ca. 50hrs up to more than 288hrs with zero-order kinetics. Incorporating higher amounts of lipids resulted in more delayed drug release. Importantly, the suitability of VPGs for the delivery of macromolecular compounds was demonstrated in this Chapter.

In the following chapters, three protein models (EPO (Chapter V), G-CSF (Chapter VI), and MAB (Chapter VII)) were loaded into the VPG matrices using the DAC method. The preparation protocol established by Massing [147] was optimized by including cooling steps in-between the multiple DAC runs to control the system temperature. Under microscopic observation, densely packed vesicle structures were observed for the prepared VPGs. Texture analysis and rheological test were performed to determine the gel characters, including the gel strength and viscosity, which might potentially influence the release behaviour of the system. It was found that the gel strength and viscosity increased with increasing the lipid content within the VPGs. The gel strength and viscosity were also influenced by varying the loading amount of proteins and changing the lipid compositions within the matrices. Moreover, the buffer ingredients contributed to the various values of the gel strength and viscosity of the VPGs containing the same content of lipids but various kinds of proteins.

SDS-PAGE was performed to examine the influence of the formulation process by using DAC on the stability of various proteins. The presence of the matrix material-lipids has shown influence on the protein bands of the electrophoresis results. Thus various extraction protocols for each protein were assessed aimed to eliminate the influence of the lipids. A case-by-case study was necessarily performed to exclude the influence of the addition of the extraction agents due to the different characters of various proteins. In general, EPO, G-CSF, and MAB all maintained the integrity within the matrices after the loading process.

In vitro release tests of VPGs loaded with various proteins were performed using the established in vitro release system. To determine the concentration of the released protein from VPGs, a quantification method for each protein was needed in presence of unknown contents of lipids. For EPO (Chapter V), the addition of chloroform to dissolve the lipids was applied to the release fractions prior to the RP-HPLC quantification of EPO. The quantification in combination with the extraction protocol showed robustness against the variation of the protein concentration as well as the variation of the lipid content and lipid composition within the release samples. Sustained release of EPO was observed from various VPG-formulations over a time period of 280hours to over 400hrs. A close to zero-order kinetic was found without initial burst. Increasing the lipid content from 300mg/g to 550mg/g resulted in delayed

release of EPO from VPGs whereas the lipid content had little effect on the release profiles in the range from 400mg/g to 500mg/g. Furthermore, the lipid charge also had strong influence on the release behavior. Incorporating 20% of positively charged lipids (DOTAP) into VPGs resulted in a strongly accelerated release rate while adding 10% of negatively charged lipids (DPPA) delivered the incorporated protein in a comparable manner to the uncharged formulation. The lipid content released from VPGs was determined to study the matrix erosion which was found to be the dominant mechanism in controlling the EPO release from the matrices. The in vitro released EPO was analyzed with SDS-PAGE and was found to be unchanged as compared to standard materials. The release fractions were shown containing free EPO and EPO liposomes (in the range up to 300nm).

For the quantification of G-CSF (Chapter VI), a RP-HPLC method was developed where N,N-Dimethylformamid was used to precipitate the lipids in the release samples. The extraction method worked under the conditions of low pH value (pH 3.5) which favoured the stability of G-CSF. The in vitro release tests have demonstrated the potential of VPGs for sustained delivery of growth factors. A sustained release of G-CSF from various VPG-formulations was observed following zero-order kinetic too. Increasing the lipid content within the matrices resulted in slower release rate. About 63% of the total entrapped G-CSF was delivered from the VPGs containing 400mg/g lipids and 4.0mg/g G-CSF over 408hrs (17 days) whereas VPGs containing 450mg/g lipids delivered 43% and VPGs containing 490mg/g lipids delivered 35% within the same period. Moreover, VPGs containing a higher content of G-CSF (8.4mg/g) revealed a slower release of 11% within 408hrs (17 days). However, the in vitro release conditions (pH 7.4, at 37°C) did not favor the stability of G-CSF, which contributed to the incomplete release of G-CSF from VPGs. To reveal the suitability of VPGs for the local application, G-CSF loaded VPGs will now be implanted to the rupture of tendons in vivo. The experiment will be performed using rats as animal model. The healing effect (e.g. histology) and the pharmacokinetics of the G-CSF VPGs will be evaluated. ELISA (Quantikine G-CSF Immunoassay) was developed for the quantification of G-CSF in the serum samples. However, the in vivo studies can not be included into this thesis since the authorization by the clinical committee had been delayed.

The study on the EPO VPGs and G-CSF VPGs has shown the potential of the system for the delivery of cytokines. In order to investigate the feasibility of the VPGs, the monoclonal antibody (MAB) loaded VPGs were developed and evaluated in the Chapter VII. A micro BCA assay was used for the determination of MAB content in the VPGs' release fractions. Sodium deoxycholate was added to eliminate the influence of lipids within the samples prior to the assay. The release profiles of MAB from VPGs were little influenced by the lipid and protein content within the formulations. Sustained release of MAB was observed over 1000hrs (42days) following zero-order kinetics. Again, it was shown that erosion was the dominating mechanism controlling the protein release from VPGs. In addition, the protein integrity was maintained in the release fractions analyzed by SDS-PAGE.

In summary, the development of VPGs can be considered as the most promising strategy for sustained local protein delivery. Accordingly, major achievements were reached in the context: First, the manufacture process was shown to be suitable for the production of VPGs with loading of proteins. Second, the suitability of the lipid based VPG system was revealed in vitro for the sustained delivery of various kinds of proteins. A long term release (over a couple of weeks) was observed following the zero-order kinetics. As conclusion, the knowledge obtained so far should enable to transfer the developed system to a variety of other pharmaceutical proteins.

CHAPTER IX: REFERENCES

[1]. Pavlou, A. K., and Reichert, J. M. Recombinant protein therapeutics - success rates, market trends and values to 2010, Nature Biotechnology *22*, (2004) 1513-1519.

[2]. Fu, K., Klibanov, A. M., and Langer, R. Protein stability in controlled-release systems, Nat Biotechnol *18*, (2000) 24-25.

[3]. Cleland, J. L., Daugherty, A., and Mrsny, R. Emerging protein delivery methods, Curr Opin Biotechnol *12*, (2001) 212-219.

[4]. van de Weert, M., Hennink, W. E., and Jiskoot, W. Protein instability in poly(lactic-co-glycolic acid) microparticles, Pharm Res *17*, (2000) 1159-1167.

[5]. Weiner, A. L. Liposomes for protein delivery: selecting manufacture and development processes, Immunomethods *4*, (1994) 201-209.

[6]. Martins, S., Sarmento, B., Ferreira, D. C., and Souto, E. B. Lipid-based colloidal carriers for peptide and protein delivery--liposomes versus lipid nanoparticles, Int J Nanomedicine *2*, (2007) 595-607.

[7]. Schulze, S., and Winter, G. Lipid extrudates as novel sustained release systems for pharmaceutical proteins, Journal of Controlled Release *134*, (2009) 177-185.

[8]. Anderson, P. M., Hanson, D. C., Hasz, D. E., Halet, M. R., Blazar, B. R., and Ochoa, A. C. Cytokines in liposomes: preliminary studies with IL-1, IL-2, IL-6, GM-CSF and interferon-gamma, Cytokine *6*, (1994) 92-101.

[9]. Ye, Q., Asherman, J., Stevenson, M., Brownson, E., and Katre, N. V. DepoFoam (TM) technology: a vehicle for controlled delivery of protein and peptide drugs, Journal of Controlled Release *64*, (2000) 155-166.

[10]. Brandl, M., Drechsler, M., Bachmann, D., Tardi, C., Schmidtgen, M., and Bauer, K. H. Preparation and characterization of semi-solid phospholipid dispersions and dilutions thereof, International Journal of Pharmaceutics *170*, (1998) 187-199.

[11]. Tardi, C., Brandl, M., and Schubert, R. Erosion and controlled release properties of semisolid vesicular phospholipid dispersions, J Control Release *55*, (1998) 261-270.

[12]. Kaiser, N., Kimpfler, A., Massing, U., Burger, A. M., Fiebig, H. H., Brandl, M., and Schubert, R. 5-Fluorouracil in vesicular phospholipid gels for anticancer treatment: entrapment and release properties, International Journal of Pharmaceutics *256*, (2003) 123-131.

[13]. Grohganz, H., Tho, I., and Brandl, M. Development and in vitro evaluation of a liposome based implant formulation for the decapeptide cetrorelix, European Journal of Pharmaceutics and Biopharmaceutics *59*, (2005) 439-448.

[14]. Banga, A. K. Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems, Second Edition, Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems, Second Edition, (2006).

[15]. Wang, W. Instability, stabilization, and formulation of liquid protein pharmaceuticals, Int J Pharm *185*, (1999) 129-188.

[16]. Li, S., Schoneich, C., and Borchardt, R. T. Chemical instability of protein pharmaceuticals: Mechanisms of oxidation and strategies for stabilization, Biotechnol Bioeng *48*, (1995) 490-500.

[17]. Senel, S., Kremer, M., Nagy, K., and Squier, C. Delivery of bioactive peptides and proteins across oral (buccal) mucosa, Curr Pharm Biotechnol *2*, (2001) 175-186.
[18]. Zuidema, J., Kadir, F., Titulaer, H. A. C., and Oussoren, C. RELEASE AND ABSORPTION RATES OF INTRAMUSCULARLY AND SUBCUTANEOUSLY INJECTED PHARMACEUTICALS (II), International Journal of Pharmaceutics *105*, (1994) 189-207.
[19]. Tice, T. R., and Tabibi, S. E. PARENTERAL DRUG DELIVERY INJECTABLES, Kydonieus, A. (Ed.). Treatise on Controlled Drug Delivery: Fundamentals, Optimization, Applications. Xi+553p. Marcel Dekker, Inc.: New York, New York, USA; Basel, Switzerland. Illus, (1992) 315-339.
[20]. Wang, W. Oral protein drug delivery, J Drug Target *4*, (1996) 195-232.
[21]. Lee, H. J. Protein drug oral delivery: the recent progress, Arch Pharm Res *25*, (2002) 572-584.
[22]. Pantzar, N., Lundin, S., and Westrom, B. R. Different properties of the paracellular pathway account for the regional small intestinal permeability to the peptide desmopressin, J Pharm Sci *84*, (1995) 1245-1248.
[23]. Morishita, M., and Peppas, N. A. Is the oral route possible for peptide and protein drug delivery?, Drug Discov Today *11*, (2006) 905-910.
[24]. Ashford, M., and Fell, J. T. Targeting drugs to the colon: delivery systems for oral administration, J Drug Target *2*, (1994) 241-257.
[25]. Saffran, M., Kumar, G. S., Savariar, C., Burnham, J. C., Williams, F., and Neckers, D. C. A new approach to the oral administration of insulin and other peptide drugs, Science *233*, (1986) 1081-1084.
[26]. Milstein, S. J., Leipold, H., Sarubbi, D., Leone-Bay, A., Mlynek, G. M., Robinson, J. R., Kasimova, M., and Freire, E. Partially unfolded proteins efficiently penetrate cell membranes--implications for oral drug delivery, J Control Release *53*, (1998) 259-267.
[27]. Bernkop-Schnurch, A. The use of inhibitory agents to overcome the enzymatic barrier to perorally administered therapeutic peptides and proteins, Journal of Controlled Release *52*, (1998) 1-16.
[28]. Ziv, E., Kidron, M., Raz, I., Krausz, M., Blatt, Y., Rotman, A., and Bar-On, H. Oral administration of insulin in solid form to nondiabetic and diabetic dogs, J Pharm Sci *83*, (1994) 792-794.
[29]. Kimura, T., Sato, K., Sugimoto, K., Tao, R., Murakami, T., Kurosaki, Y., and Nakayama, T. Oral administration of insulin as poly(vinyl alcohol)-gel spheres in diabetic rats, Biological & Pharmaceutical Bulletin *19*, (1996) 897-900.
[30]. Amsden, B. G., and Goosen, M. F. A. TRANSDERMAL DELIVERY OF PEPTIDE AND PROTEIN DRUGS - AN OVERVIEW, Aiche Journal *41*, (1995) 1972-1997.
[31]. Celebi, N., Erden, N., Gonul, B., and Koz, M. EFFECTS OF EPIDERMAL GROWTH-FACTOR DOSAGE FORMS ON DERMAL WOUND STRENGTH IN MICE, Journal of Pharmacy and Pharmacology *46*, (1994) 386-387.
[32]. Kickhofen, B., Wokalek, H., Scheel, D., and Ruh, H. Chemical and physical properties of a hydrogel wound dressing, Biomaterials *7*, (1986) 67-72.
[33]. Mezei, M., and Gulasekharam, V. Liposomes--a selective drug delivery system for the topical route of administration: gel dosage form, J Pharm Pharmacol *34*, (1982) 473-474.
[34]. Patton, J. S., Trinchero, P., and Platz, R. M. BIOAVAILABILITY OF PULMONARY DELIVERED PEPTIDES AND PROTEINS - ALPHA-

INTERFERON, CALCITONINS AND PARATHYROID HORMONES, Journal of Controlled Release *28*, (1994) 79-85.
[35]. Hoover, J. L., Rush, B. D., Wilkinson, K. F., Day, J. S., Burton, P. S., Vidmar, T. J., and Ruwart, M. J. PEPTIDES ARE BETTER ABSORBED FROM THE LUNG THAN THE GUT IN THE RAT, Pharmaceutical Research *9*, (1992) 1103-1106.
[36]. Nakada, Y., Miyake, M., and Awata, N. SOME FACTORS AFFECTING THE VAGINAL ABSORPTION OF HUMAN CALCITONIN IN RATS, International Journal of Pharmaceutics *89*, (1993) 169-175.
[37]. Segers, V. F. M., and Lee, R. T. Local delivery of proteins and the use of self-assembling peptides, Drug Discovery Today *12*, (2007) 561-568.
[38]. Gombotz, W. R., and Pettit, D. K. Biodegradable polymers for protein and peptide drug delivery, Bioconjug Chem *6*, (1995) 332-351.
[39]. Sinha, V. R., and Trehan, A. Biodegradable microspheres for protein delivery, J Control Release *90*, (2003) 261-280.
[40]. Morlock, M., Koll, H., Winter, G., and Kissel, T. Microencapsulation of rh-erythropoietin, using biodegradable poly(D,L-lactide-co-glycolide): Protein stability and the effects of stabilizing excipients, European Journal of Pharmaceutics and Biopharmaceutics *43*, (1997) 29-36.
[41]. Wang, P. Y. LIPIDS AS EXCIPIENT IN SUSTAINED-RELEASE INSULIN IMPLANTS, International Journal of Pharmaceutics *54*, (1989) 223-230.
[42]. Vogelhuber, W., Magni, E., Gazzaniga, A., and Gopferich, A. Monolithic glyceryl trimyristate matrices for parenteral drug release applications, European Journal of Pharmaceutics and Biopharmaceutics *55*, (2003) 133-138.
[43]. Reithmeier, H., Herrmann, J., and Gopferich, A. Development and characterization of lipid microparticles as a drug carrier for somatostatin, Int J Pharm *218*, (2001) 133-143.
[44]. Kim, S., and Howell, S. B. Multivesicular liposomes containing cytarabine entrapped in the presence of hydrochloric acid for intracavitary chemotherapy, Cancer Treat Rep *71*, (1987) 705-711.
[45]. Angst, M. S., and Drover, D. R. Pharmacology of drugs formulated with DepoFoam: a sustained release drug delivery system for parenteral administration using multivesicular liposome technology, Clin Pharmacokinet *45*, (2006) 1153-1176.
[46]. Mantripragada, S. A lipid based depot (DepoFoam technology) for sustained release drug delivery, Prog Lipid Res *41*, (2002) 392-406.
[47]. Howell, S. B. Clinical applications of a novel sustained-release injectable drug delivery system: DepoFoam technology, Cancer J *7*, (2001) 219-227.
[48]. Ye, Q., Asherman, J., Stevenson, M., Brownson, E., and Katre, N. V. DepoFoam technology: a vehicle for controlled delivery of protein and peptide drugs, J Control Release *64*, (2000) 155-166.
[49]. Katre, N. V., Asherman, J., Schaefer, H., and Hora, M. Multivesicular liposome (DepoFoam) technology for the sustained delivery of insulin-like growth factor-I (IGF-I), J Pharm Sci *87*, (1998) 1341-1346.
[50]. Brandl, M. Vesicular phospholipid gels: a technology platform, J Liposome Res *17*, (2007) 15-26.
[51]. Grohganz, H., Tho, I., and Brandl, M. Development and in vitro evaluation of a liposome based implant formulation for the decapeptide cetrorelix, Eur J Pharm Biopharm *59*, (2005) 439-448.

[52]. Asano, M., Yoshida, M., Omichi, H., and Yamanaka, H. BIODEGRADABLE POLYMERS FOR APPLICATION IN DRUG DELIVERY SYSTEMS, Membrane *17*, (1992) 216-227.
[53]. Sung, Y. K., and Kim, S. W. Advances in biodegradable polymers for drug delivery systems, Korea Polymer Journal *8*, (2000) 199-208.
[54]. Emami, J., Hamishehkar, H., Najafabadi, A. R., Gilani, K., Minaiyan, M., Mahdavi, H., and Nokhodchi, A. A Novel Approach to Prepare Insulin-Loaded Poly (Lactic-Co-Glycolic Acid) Microcapsules and the Protein Stability Study, Journal of Pharmaceutical Sciences *98*, (2009) 1712-1731.
[55]. Crotts, G., and Park, T. G. Protein delivery from poly(lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues, J Microencapsul *15*, (1998) 699-713.
[56]. Deshpande, A. A., Heller, J., and Gurny, R. Bioerodible polymers for ocular drug delivery, Crit Rev Ther Drug Carrier Syst *15*, (1998) 381-420.
[57]. Park, T. G., Yong Lee, H., and Sung Nam, Y. A new preparation method for protein loaded poly(D, L-lactic-co-glycolic acid) microspheres and protein release mechanism study, J Control Release *55*, (1998) 181-191.
[58]. Perez, C., Castellanos, I. J., Costantino, H. R., Al-Azzam, W., and Griebenow, K. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers, Journal of Pharmacy and Pharmacology *54*, (2002) 301-313.
[59]. QuintanarGuerrero, D., Allemann, E., Fessi, H., and Doelker, E. Applications of the ion-pair concept to hydrophilic substances with special emphasis on peptides, Pharmaceutical Research *14*, (1997) 119-127.
[60]. Yoo, H. S., Choi, H. K., and Park, T. G. Protein-fatty acid complex for enhanced loading and stability within biodegradable nanoparticles, Journal of Pharmaceutical Sciences *90*, (2001) 194-201.
[61]. Schwendeman, S. P. Recent advances in the stabilization of proteins encapsulated in injectable PLGA delivery systems, Crit Rev Ther Drug Carrier Syst *19*, (2002) 73-98.
[62]. Fu, K., Pack, D. W., Klibanov, A. M., and Langer, R. Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres, Pharm Res *17*, (2000) 100-106.
[63]. Brunner, A., Mader, K., and Gopferich, A. pH and osmotic pressure inside biodegradable microspheres during erosion, Pharm Res *16*, (1999) 847-853.
[64]. Lucke, A., Kiermaier, J., and Gopferich, A. Peptide acylation by poly(alpha-hydroxy esters), Pharm Res *19*, (2002) 175-181.
[65]. Almeida, A. J., Runge, S., and Muller, R. H. Peptide-loaded solid lipid nanoparticles (SLN): Influence of production parameters, International Journal of Pharmaceutics *149*, (1997) 255-265.
[66]. Blake, C. C., Koenig, D. F., Mair, G. A., North, A. C., Phillips, D. C., and Sarma, V. R. Structure of hen egg-white lysozyme. A three-dimensional Fourier synthesis at 2 Angstrom resolution, Nature *206*, (1965) 757-761.
[67]. Bodmer, D., Kissel, T., and Traechslin, E. FACTORS INFLUENCING THE RELEASE OF PEPTIDES AND PROTEINS FROM BIODEGRADABLE PARENTERAL DEPOT SYSTEMS, Journal of Controlled Release *21*, (1992) 129-137.
[68]. Boonyaratanakornkit, B. B., Park, C. B., and Clark, D. S. Pressure effects on intra- and intermolecular interactions within proteins, Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology *1595*, (2002) 235-249.

[69]. Frokjaer, S., and Otzen, D. E. Protein drug stability: a formulation challenge, Nat Rev Drug Discov *4*, (2005) 298-306.
[70]. Gopferich, A. Mechanisms of polymer degradation and erosion, Biomaterials *17*, (1996) 103-114.
[71]. Manning, M. C., Patel, K., and Borchardt, R. T. Stability of protein pharmaceuticals, Pharm Res *6*, (1989) 903-918.
[72]. Chen, S. C., Wu, Y. C., Mi, F. L., Lin, Y. H., Yu, L. C., and Sung, H. W. A novel pH-sensitive hydrogel composed of N,O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery, J Control Release *96*, (2004) 285-300.
[73]. Van Tomme, S. R., Storm, G., and Hennink, W. E. In situ gelling hydrogels for pharmaceutical and biomedical applications, Int J Pharm *355*, (2008) 1-18.
[74]. Hennink, W. E., and van Nostrum, C. F. Novel crosslinking methods to design hydrogels, Adv Drug Deliv Rev *54*, (2002) 13-36.
[75]. Peppas, N. A., Wood, K. M., and Blanchette, J. O. Hydrogels for oral delivery of therapeutic proteins, Expert Opin Biol Ther *4*, (2004) 881-887.
[76]. Corkhill, P. H., Hamilton, C. J., and Tighe, B. J. SYNTHETIC HYDROGELS .6. HYDROGEL COMPOSITES AS WOUND DRESSINGS AND IMPLANT MATERIALS, Biomaterials *10*, (1989) 3-10.
[77]. Qiu, B., Stefanos, S., Ma, J. L., Lalloo, A., Perry, B. A., Leibowitz, M. J., Sinko, P. J., and Stein, S. A hydrogel prepared by in situ cross-linking of a thiol-containing poly(ethylene glycol)-based copolymer: a new biomaterial for protein drug delivery, Biomaterials *24*, (2003) 11-18.
[78]. Lee, F., Chung, J. E., and Kurisawa, M. An injectable hyaluronic acid-tyramine hydrogel system for protein delivery, J Control Release *134*, (2009) 186-193.
[79]. Kashyap, N., Kumar, N., and Kumar, M. N. Hydrogels for pharmaceutical and biomedical applications, Crit Rev Ther Drug Carrier Syst *22*, (2005) 107-149.
[80]. Chung, H. J., Lee, Y., and Park, T. G. Thermo-sensitive and biodegradable hydrogels based on stereocomplexed Pluronic multi-block copolymers for controlled protein delivery, J Control Release *127*, (2008) 22-30.
[81]. Lectures to the Laity: War and the Expanding Frontiers of Medicine, Bull N Y Acad Med *19*, (1943) 865.
[82]. Delgado, M., Spanka, C., Kerwin, L. D., Wentworth, P., Jr., and Janda, K. D. A tunable hydrogel for encapsulation and controlled release of bioactive proteins, Biomacromolecules *3*, (2002) 262-271.
[83]. Fahy, E., Subramaniam, S., Murphy, R. C., Nishijima, M., Raetz, C. R., Shimizu, T., Spener, F., van Meer, G., Wakelam, M. J., and Dennis, E. A. Update of the LIPID MAPS comprehensive classification system for lipids, J Lipid Res *50 Suppl*, (2009) S9-14.
[84]. Bangham, A. D., Standish, M. M., and Watkins, J. C. Diffusion of univalent ions across the lamellae of swollen phospholipids, J Mol Biol *13*, (1965) 238-252.
[85]. Gabizon, A., Goren, D., Cohen, R., and Barenholz, Y. Development of liposomal anthracyclines: from basics to clinical applications, Journal of Controlled Release *53*, (1998) 275-279.
[86]. Storm, G., Koppenhagen, F., Heeremans, A., Vingerhoeds, M., Woodle, M. C., and Crommelin, D. J. A. NOVEL DEVELOPMENTS IN LIPOSOMAL DELIVERY OF PEPTIDES AND PROTEINS, Journal of Controlled Release *36*, (1995) 19-24.
[87]. Cortesina, G., De Stefani, A., Giovarelli, M., Barioglio, M. G., Cavallo, G. P., Jemma, C., and Forni, G. Treatment of recurrent squamous cell carcinoma of

the head and neck with low doses of interleukin-2 injected perilymphatically, Cancer *62*, (1988) 2482-2485.

[88]. Yoshida, S., Tanaka, R., Takai, N., and Ono, K. Local administration of autologous lymphokine-activated killer cells and recombinant interleukin 2 to patients with malignant brain tumors, Cancer Res *48*, (1988) 5011-5016.

[89]. BeduAddo, F. K., and Huang, L. Effect of matrix lipid chain length on liposomes containing cholesterol and ganglioside G(M1): Implications in drug delivery, Journal of Pharmaceutical Sciences *85*, (1996) 714-719.

[90]. Mohl, S., and Winter, G. Continuous release of Rh-interferon (alpha-2a from triglyceride implants: storage stability of the dosage forms, Pharm Dev Technol *11*, (2006) 103-110.

[91]. Kaewvichit, S., and Tucker, I. G. The release of macromolecules from fatty acid matrices: complete factorial study of factors affecting release, J Pharm Pharmacol *46*, (1994) 708-713.

[92]. Mohl, S., and Winter, G. Continuous release of rh-interferon alpha-2a from triglyceride matrices, J Control Release *97*, (2004) 67-78.

[93]. Vogelhuber, W., Magni, E., Mouro, M., Spruss, T., Guse, C., Gazzaniga, A., and Gopferich, A. Monolithic triglyceride matrices: A controlled-release system for proteins, Pharmaceutical Development and Technology *8*, (2003) 71-79.

[94]. Koennings, S., Garcion, E., Faisant, N., Menei, P., Benoit, J. P., and Goepferich, A. In vitro investigation of lipid implants as a controlled release system for interleukin-18, International Journal of Pharmaceutics *314*, (2006) 145-152.

[95]. Kent, J. S. (1984) Cholesterol matrix delivery system for sustained release of macromolecules, p 8 pp, (Syntex (U.S.A.), Inc., USA). Application: US
US.

[96]. Guse, C., Koennings, S., Kreye, F., Siepmann, F., Goepferich, A., and Siepmann, J. Drug release from lipid-based implants: Elucidation of the underlying mass transport mechanisms, Int. J. Pharm. *314*, (2006) 137-144.

[97]. Mohl, S. (2003) The Development of a Sustained and Controlled Release Device for Pharmaceutical Proteins based on Lipid Implants, p No pp given.

[98]. Herrmann, S., Mohl, S., Siepmann, F., Siepmann, J., and Winter, G. New insight into the role of polyethylene glycol acting as protein release modifier in lipidic implants, Pharm. Res. *24*, (2007) 1527-1537.

[99]. Schulze, S., and Winter, G. Lipid extrudates as novel sustained release systems for pharmaceutical proteins, J. Controlled Release *134*, (2009) 177-185.

[100]. Herrmann, S. (2007) Lipidic Implants for Pharmaceutical Proteins. Mechanisms of Release and Development of Extruded Devices, p No pp.

[101]. Herrmann, S., Winter, G., Mohl, S., Siepmann, F., and Siepmann, J. Mechanisms controlling protein release from lipidic implants: Effects of PEG addition, J. Controlled Release *118*, (2007) 161-168.

[102]. Schwab, M., Kessler, B., Wolf, E., Jordan, G., Mohl, S., and Winter, G. Correlation of in vivo and in vitro release data for rh-INFalpha lipid implants, Eur. J. Pharm. Biopharm. *70*, (2008) 690-694.

[103]. Kim, S., Turker, M. S., Chi, E. Y., Sela, S., and Martin, G. M. Preparation of multivesicular liposomes, Biochim. Biophys. Acta, Biomembr. *728*, (1983) 339-348.

[104]. Mantripragada, S. A lipid based depot (DepoFoam technology) for sustained release drug delivery, Prog. Lipid Res. *41*, (2002) 392-406.

[105]. Angst Martin, S., and Drover David, R. Pharmacology of drugs formulated with DepoFoam: a sustained release drug delivery system for parenteral administration using multivesicular liposome technology, Clin Pharmacokinet *45*, (2006) 1153-1176.

[106]. Asherman, J., Ye, Q., Stevenson, M., and Katre, N. Depofoam technology: an effective vehicle for controlled delivery of proteins and peptides, Proc. Int. Symp. Controlled Release Bioact. Mater. *25th*, (1998) 503.

[107]. Katre, N. V., Asherman, J., Schaefer, H., and Hora, M. Multivesicular Liposome (DepoFoam) Technology for the Sustained Delivery of Insulin-like Growth Factor-I (IGF-I), J. Pharm. Sci. *87*, (1998) 1341-1346.

[108]. Toliyat, T., and Khorasanirad, Z. Development of a Depofoam technology for the sustained delivery of desferrioxamine mesylate, Daru, J. Fac. Pharm., Tehran Univ. Med. Sci. *11*, (2003) 88-94.

[109]. Ye, Q., Asherman, J., Stevenson, M., Brownson, E., and Katre, N. V. DepoFoam technology: a vehicle for controlled delivery of protein and peptide drugs, J. Controlled Release *64*, (2000) 155-166.

[110]. Katre, N. V. Lipid-based multivesicular carriers for sustained delivery of therapeutic proteins and peptides, BioPharm (Duluth, MN, U. S.), (2001) 8-9, 11.

[111]. Sankaram, M. B., and Kim, S. (1996) Preparation of multivesicular liposomes for controlled release of active agents, p 34 pp, (Depotech Corporation, USA). Application: WO

[112]. Kim, S., and Howell, S. B. Multivesicular liposomes containing cytarabine for slow-release Sc administration, Cancer Treat Rep *71*, (1987) 447-450.

[113]. Mantripragada, S. B., and Howell, S. B. Sustained-release drug delivery with DepoFoam, Drug Delivery Syst. Cancer Ther., (2004) 247-262.

[114]. Langston, M. V., Ramprasad, M. P., Asherman, J. T., Katre, N. V., and Kararli, T. T. Increased triglyceride stabilizes multivesicular liposomes for sustained delivery of encapsulated myelopoietin, Proc. - 28th Int. Symp. Controlled Release Bioact. Mater. 4th Consum. Diversified Prod. Conf. *2*, (2001) 1017-1018.

[115]. Willis, R. C. (1998) Method for utilizing neutral lipids to modify in vivo release from multivesicular liposomes, p 94 pp, (Depotech Corporation, USA). Application: WO

[116]. Ye, Q., Katre, N., and Sankaram, M. (1999) Modulation of drug loading in multivesicular liposomes, p 54 pp, (Depotech Corporation, USA). Application: WO

[117]. Willis, R. C., Gai, W., McAllister, D. L., and Samaniego, A. C. Control of release by multivesicular liposomes, Proc. Int. Symp. Controlled Release Bioact. Mater. *25th*, (1998) 390-391.

[118]. Katre, N. V. Multivesicular liposomes (DepoFoam) for sustained delivery of therapeutics, Abstracts of Papers, 226th ACS National Meeting, New York, NY, United States, September 7-11, 2003, (2003) PMSE-006.

[119]. Jain, A. K., Chalasani, K. B., Khar, R. K., Ahmed, F. J., and Diwan, P. V. Muco-adhesive multivesicular liposomes as an effective carrier for transmucosal insulin delivery, J. Drug Targeting *15*, (2007) 417-427.

[120]. Kim, S., and Howell, S. B. (1998) Multivesicular liposomes having a biologically active substance encapsulated therein in the presence of a hydrochloride, pp 12 pp , Cont -in-part of U S Ser No 352,342, abandoned, (Depotech Corporation, USA). Application: US

[121]. Shirley, B. A., Hora, M., Ye, Q., Katre, N., and Asherman, J. (1999) High and low load formulations of IGF-I in multivesicular liposomes, p 59 pp, (Depotech Corporation, USA; Chiron Corporation). Application: WO
[122]. Angst, M. S., and Drover, D. R. Pharmacology of drugs formulated with DepoFoam: a sustained release drug delivery system for parenteral administration using multivesicular liposome technology, Clin. Pharmacokinet. *45*, (2006) 1153-1176.
[123]. Brandl, M. Liposomes as drug carriers: a technological approach, Biotechnol. Annu. Rev. *7*, (2001) 59-85.
[124]. Newman, M. S., Colbern, G. T., Wirking, P. K., Engbers, C., and Amantea, M. A. Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long-circulating, pegylated liposomes (SPI-077) in tumor-bearing mice. [Erratum to document cited in CA130:227670], Cancer Chemother. Pharmacol. *43*, (1999) 524.
[125]. Freise, J., Mueller, W. H., Magerstedt, P., and Schmoll, H. J. Pharmacokinetics of liposome encapsulated cisplatin in rats, Arch. Int. Pharmacodyn. Ther. *258*, (1982) 180-192.
[126]. Xiao, C., Qi, X., Aini, W. e., and Wei, S. preparation of cisplatin multivesicular liposomes and its in vitro release properties, Yaoxue Xuebao *38*, (2003) 133-137.
[127]. Chamberlain, M. C., Khatibi, S., Kim, J. C., Howell, S. B., Chatelut, E., and Kim, S. Treatment of leptomeningeal metastasis with intraventricular administration of depot cytarabine (DTC 101). A phase I study, Arch Neurol *50*, (1993) 261-264.
[128]. Kim, S., and Howell, S. B. Multivesicular liposomes containing cytarabine entrapped in the presence of hydrochloric acid for intracavitary chemotherapy, Cancer Treat. Rep. *71*, (1987) 705-711.
[129]. Kohn, F. R., Malkmus, S. A., Brownson, E. A., Rossi, S. S., and Yaksh, T. L. Fate of the predominant phospholipid component of DepoFoam drug delivery matrix after intrathecal administration of sustained-release encapsulated cytarabine in rats, Drug Delivery *5*, (1998) 143-151.
[130]. Viscusi, E. R. Liposomal drug delivery for postoperative pain management, Reg. Anesth. Pain Med. *30*, (2005) 491-496.
[131]. Yaksh, T. L., Provencher, J. C., Rathbun, M. L., and Kohn, F. R. Pharmacokinetics and efficacy of epidurally delivered sustained-release encapsulated morphine in dogs, Anesthesiology *90*, (1999) 1402-1412.
[132]. Yaksh, T. L., Provencher, J. C., Rathbun, M. L., Myers, R. R., Powell, H., Richter, P., and Kohn, F. R. Safety assessment of encapsulated morphine delivered epidurally in a sustained-release multivesicular liposome preparation in dogs, Drug Delivery *7*, (2000) 27-36.
[133]. Howell, S. B., and Kim, S. (1988) Multivesicular pharmaceutical liposomes containing active agents encapsulated in the presence of hydrochlorides, p 9 pp, (Clayton Foundation for Research, USA). Application: EP
[134]. Ramprasad, M. P., Amini, A., Kararli, T., and Katre, N. V. The sustained granulopoietic effect of progenipoietin encapsulated in multivesicular liposomes, Int. J. Pharm. *261*, (2003) 93-103.
[135]. Langston, M. V., Ramprasad, M. P., Kararli, T. T., Galluppi, G. R., and Katre, N. V. Modulation of the sustained delivery of myelopoietin (Leridistim) encapsulated in multivesicular liposomes (DepoFoam), J. Controlled Release *89*, (2003) 87-99.

[136]. Qiu, J., Wei, X.-h., Geng, F., Liu, R., Zhang, J.-w., and Xu, Y.-h. Multivesicular liposome formulations for the sustained delivery of interferon alpha -2b, Acta Pharmacol. Sin. *26*, (2005) 1395-1401.
[137]. Bonetti, A., and Kim, S. Pharmacokinetics of an extended-release human interferon alpha -2b formulation, Cancer Chemother. Pharmacol. *33*, (1993) 258-261.
[138]. Brandl, M., and Reszka, R. Preparation and characterization of phospholipid membrane gels as depot formulations for potential use as implants, Proc. Int. Symp. Controlled Release Bioact. Mater. *22nd*, (1995) 472-473.
[139]. Brandl, M., Bachmann, D., Drechsler, M., and Bauer, K. H. Liposome preparation by a new high pressure homogenizer Gaulin Micron LAB 40, Drug Dev. Ind. Pharm. *16*, (1990) 2167-2191.
[140]. Bandl, M., Tardi, C., Dreschsler, M., Bachmann, D., Reszka, R., Bauer, K. H., and Schubert, R. Three-dimensional liposome networks: freeze fracture electron microscopical evaluation of their structure and in vitro analysis of release of hydrophilic markers, Adv. Drug Delivery Rev. *24*, (1997) 161-164.
[141]. Brandl, M. M., Bachmann, D., Drechsler, M., and Bauer, K. H. Liposome preparation using high-pressure homogenizers, Liposome Technol. (2nd Ed.) *1*, (1993) 49-65.
[142]. Bender, J., Frey, C., Schubert, R., and Brandl, M. Highly concentrated semisolid phospholipid dispersions as intermediate during liposome preparation. Study on influence of the valve geometry on product properties, Emulsions Nanosuspensions Formulation Poorly Soluble Drugs, Based Invited Lect. Commun. Colloidal Drug Carriers Expert Meet., 3rd, (1998) 346-349.
[143]. Brandl, M., and Massing, U. Vesicular phospholipid gels, Liposomes (2nd Ed.), (2003) 353-372.
[144]. Lasch, J., Weissig, V., and Brandl, M. Preparation of liposomes, Liposomes (2nd Ed.), (2003) 3-29.
[145]. Kint, D., Seeley, G., Gio-Batta, M., and Burgess, A. Structure and Properties of Epoxy-Based Layered Silicate Nanocomposites, J. Macromol. Sci., Part B Phys. *44*, (2005) 1021-1040.
[146]. Wang, G., Chen, X. Y., Huang, R., and Zhang, L. Nano-CaCO3/polypropylene composites made with ultra-high-speed mixer, J. Mater. Sci. Lett. *21*, (2002) 985-986.
[147]. Massing, U., Cicko, S., and Ziroli, V. Dual asymmetric centrifugation (DAC)--a new technique for liposome preparation, J Control Release *125*, (2008) 16-24.
[148]. Karst, C. Neither shaken nor stirred: mixing with the dual asymmetric centrifuge, GIT Labor-Fachz. *45*, (2001) 836-837.
[149]. Massing, U. (2006) Preparation of lipid based nano-particles with a dual asymmetric centrifuge, p 40 pp, (KTB Tumorforschungsgesellschaft mbH, Germany). Application: EP
[150]. Bender, J., Michaelis, W., and Schubert, R. Morphological and Thermal Properties of Vesicular Phospholipid Gels Studied by DSC, Rheometry and Electron Microscopy, J. Therm. Anal. Calorim. *68*, (2002) 603-612.
[151]. Brandl, M., Drechsler, M., Bachmann, D., and Bauer, K.-H. Morphology of semisolid aqueous phosphatidylcholine dispersions, a freeze fracture electron microscopy study, Chem. Phys. Lipids *87*, (1997) 65-72.
[152]. Brandl, M., Drechsler, M., Bachmann, D., Tardi, C., Schmidtgen, M., and Bauer, K.-H. Preparation and characterization of semi-solid phospholipid dispersions and dilutions thereof, Int. J. Pharm. *170*, (1998) 187-199.

[153]. Moog, R., Burger, A. M., Brandl, M., Schuler, J., Schubert, R., Unger, C., Fiebig, H. H., and Massing, U. Change in pharmacokinetic and pharmacodynamic behavior of gemcitabine in human tumor xenografts upon entrapment in vesicular phospholipid gels, Cancer Chemother Pharmacol *49*, (2002) 356-366.
[154]. Tardi, C., Drechsler, M., Bauer, K. H., and Brandl, M. Steam sterilisation of vesicular phospholipid gels, Int J Pharm *217*, (2001) 161-172.
[155]. Guthlein, F., Burger, A. M., Brandl, M., Fiebig, H. H., Schubert, R., Unger, C., and Massing, U. Pharmacokinetics and antitumor activity of vincristine entrapped in vesicular phospholipid gels, Anticancer Drugs *13*, (2002) 797-805.
[156]. Fancher, T. L., Wun, T., Hotz, C. S., and Henderson, M. C. Jumpstarting academic careers with a novel intern research rotation: the AIMS rotation, Am J Med *122*, (2009) 1061-1066.
[157]. Segers, V. F. M., and Lee, R. T. Local delivery of proteins and the use of self-assembling peptides, Drug Discovery Today *12*, (2007) 561-568.
[158]. Randolph, G. J., and Furie, M. B. A soluble gradient of endogenous monocyte chemoattractant protein-1 promotes the transendothelial migration of monocytes in vitro, J Immunol *155*, (1995) 3610-3618.
[159]. Kaiser, N., Kimpfler, A., Massing, U., Burger, A. M., Fiebig, H. H., Brandl, M., and Schubert, R. 5-Fluorouracil in vesicular phospholipid gels for anticancer treatment: entrapment and release properties, Int J Pharm *256*, (2003) 123-131.
[160]. Grohganz, H., Schlafli, O., Rischer, M., and Brandl, M. Development and validation of a HPLC method for routine quantification of the decapeptide Cetrorelix in liposome dispersions, J Pharm Biomed Anal *34*, (2004) 963-969.
[161]. Grohganz, H., Ziroli, V., Massing, U., and Brandl, M. Quantification of various phosphatidylcholines in liposomes by enzymatic assay, AAPS PharmSciTech *4*, (2003) E63.
[162]. Farkas, E., Schubert, R., and Zelko, R. Effect of beta -sitosterol on the characteristics of vesicular gels containing chlorhexidine, Int. J. Pharm. *278*, (2004) 63-70.
[163]. Farkas, E., Schubert, R., and Zelko, R. Effect of beta -sitosterol concentration and high pressure homogenization on the chlorhexidine release from vesicular gels, Int. J. Pharm. *307*, (2006) 51-55.
[164]. Rawat, M., Singh, D., and Saraf, S. Lipid carriers: a versatile delivery vehicle for proteins and peptides, Yakugaku Zasshi *128*, (2008) 269-280.
[165]. Brandl, M., Tardi, C., Drechsler, M., Bachmann, D., Reszka, R., Bauer, K. H., and Schubert, R. Three-dimensional liposome networks: Freeze fracture electron microscopical evaluation of their structure and in vitro analysis of release of hydrophilic markers, Advanced Drug Delivery Reviews *24*, (1997) 161-164.
[166]. Grohganz, H., Rischer, M., and Brandl, M. Adsorption of the decapeptide Cetrorelix depends both on the composition of dissolution medium and the type of solid surface, Eur J Pharm Sci *21*, (2004) 191-196.
[167]. Rankin, J. C., and Jeanes, A. Evaluation of the periodate oxidation method for structural analysis of dextrans, J. Am. Chem. Soc. *76*, (1954) 4435-4441.
[168]. Thorball, N. FITC-dextran tracers in microcirculatory and permeability studies using combined fluorescence stereo microscopy, fluorescence light microscopy and electron microscopy, Histochemistry *71*, (1981) 209-233.

[169]. Cole, L., Coleman, J., Evans, D., and Hawes, C. Internalization of fluorescein isothiocyanate and fluorescein isothiocyanate-dextran by suspension-cultured plant cells, J. Cell Sci. *96*, (1990) 721-730.
[170]. Oliver, J. M., Berlin, R. D., and Davis, B. H. Use of horseradish peroxidase and fluorescent dextrans to study fluid pinocytosis in leukocytes, Methods Enzymol. *108*, (1984) 336-347.
[171]. Chretien, C., and Chaumeil, J. C. Release of a macromolecular drug from alginate-impregnated microspheres, Int. J. Pharm. *304*, (2005) 18-28.
[172]. Qi, X.-R., Maitani, Y., Shimoda, N., Sakaguchi, K., and Nagai, T. Evaluation of liposomal erythropoietin prepared with reverse-phase evaporation vesicle method by subcutaneous administration in rats, Chem. Pharm. Bull. *43*, (1995) 295-299.
[173]. Bonate, P. L. (2003) Biotechnology and Biopharmaceuticals: Transforming Proteins and Genes into Drugs by Rodney J. Y. Ho and Milo Gibaldi, Vol. 20.
[174]. Lin, F. K. Development of recombinant human erythropoietin, Immune Consequences Trauma, Shock Sepsis Mech. Ther. Approaches, [Int. Congr.], 3rd *2*, (1996) 794-801.
[175]. Zhou, G., Luo, G., Zhou, Y., Zhou, K., and Song, Q. Characterization of recombinant human erythropoietin by high performance capillary electrophoresis and capillary electrophoresis electrospray-ionization mass spectrometry, Fenxi Huaxue *26*, (1998) 249-253.
[176]. Zhou, G., Luo, G., Zhou, Y., Zhou, K., and Zhang, J. Characterization of recombinant human erythropoietin by matrix-assisted laser desorption ionization/time of flight mass spectrometry, Zhongguo Yaoke Daxue Xuebao *29*, (1998) 124-127.
[177]. Haroon, Z. A., Amin, K., Jiang, X., and Arcasoy, M. O. A novel role for erythropoietin during fibrin-induced wound-healing response, Am. J. Pathol. *163*, (2003) 993-1000.
[178]. Siren, A. L., Fratelli, M., Brines, M., Goemans, C., Casagrande, S., Lewczuk, P., Keenan, S., Gleiter, C., Pasquali, C., Capobianco, A., Mennini, T., Heumann, R., Cerami, A., Ehrenreich, H., and Ghezzi, P. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress, Proc Natl Acad Sci U S A *98*, (2001) 4044-4049.
[179]. Lacombe, C., Mayreux, P., and Casadevall, N. Erythropoetin, Nephrologie *12*, (1991) 221-226.
[180]. Duarte, R. F., and Frank, D. A. The synergy between stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF): Molecular basis and clinical relevance, Leuk. Lymphoma *43*, (2002) 1179-1187.
[181]. Mroczko, B., and Szmitkowski, M. Hematopoietic cytokines as tumor markers, Clin. Chem. Lab. Med. *42*, (2004) 1347-1354.
[182]. Roberts, A. W. G-CSF: A key regulator of neutrophil production, but that's not all!, Growth Factors *23*, (2005) 33-41.
[183]. Anderlini, P., and Korbling, M. The use of mobilized peripheral blood stem cells from normal donors for allografting, Stem Cells *15*, (1997) 9-17.
[184]. Layton, J. E., Hockman, H., Sheridan, W. P., and Morstyn, G. Evidence for a novel in vivo control mechanism of granulopoiesis: mature cell-related control of a regulatory growth factor, Blood *74*, (1989) 1303-1307.
[185]. Soerli, J., Barfod, L., Lavstsen, T., Bernasconi, N. L., Lanzavecchia, A., and Hviid, L. Human monoclonal IgG selection of Plasmodium falciparum for the expression of placental malaria-specific variant surface antigens, Parasite Immunol. *31*, (2009) 341-346.

[186]. D'Agostino, B., Bellofiore, P., De Martino, T., Punzo, C., Rivieccio, V., and Verdoliva, A. Affinity purification of IgG monoclonal antibodies using the D-PAM synthetic ligand: chromatographic comparison with protein A and thermodynamic investigation of the D-PAM/IgG interaction, J. Immunol. Methods *333*, (2008) 126-138.
[187]. Bilgicer, B., Thomas, S. W., Shaw, B. F., Kaufman, G. K., Krishnamurthy, V. M., Estroff, L. A., Yang, J., and Whitesides, G. M. A Non-Chromatographic Method for the Purification of a Bivalently Active Monoclonal IgG Antibody from Biological Fluids, J. Am. Chem. Soc. *131*, (2009) 9361-9367.
[188]. Scott, M. G., Shackelford, P. G., Briles, D. E., and Nahm, M. H. Human IgG subclasses and their relation to carbohydrate antigen immunocompetence, Diagn Clin Immunol *5*, (1988) 241-248.
[189]. Davis, J. M., Arakawa, T., Strickland, T. W., and Yphantis, D. A. Characterization of recombinant human erythropoietin produced in Chinese hamster ovary cells, Biochemistry *26*, (1987) 2633-2638.
[190]. Brems David, N. The kinetics of G-CSF folding, Protein Sci *11*, (2002) 2504-2511.
[191]. Kondo, T., and Suzuki, S. (1994) Introduction to Science of Colloids and Interfaces.
[192]. Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. Fluorometric assay of proteins in the nanogram range, Arch. Biochem. Biophys. *155*, (1973) 213-220.
[193]. Horowitz, P. M. Rapid fluorescamine based protein assay usable in the presence of interfering substances, J. Chromatogr. *319*, (1985) 446-449.
[194]. Lorenzen, A., and Kennedy, S. W. A fluorescence-based protein assay for use with a microplate reader, Anal. Biochem. *214*, (1993) 346-348.
[195]. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent, J. Biol. Chem. *193*, (1951) 265-275.
[196]. You, W. W., Haugland, R. P., Ryan, D. K., and Haughland, R. P. 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde, a reagent with broad dynamic range for the assay of proteins and lipoproteins in solution, Anal. Biochem. *244*, (1997) 277-282.
[197]. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem *72*, (1976) 248-254.
[198]. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. Measurement of protein using bicinchoninic acid, Anal Biochem *150*, (1985) 76-85.
[199]. Smith, P. K. (1989) Measurement of protein using bicinchoninic acid, pp 2 pp Cont of U S Ser No 618,727, abandoned, (Pierce Chemical Co., USA). Application: US
US.
[200]. Noble, J. E., Knight, A. E., Reason, A. J., Di Matola, A., and Bailey, M. J. A. A comparison of protein quantitation assays for biopharmaceutical applications, Mol. Biotechnol. *37*, (2007) 99-111.
[201]. Kessler, R. J., and Fanestil, D. D. Interference by lipids in the determination of protein using bicinchoninic acid, Anal. Biochem. *159*, (1986) 138-142.
[202]. Takayama, M., Itoh, S., Nagasaki, T., and Tanimizu, I. A new enzymatic method for determination of serum choline-containing phospholipids, Clin Chim Acta *79*, (1977) 93-98.

[203]. Duconge, J., Rodriguez-Vera, L., Valenzuela, C., Alvarez, D., Ramirez, O., de la Luz-Hernandez Kathya, R., Rabeza-Legon Estela, Y., Casaco, A., and Fernandez-Sanchez, E. Pharmacokinetic comparison of two recombinant human granulocyte colony-stimulating factor after subcutaneous administration in rabbits, Eur J Pharm Biopharm *61*, (2005) 142-148.
[204]. Qi, X. R., Maitani, Y., Shimoda, N., Sakaguchi, K., and Nagai, T. Evaluation of liposomal erythropoietin prepared with reverse-phase evaporation vesicle method by subcutaneous administration in rats, Chem Pharm Bull (Tokyo) *43*, (1995) 295-299.
[205]. Gonzalez-Ros, J. M., Paraschos, A., Farach, M. C., and Martinez-Carrion, M. Characterization of acetylcholine receptor isolated from Torpedo californica electroplax through the use of an easily removable detergent, beta-D-octylglucopyranoside, Biochim Biophys Acta *643*, (1981) 407-420.
[206]. Hermeling, S., Schellekens, H., Crommelin, D. J. A., and Jiskoot, W. Micelle-Associated Protein in Epoetin Formulations: A Risk Factor for Immunogenicity?, Pharm. Res. *20*, (2003) 1903-1907.
[207]. Sah, H. Protein instability toward organic solvent/water emulsification: implications for protein microencapsulation into microspheres, PDA J Pharm Sci Technol *53*, (1999) 3-10.
[208]. Kim, H. K., and Park, T. G. Microencapsulation of human growth hormone within biodegradable polyester microspheres: protein aggregation stability and incomplete release mechanism, Biotechnol Bioeng *65*, (1999) 659-667.
[209]. van de Weert, M., Hoechstetter, J., Hennink, W. E., and Crommelin, D. J. The effect of a water/organic solvent interface on the structural stability of lysozyme, J Control Release *68*, (2000) 351-359.
[210]. Masuoka, K., Ishihara, M., Asazuma, T., Hattori, H., Matsui, T., Takase, B., Kanatani, Y., Fujita, M., Saito, Y., Yura, H., Fujikawa, K., and Nemoto, K. The interaction of chitosan with fibroblast growth factor-2 and its protection from inactivation, Biomaterials *26*, (2005) 3277-3284.
[211]. Gospodarowicz, D. Fibroblast growth factor and its involvement in developmental processes, Curr Top Dev Biol *24*, (1990) 57-93.
[212]. Choi, S. H., and Park, T. G. G-CSF loaded biodegradable PLGA nanoparticles prepared by a single oil-in-water emulsion method, Int J Pharm *311*, (2006) 223-228.
[213]. Elisseeff, J., McIntosh, W., Fu, K., Blunk, B. T., and Langer, R. Controlled-release of IGF-I and TGF-beta1 in a photopolymerizing hydrogel for cartilage tissue engineering, J Orthop Res *19*, (2001) 1098-1104.
[214]. Norton, L. W., Tegnell, E., Toporek, S. S., and Reichert, W. M. In vitro characterization of vascular endothelial growth factor and dexamethasone releasing hydrogels for implantable probe coatings, Biomaterials *26*, (2005) 3285-3297.
[215]. Richardson, T. P., Peters, M. C., Ennett, A. B., and Mooney, D. J. Polymeric system for dual growth factor delivery, Nat Biotechnol *19*, (2001) 1029-1034.
[216]. Layman, H., Sacasa, M., Murphy, A. E., Murphy, A. M., Pham, S. M., and Andreopoulos, F. M. Co-delivery of FGF-2 and G-CSF from gelatin-based hydrogels as angiogenic therapy in a murine critical limb ischemic model, Acta Biomater. *5*, (2009) 230-239.
[217]. Perez, C., Castellanos, I. J., Costantino, H. R., Al-Azzam, W., and Griebenow, K. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers, J. Pharm. Pharmacol. *54*, (2002) 301-313.

[218]. Fu, K., Griebenow, K., Hsieh, L., Klibanov, A. M., and LangerR. FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres, J Control Release *58*, (1999) 357-366.
[219]. van de Weert, M., van 't Hof, R., van der Weerd, J., Heeren, R. M. A., Posthuma, G., Hennink, W. E., and Crommelin, D. J. A. Lysozyme distribution and conformation in a biodegradable polymer matrix as determined by FTIR techniques, J. Controlled Release *68*, (2000) 31-40.
[220]. Yang, T.-H., Dong, A., Meyer, J., Johnson, O. L., Cleland, J. L., and Carpenter, J. F. Use of Infrared Spectroscopy to Assess Secondary Structure of Human Growth Hormone within Biodegradable Microspheres, J. Pharm. Sci. *88*, (1999) 161-165.
[221]. Anderson, P. M., Hanson, D. C., Hasz, D. E., Halet, M. R., Blazar, B. R., and Ochoa, A. C. Cytokines in liposomes: preliminary studies with IL-1- IL-2, IL-6, GM-CSF and interferon-gamma, Cytokine (Philadelphia) *6*, (1994) 92-101.
[222]. Meyer, J., Whitcomb, L., and Collins, D. Efficient encapsulation of proteins within liposomes for slow release in vivo, Biochem. Biophys. Res. Commun. *199*, (1994) 433-438.
[223]. Chi, E. Y., Krishnan, S., Kendrick, B. S., Chang, B. S., Carpenter, J. F., and Randolph, T. W. Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor, Protein Sci. *12*, (2003) 903-913.
[224]. Sah, H. Protein instability toward organic solvent/water emulsification: implications for protein microencapsulation into microspheres, PDA J. Pharm. Sci. Technol. *53*, (1999) 3-10.
[225]. Chi, E. Y., Krishnan, S., Kendrick, B. S., Chang, B. S., Carpenter, J. F., and Randolph, T. W. Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor, Protein Sci *12*, (2003) 903-913.
[226]. Chung, L. A. A fluorescamine assay for membrane protein and peptide samples with non-amino-containing lipids, Anal. Biochem. *248*, (1997) 195-201.
[227]. Braune, C., von Eisenhart-Rothe, R., Welsch, F., Teufel, M., and Jaeger, A. Mid-term results and quantitative comparison of postoperative shoulder function in traumatic and non-traumatic rotator cuff tears, Arch Orthop Trauma Surg *123*, (2003) 419-424.
[228]. Visotsky Jeffrey, L., Basamania, C., Seebauer, L., Rockwood Charles, A., and Jensen Kirk, L. Cuff tear arthropathy: pathogenesis, classification, and algorithm for treatment, J Bone Joint Surg Am *86-A Suppl 2*, (2004) 35-40.
[229]. Molloy, T., Wang, Y., and Murrell, G. The roles of growth factors in tendon and ligament healing, Sports Med *33*, (2003) 381-394.
[230]. Chan, B. P., Fu, S., Qin, L., Lee, K., Rolf, C. G., and Chan, K. Effects of basic fibroblast growth factor (bFGF) on early stages of tendon healing: a rat patellar tendon model, Acta Orthop Scand *71*, (2000) 513-518.
[231]. Frid, M. G., Brunetti, J. A., Burke, D. L., Carpenter, T. C., Davie, N. J., Reeves, J. T., Roedersheimer, M. T., van Rooijen, N., and Stenmark, K. R. Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage, Am. J. Pathol. *168*, (2006) 659-669.
[232]. Sugiyama, K., Ishii, G., Ochiai, A., and Esumi, H. Improvement of the breaking strength of wound by combined treatment with recombinant human G-CSF,

recombinant human M-CSF, and a TGF-beta 1 receptor kinase inhibitor in rat skin, Cancer Sci. *99*, (2008) 1021-1028.

[233]. Cao, R., Brakenhielm, E., Pawliuk, R., Wariaro, D., Post, M. J., Wahlberg, E., Leboulch, P., and Cao, Y. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2, Nat Med *9*, (2003) 604-613.

[234]. Tabata, Y., Hijikata, S., Muniruzzaman, M., and Ikada, Y. Neovascularization effect of biodegradable gelatin microspheres incorporating basic fibroblast growth factor, J. Biomater. Sci., Polym. Ed. *10*, (1999) 79-94.

[235]. Marks, M. G., Doillon, C., and Silver, F. H. Effects of fibroblasts and basic fibroblast growth factor on facilitation of dermal wound healing by type I collagen matrices, J Biomed Mater Res *25*, (1991) 683-696.

[236]. Berscht, P. C., Nies, B., Liebendoerfer, A., and Kreuter, J. Incorporation of basic fibroblast growth factor into methylpyrrolidinone chitosan fleeces and determination of the in vitro release characteristics, Biomaterials *15*, (1994) 593-600.

[237]. Pieper, J. S., Hafmans, T., Van Wachem, P. B., Van Luyn, M. J. A., Brouwer, L. A., Veerkamp, J. H., and Van Kuppevelt, T. H. Loading of collagen-heparan sulfate matrices with bFGF promotes angiogenesis and tissue generation in rats, J. Biomed. Mater. Res. *62*, (2002) 185-194.

[238]. Obara, K., Ishihara, M., Ishizuka, T., Fujita, M., Ozeki, Y., Maehara, T., Saito, Y., Yura, H., Matsui, T., Hattori, H., Kikuchi, M., and Kurita, A. Photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 stimulates wound healing in healing-impaired db/db mice, Biomaterials *24*, (2003) 3437-3444.

[239]. Ono, I., Tateshita, T., and Inoue, M. Effects of a collagen matrix containing basic fibroblast growth factor on wound contraction, J. Biomed. Mater. Res. *48*, (1999) 621-630.

[240]. Richa, E., Papari, M., Allen, J., Martinez, G., Wickrema, A., Anastasi, J., Van Besien, K., and Artz, A. Older age but not donor health impairs allogeneic granulocyte colony-stimulating factor (G-CSF) peripheral blood stem cell mobilization, Biol. Blood Marrow Transplant. *15*, (2009) 1394-1399.

[241]. Ohdo, S., Arata, N., Furukubo, T., Yukawa, E., Higuchi, S., Nakano, S., and Ogawa, N. Chronopharmacology of granulocyte colony-stimulating factor in mice, J Pharmacol Exp Ther *285*, (1998) 242-246.

[242]. De Souza, R., Zahedi, P., Allen, C. J., and Piquette-Miller, M. Biocompatibility of injectable chitosan-phospholipid implant systems, Biomaterials *30*, (2009) 3818-3824.

[243]. Assil, K. K., and Weinreb, R. N. Multivesicular liposomes. Sustained release of the antimetabolite cytarabine in the eye, Arch Ophthalmol *105*, (1987) 400-403.

[244]. Kimura, M., Takai, M., and Ishihara, K. Biocompatibility and drug release behavior of spontaneously formed phospholipid polymer hydrogels, J Biomed Mater Res A *80*, (2007) 45-54.

[245]. Collier, J. H., and Messersmith, P. B. Phospholipid strategies in biomineralization and biomaterials research, Annu. Rev. Mater. Res. *31*, (2001) 237-263.

[246]. Sakaki, S., Tsuchida, M., Iwasaki, Y., and Ishihara, K. A water-soluble phospholipid polymer as a new biocompatible synthetic DNA carrier, Bull. Chem. Soc. Jpn. *77*, (2004) 2283-2288.

[247]. Barton Elisabeth, R., Gimbel Jonathan, A., Williams Gerald, R., and Soslowsky Louis, J. Rat supraspinatus muscle atrophy after tendon detachment, J Orthop Res *23*, (2005) 259-265.
[248]. Gimbel Jonathan, A., Van Kleunen Jonathan, P., Mehta, S., Perry Stephanie, M., Williams Gerald, R., and Soslowsky Louis, J. Supraspinatus tendon organizational and mechanical properties in a chronic rotator cuff tear animal model, J Biomech *37*, (2004) 739-749.
[249]. Theeuwes, F., and Yum, S. I. Principles of the design and operation of generic osmotic pumps for the delivery of semisolid or liquid drug formulations, Ann. Biomed. Eng. *4*, (1976) 343-353.
[250]. Landau, C., Jacobs, A. K., and Haudenschild, C. C. Intrapericardial basic fibroblast growth factor induces myocardial angiogenesis in a rabbit model of chronic ischemia, Am Heart J *129*, (1995) 924-931.
[251]. Kotto-Kome, A. C., Fox, S. E., Lu, W., Yang, B.-B., Christensen, R. D., and Calhoun, D. A. Evidence that the granulocyte colony-stimulating factor (G-CSF) receptor plays a role in the pharmacokinetics of G-CSF and PegG-CSF using a G-CSF-R KO model, Pharmacol. Res. *50*, (2004) 55-58.
[252]. Piggee, C. Therapeutic antibodies coming through the pipeline, Anal Chem *80*, (2008) 2305-2310.
[253]. Grainger, D. W. Controlled-release and local delivery of therapeutic antibodies, Expert Opin Biol Ther *4*, (2004) 1029-1044.
[254]. Wang, W., Singh, S., Zeng, D. L., King, K., and Nema, S. Antibody structure, instability, and formulation, J Pharm Sci *96*, (2007) 1-26.
[255]. You, W. W., Haugland, R. P., Ryan, D. K., and Haugland, N. P. 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde, a reagent with broad dynamic range for the assay of proteins and lipoproteins in solution, Anal. Biochem. *244*, (1997) 277-282.
[256]. Luo, S., Takano, M., and Asakawa, T. Measurement of solubilized membrane-protein, using bicinchoninic acid (BCA): A device for elimination of interfering substances, Japanese Journal of Pharmacology *85*, (2001) 185P.
[257]. Brown, R. E., Jarvis, K. L., and Hyland, K. J. PROTEIN MEASUREMENT USING BICINCHONINIC ACID - ELIMINATION OF INTERFERING SUBSTANCES, Anal. Biochem. *180*, (1989) 136-139.
[258]. Kessler, R. J., and Fanestil, D. D. INTERFERENCE BY LIPIDS IN THE DETERMINATION OF PROTEIN USING BICINCHONINIC ACID, Anal. Biochem. *159*, (1986) 138-142.

PRESENTATIONTS AND PUBLICATIONS ASSOCIATED WITH THIS THESIS

ARTICLES

W. Tian, S. Schulze, M. Brandl, G. Winter, A lipid based depot formulation for erythropoietin (EPO): development and in vitro evaluation. *Journal of Controlled Release.*: in press (2010).

POSTER PRESENTATIONS

W. Tian, S. Herrmann, M. Brandl, G. Winter, Sustained release formulation of pharmaceutical proteins based on vesicular phospholipid gels. Science to Market Meeting, Hannover, Germany, 2009.

W. Tian, S. Herrmann, M. Brandl, G. Winter, Macromolecular drug loaded vesicular phospholipid gels: preparation and characterization. 2nd Midnight Sun Meeting on Drug Transport and Delivery, Tromso, Norway, 2008.

W. Tian, S. Herrmann, M. Brandl, G. Winter, Texture analysis as an analytical tool for the characterization of FITC-Dextran loaded vesicular phospholipid gels, 6th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Barcelona, 2008.

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