Dissertation

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

Virus-Like Particle Based Vaccines: Stabilization by Freeze-Drying and Development of Sustained Release Devices

vorgelegt von

Rainer Albert Lang
aus Mediasch (Rumänien)

2008

Erklärung

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

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

München, den 14. September 2008

Rainer Albert Lang

Dissertation eingereicht am:	15. September 2008
1. Gutachter:	Prof. Dr. Gerhard Winter
2. Gutachter:	Prof. Dr. Wolfgang Frieß
Tag der mündlichen Prüfung:	6. Oktober 2008

For my family

Acknowledgements

This thesis has been prepared between October 2004 and April 2008 at the Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University Munich under the supervision of Prof. Dr. Gerhard Winter.

First of all I want to express my deepest gratitude to Prof. Dr. Gerhard Winter for giving me the opportunity to join his research group and to prepare this thesis. I am particularly grateful for his highly professional guidance of my work and the inspiring discussions. Furthermore, I appreciated the pleasant working atmosphere and the numerous social activities which he organized together with Prof. Dr. Wolfgang Frieß for the whole working group.

I am very thankful for the financial and scientific support provided by Cytos Biotechnology AG as well as for offering me the chance to being involved in this very interesting projects. I like to thank Benedetta Dorigo, Kathrin Schwarz, Yixin Pfister-Fu, Frank Hennecke, Alain Tissot, Partik Maurer, Matthias Kinzler, Stefan Landolt, Martin Sperrle and all the other collegues at Cytos Biotechnology AG for their great support and assistance. Special thanks go to Lorenz Vogt and Alexander Zürcher for their professional and enthusiastic guidance, their scientific input, countless discussions, and of course for the pleasant, amicable teamwork. I always enjoyed the outstandingly obliging, warmhearted working climate at Cytos Biotechnology AG.

Further, I would like to thank Prof. Dr. Wolfgang Friess for taking his time to survey this work and for the great working spirit being present at the institute.

The team of Wyatt Technology Europe shall be acknowledged for their always uncomplicated, promt help with keeping the AF4 system alive.

Within the campus I am very grateful to PD Dr. Stefan Zahler for assisting me with fluorescence microscopy, Wolfgang Wünschheim for his help with the powder diffractometer and Christian Minke for taking the SEM pictures.

I am very thankfull to Alice Hirschmann, Particia Plath and Julia El-Hayek for their great assistance with several experiments, for the work in the cleanroom and for providing me with literature.

Many thanks go to all the collegues from the research groups of Prof. Dr. Gerhard Winter and Prof. Dr. Wolfgang Frieß who shared the time with me at the department. I highly appreciated their help, our interesting discussions and the great time we had. Special thanks go to Kathrin Schersch, Sandra Herrmann, Stefan Gottschalk, Martin Schwab and Jan Zillies for their great support and the extremely cool time we had in- and outside the lab. Furthemore, I like to thank Julia El-Hayek, Ahmed Youssef and Stefan Gottschalk for the great cigarette breaks. Especially, I want to highlight Stefan Gottschalk, who joined all those years the lab with me. Stefan, many thanks for your continous help, the perfect collaboration, and for sharing a great taste of music. Cheers!

I wish all of you all the best for your future and I am looking forward to meeting you here and there.

Last but not least, I am deeply thankful to my family, my parents Christine and Albert, my sister Erna, my cohabitee Verena, and my children Luna, Lorenz and Kilian, this work goes out to you.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
ACE	Angiotensin-converting-enzyme
AF4	Asymmetrical flow field-flow fractionation
API	Active pharmaceutical ingredient
Ar	Argon
AUC	Area under the curve
BCA	Bicinchoninic acid
BCP	Bromochloropropane
BSA	Bovine serum albumin
CLSM	Confocal laser scanning microscopy
CPG	Cytosine-phosphate-guanosine
CTL	Cytotoxic T-lymphocyte
CVLP	Chimeric virus-like particles
DEPC	Diethyl pyrocarbonate
DLS	Dynamic light scattering
DMF	Dimethylformamide
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EP	European Pharmacopoeia
ETFE	Ethylenetetrafluoroethylene
EVAc	Ethylene-vinyl acetate
FACS	Fluorescence-activated cell sorting
FD	Freeze-drying
FDA	U.S. Food and Drug Administration
FFF	Field-flow fractionation
FITC	Fluorescein isothiocyanate
FT	Freeze-thawing
G-CSF	Granulocyte colony stimulating factor
GuaHCl	Guanidinium chloride

HBV	Hepatitis B virus
HBsAg	Hepatitis B virus surface antigen
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPV	Human papilloma virus
HSA	Human serum albumin
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
Ig	Immunoglobulin
KF	Karl Fischer
LDS	Lithium dodecyl sulfate
lmw	low molecular weight
M	Mannitol
MALLS	Multi angle laser light-scattering
MW	Molecular weight
MWCO	Molecular weight cut-off
NA	not applicable
NCVP	Norwalk virus capsid protein
ND	not determined
NNLS	Non-negatively constrained least squares
NTU	Nephelometric Turbidity Unit
NV	Norwalk virus
NVCP	Norwalk virus capsid protein
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCL	Polycaprolactone
PCS	Photon correlation spectroscopy
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PELA	Poly (lactide-co-glycolide)-poly(ethylene glycol)
PES	Polyethersulfone

PDMS	Polydimethylsiloxane
PI	Polydisperisty index
PIBCA	Poly (isobutylcyanoacrylate)
PLA	Poly (lactide)
PLGA	Poly (lactide-co-glycolide)
PP	Polypropylene
PVA	Polyvinyl alcohol
PVDF	Polyvinylidene difluoride
Qb	RNA bacteriophage Qb
QELS	Quasi-elastic light scattering
rcf	relative centrifugal force
RH	Relative humidity
RM	Residual moisture
RNA	Ribonucleic acid
RNase	Ribunuclease
RP-HPLC	Reversed phase HPLC
rpm	revolutions per minute
RSA	Rabbit serum albumin
RT	Room temperature
SAMSA fluorescein	((2-(and-3)-S-(acetylmercapto)succinoyl)amino) fluorescein
SDS	Sodium dodecyl sulfate
SE-HPLC	Size exclusion HPLC
SEM	Scanning electron microscopy
T	Trehalose
Tc	Collapsing temperature
TEM	Transmission electron microscopy
Tg	Glass transition temperature
Tg'	Glass transition temperature of maximally freeze-concentrated solution
tRNA	transfer ribonucleic acid
VLP	Virus-like particle
WHO	World Health Organization
XRD	X-ray powder diffraction

1 GENERAL INTRODUCTION TO THE THESIS

1.1 General Background

The prevention of diseases by vaccination is one of the most significant medical achievements of mankind. More than 3 million deaths per year are currently prevented by vaccines [Ulmer et al., 2006]. Antiviral vaccines garner the largest share of the market with nineteen billion dollars and a positive economic impact of about a billion dollars per year [Fox, 2007].

The most potent vaccines used in the past decades consisted of live attenuated or inactivated forms of whole pathogens [Russo et al., 1997; Vollmar et al., 2005]. Although inactivated and attenuated vaccines are relatively safe, there is a small but present risk of reversion to virulent, aggressive phenotypes in vivo which can cause disease [Russo et al., 1997]. This was observed for the Sabin attenuated poliovirus vaccine [Murdin et al., 1996]. Additionally, non-infectious subunits of pathogens might be poorly immunogenic and might have to be formulated with immune-stimulating adjuvants [Kaufmann, 2004]. Unfortunately, many potent adjuvants are rather toxic or at least painful and are not allowed in human use [Singh et al., 1999].

Thus, significant efforts have been taken to develop more potent but safer vaccines and adjuvants with improved compatibility to humans. A new class of vaccines of growing interest are virus-like particles (VLP). They consist of one or several viral proteins, recombinantly expressed in cell culture systems, which spontaneously assemble into supramolecular, highly repetitive, icosahedral or rod-like structures. Of capital importance is that VLP do not contain any genetic information of the virulent phenotype which renders the replication in the host impossible [Kaufmann, 2004].

Due to their particular structure with highly repetitive, organised epitopes on their surface, VLP are able to induce strong and long-lasting humoral and cell-mediated immune responses in the absence of adjuvants [Lechner et al., 2002; Kaufmann, 2004]. Several viral antigens have been expressed as VLP and have been tested in clinical trials, including some that were recently approved for human use (Table 1.1.1). The high economic value of this new class of vaccines was

demonstrated by Gardasil® (Merck & Co., Inc.), a vaccine for the prophylaxis of human papillomavirus (HPV) infection, which achieved blockbuster status within the first year of approval°.

Table 1.1.1 Commercialized VLP based vaccines and selection of VLP vaccines recently tested in clinical trials.

Vaccine	Indication	Stage of clinical development	Company or institute
HBsAg VLP (Engerix-B®)	Prophylactic B cell vaccine for HBV infection	Approved	GlaxoSmithKline*
HPV L1 capsid VLP: 6 / 11 / 16 / 18 (Gardasil®)	Prophylactic B cell vaccine for HPV infection	Approved	Merck & Co., Inc.#
HPV L1 (16 / 18) capsid VLP (Cervarix®)	Prophylactic B cell vaccine for HPV infection	Approved	GlaxoSmithKline*
Norwalk virus capsid protein VLP (NVCP-VLP)	Prophylactic B cell vaccine for NV infect. gastroenteritis	Phase I completed	Baylor College of Medicine&
Nicotine derivative covalently linked to Immunodrug™ carrier Qbeta (bacteriophage Qb capsid VLP) (NIC002)	Therapeutic B cell vaccine for smoking addiction	Phase I and II completed	Cytos Biotechnology AG+ (in collaboration with Novartis)
Chemically synthesized angiotensin II coupled to Immunodrug™ carrier Qbeta (CYT006-AngQb)	Therapeutic B cell vaccine for treatment of hypertension	Combined phase I / II completed	Cytos Biotechnology AG+
Peptide of TNF-α covalently linked to Immunodrug™ carrier Qbeta (CYT007-TNFQb)	Therapeutic B cell vaccine for treatment of psoriasis	Combined phase I / II ongoing	Cytos Biotechnology AG+
Immunodrug™ carrier QbG10 (Qb capsid VLP filled with immunostimulatory DNA sequence G10) (CYT003-QbG10)	Therapeutic T cell vaccine for treatment of perennial allergy to house dust mite and / or cat	Phase I and II completed	Cytos Biotechnology AG+
Melan-A/MART-1 protein coupled to the Immunodrug™ carrier QbG10 (CYT004-MelQbG10)	Therapeutic T cell vaccine for treatment of malignant melanoma	Phase I and II completed	Cytos Biotechnology AG+
Immunodrug™ carrier QbG10 together with grass pollen allergen (CYT005-AllQbG10)	Therapeutic T cell vaccine for treatment of grass pollen allergy	Phase I and II completed	Cytos Biotechnology AG+

° http://blog.vaccineethics.org/2007/10/gardasil-rotateq-sales-top-merck.html

Vaccine	Indication	Stage of clinical development	Company or institute
Fragment of the β-amyloid-protein coupled to Immunodrug™ carrier Qbeta (CAD106)	Therapeutic B cell vaccine for treatment of Alzheimer's disease	Phase I ongoing	Cytos Biotechnology AG[+] (in collaboration with Novartis)
HIV p17 / p24:Ty VLP	Therapeutic T cell vaccine for the treatment of HIV infections	Phase I completed	National Institute of Allergy and Infectious Diseases (NIAID)[~]
HPV 16 L1 / E7 VLP: chimeric HPV capsid / cytotoxic T cell (CTL) epitope VLP (CVLP)	Therapeutic T cell vaccine for cervical dysplasias	Phase I and II completed	MediGene AG[§] (in collaboration with Virionics Corp.)
H5N1 VLP influenza vaccine	Prophylactic B cell vaccine for H5N1 clade 2 influenza virus infection	Phase I and II ongoing	Novavax[$]
HPV L1 capsid VLP: 6 / 11 /16 / 18	Prophylactic B cell vaccine for HPV infection in HIV infected children	Phase I completed phase II ongoing	NIAID[~]

* http://www.gsk.com
§ http://www.medigene.de
$ http://www.novavax.com
# http://www.merckvaccines.com
~ http://www.niaid.nih.gov
+ http://www.cytos.com
& http://www.bcm.edu

Additionally, VLP not only act as carriers of immunological epitopes derived from microbial pathogens, but they have also been successfully used to present self-antigens or non-immunogenic small molecular weight molecules to the immune system and to overcome B cell tolerance [Lechner et al., 2002]. Based on this principle, Cytos Biotechnology AG developed several therapeutical vaccines based on VLP built from recombinantly expressed coat proteins of the bacteriophage Qb. For example, the therapeutical treatment of chronic diseases such as hypertension (e.g. angiotensin as antigen) [Ambuehl et al., 2007] and drug addiction like smoking (e.g. nicotine as hapten) [Maurer et al., 2006], two of the most important preventable causes of premature death worldwide [Mackay, 2004], are attained.

However, for commercialization, the development of stable and convenient formulations is essential. In this context two major challenges of VLP vaccines have to be addressed: (1) VLP as highly complex protein drugs might be prone to many chemical and physical degradation pathways which can lead to ineffectiveness of the drug and / or side reactions [Manning et al., 1989; Brandau et al., 2003; Hermeling et al., 2004; Wang, 2005]; (2) To reach adequate immune responses

vaccines have to be applied at least two to three times which might lead to poor patient compliance [Aguado, 1993; Sinha et al., 2003].

Although a liquid formulation is preferred in terms of cost and convenience for the end user, many vaccines are not stable enough in liquid solutions to meet shelf-life requirements. Thus, in order to overcome stability issues often dried forms are produced. Freeze-drying is the most widely used technique for this purpose. [Scott et al., 1976; Adebayo et al., 1998; Rexroad et al., 2002; Sarkar et al., 2003; Zhai et al., 2004; Abdul-Fattah et al., 2007]. However, during freeze-drying the drug is exposed to diverse stress factors, ascribed to freezing and drying, which can cause significant loss of activity. Thus, the proper choice of the freeze-drying process and the accurate selection of stabilizing excipients is inevitable [Franks, 1998; Nail et al., 2002; Tang et al., 2004].

With the intention to improve the effectiveness of a vaccine and patient compliance tremendous efforts have been taken during the last decades to develop single dose vaccine devices. Although, plenty of promising systems like implants, microspheres, and in-situ forming systems have been investigated [Cohen et al., 1994; Sanchez et al., 1996; O'Hagan et al., 1998; Cleland, 1999; Dorta et al., 2002; Eliaz et al., 2002; Jaganathan et al., 2005; Katare et al., 2006], no controlled release vaccine formulation has been marketed so far. The limited success can be related to stress factors occurring during manufacture of the specific devices, storage instabilities, and the environmental changes during release. Nevertheless, due to the strong demand for stable, single dose vaccines further work in this field is valuable.

1.2 Aim and Organization of the Thesis

One main objective of the thesis was the development of stable, freeze-dried formulations for a specific VLP based vaccine, NicQb, meeting the standard requirements necessary for large scale clinical studies and commercial use.

In this context the development of an asymmetrical flow field-flow fractionation (AF4) method, as a new powerful tool, in the investigation of the physical stability of VLP is described (**Chapter 3**).

In order to determine adequate formulations all steps of a complete formulation development study, i.e. preformulation, freeze-drying and long-term stability studies were conducted. The effect of parameters like pH and salt concentration, and various excipients on the stability of the drug were investigated. Additionally, the biological activity of the drug in the final freeze-dried form was assessed (**Chapter 4**).

The second goal of the thesis was the development of sustained release devices for VLP. For this purpose, preliminary studies with PLGA and lipid based implants as surrogate formulations were carried out and their efficacy in vivo was assessed. Due to the inconvenient route of application and unsatisfactory stability of the target VLP in these devices PLGA microcapsules with an oily inner core as potentially improved formulation were scrutinized (**Chapter 5**).

1.3 References

Adebayo, A. A., Sim-Brandenburg, J. W., Emmel, H., Olaleye, D. O., and Niedrig, M.; Stability of 17D yellow fever virus vaccine using different stabilizers; *Biologicals*, 1998, 26(4), 309-316

Aguado, M. T.; Future approaches to vaccine development: Single-dose vaccines using controlled-release delivery systems; *Vaccine*, 1993, 11(5), 596-597

Ambuehl, P. M., Tissot, A. C., Fulurija, A., Maurer, P., Nussberger, J., Sabat, R., Nief, V., Schellekens, C., Sladko, K., Roubicek, K., Pfister, T., Rettenbacher, M., Volk, H. D., Wagner, F., Mueller, P., Jennings, G. T., and Bachmann, M. F.; A vaccine for hypertension based on virus-like particles: preclinical efficacy and phase I safety and immunogenicity; *Journal of Hypertension*, 2007, 25(1), 63-72

Abdul-Fattah, A. M., Truong-Le, V., Yee, L., Pan, E., Ao, Y., Kalonia, D. S., and Pikal, M. J.; Drying-Induced Variations in Physico-Chemical Properties of Amorphous Pharmaceuticals and Their Impact on Stability II: Stability of a Vaccine; *Pharmaceutical Research*, 2007, 24(4), 715-727

Brandau, D. T., Jones, L. S., Wiethoff, C. M., Rexroad, J., and Middaugh, C. R.; Thermal stability of vaccines; *Journal of Pharmaceutical Sciences*, 2003, 92(2), 218-231

Cleland, J. L.; Single-administration vaccines: controlled-release technology to mimic repeated immunizations; *Trends in Biotechnology*, 1999, 17(1), 25-29

Dorta, M. J., Santovena, A., Llabres, M., and Farina, J. B.; Potential applications of PLGA film-implants in modulating in vitro drugs release; *International Journal of Pharmaceutics*, 2002, 248(1-2), 149-156

Eliaz, R. E., and Szoka, F. C., Jr.; Robust and prolonged gene expression from injectable polymeric implants; *Gene Therapy*, 2002, 9(18), 1230-1237

Fox, J. L.; Antivirals become a broader enterprise; *Nature Biotechnology*, 2007, 25(12), 1395-1402

Franks, F.; Freeze-drying of bioproducts: putting principles into practice; *European Journal of Pharmaceutics and Biopharmaceutics*, 1998, 45(3), 221-229

Hermeling, S., Crommelin, D. J. A., Schellekens, H., and Jiskoot, W.; Structure-Immunogenicity Relationships of Therapeutic Proteins; *Pharmaceutical Research*, 2004, 21(6), 897-903

Jaganathan, K. S., Rao, Y. U. B., Singh, P., Prabakaran, D., Gupta, S., Jain, A., and Vyas, S. P.; Development of a single dose tetanus toxoid formulation based on polymeric microspheres: a comparative study of poly (-lactic-co-glycolic acid) versus chitosan microspheres; *International Journal of Pharmaceutics*, 2005, 294(1-2), 23-32

Katare, Y. K., and Panda, A. K.; Influences of excipients on in vitro release and in vivo performance of tetanus toxoid loaded polymer particles; *European Journal of Pharmaceutical Sciences*, 2006, 28(3), 179-188

Kaufmann, S. H. E.; Novel Vaccination Strategies; *WILEY-VCH Verlag GmbH & Co.KGaA*, 2004

Lechner, F., Jegerlehner, A., Tissot, A. C., Maurer, P., Sebbel, P., Renner, W. A., Jennings, G. T., and Bachmann, M. F.; Virus-like particles as a modular system for novel vaccines; *Intervirology*, 2002, 45(4-6), 212-217

Mackay, J., and Mensah, G. A.; The Atlas of Heart Disease and Stroke; *WHO Press*, 2004

Manning, M. C., Patel, K., and Borchardt, R. T.; Stability of protein pharmaceuticals; *Pharmaceutical Research*, 1989, 6(11), 903-918

Maurer, P., and Bachmann, M. F.; Therapeutic vaccines for nicotine dependence; *Current Opinion in Molecular Therapeutics*, 2006, 8(1), 11-16

Murdin, A. D., Barreto, L., and Plotkin, S.; Inactivated poliovirus vaccine: past and present experience; *Vaccine*, 1996, 14(8), 735-746

Nail, S. L., Jiang, S., Chongprasert, S., and Knopp, S. A.; Fundamentals of freeze-drying; *Pharmaceutical Biotechnology*, 2002, 14, 281-360

O'Hagan, D., Singh, M., and Gupta, R. K.; Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines; *Advanced Drug Delivery Reviews*, 1998, 32(3), 225-246

Rexroad, J., Wiethoff, C. M., Jones, L. S., and Middaugh, C. R.; Lyophilization and the thermostability of vaccines; *Cell Preservation Technology*, 2002, 1(2), 91-104

Russo, S., Turin, L., Zanella, A., Ponti, W., and Poli, G.; What's going on in vaccine technology?; *Medicinal Research Reviews*, 1997, 17(3), 277-301

Sanchez, A., Gupta, R. K., Alonso, M. J., Siber, G. R., and Langer, R.; Pulsed controlled-release system for potential use in vaccine delivery; *Journal of Pharmaceutical Sciences*, 1996, 85(6), 547-552

Sarkar, J., Sreenivasa, B. P., Singh, R. P., Dhar, P., and Bandyopadhyay, S. K.; Comparative efficacy of various chemical stabilizers on the thermostability of a live-attenuated peste des petits ruminants (PPR) vaccine; *Vaccine*, 2003, 21(32), 4728-4735

Scott, E. M., and Woodside, W.; Stability of pseudorabies virus during freeze-drying and storage: effect of suspending media; *Journal of Clinical Microbiology*, 1976, 4(1), 1-5

Singh, M., and O'Hagan, D.; Advances in vaccine adjuvants; *Nature Biotechnology*, 1999, 17(11), 1075-1081

Sinha, V. R., and Trehan, A.; Biodegradable microspheres for protein delivery; *Journal of Controlled Release*, 2003, 90(3), 261-280

Tang, X., and Pikal, M. J.; Design of freeze-drying processes for pharmaceuticals: practical advice; *Pharmaceutical Research*, 2004, 21(2), 191-200

Ulmer, J. B., Valley, U., and Rappuoli, R.; Vaccine manufacturing: challenges and solutions; *Nature Biotechnology*, 2006, 24(11), 1377-1383

Vollmar, A., and Dingermann, T.; Immunologie; *Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart*, 2005

Wang, W.; Lyophilization and development of solid protein pharmaceuticals; *International Journal of Pharmaceutics*, 2000, 203(1-2), 1-60

Wang, W.; Protein aggregation and its inhibition in biopharmaceutics; *International Journal of Pharmaceutics*, 2005, 289(1-2), 1-30

Zhai, S., Hansen, R. K., Taylor, R., Skepper, J. N., Sanches, R., and Slater, N. K. H.; Effect of Freezing Rates and Excipients on the Infectivity of a Live Viral Vaccine during Lyophilization; *Biotechnology Progress*, 2004, 20(4), 1113-1120

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Virus-Like Particles (VLP)

All VLP used in this work were provided by Cytos Biotechnology AG, Schlieren, Switzerland, as liquid, frozen and stored at -80 °C until use.

The VLP are derived from the coat protein of the bacteriophage Qb. The coat protein is recombinantly expressed in E. coli and assembles spontaneously in the bacterial cytoplasm to RNA stabilized VLP of about 30 nm diameter [Kozlovska et al., 1993]. Each VLP consists of 180 highly organized repetitive subunits of approximately 14 kDa which results in a theoretical molecular weight (MW) of the VLP of 2.5 MDa. The amino acid sequence and a model of the assembled Qb VLP based on the structure dissolved by crystallography [Golmohammadi et al., 1996] are depicted in Figure 2.1.1 and Figure 2.1.2, respectively.

A**K**LETVTLGNIG**K**DG**K**QTLVLNPRGVNPTNGVASLSQAGAVPALE**K**RVTVSVSQPSRNR**K**NY
KVQV**K**IQNPTACTANGSCDPSVTRQAYADVTFSFTQYSTDEERAFVRTELAALLASPLLIDAID
QLNPAY

Figure 2.1.1 Amino acid sequence of Qb VLP coat protein.

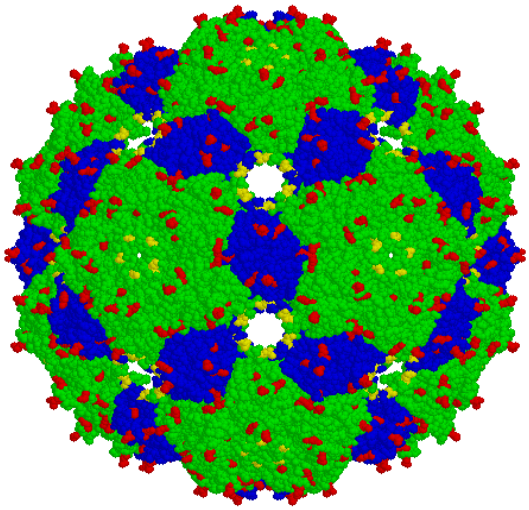


Figure 2.1.2 Model of Qb VLP.

Each of the 180 VLP subunits comprises 7 lysine residues (Figure 2.1.1). Using chemical cross-linkers, antigens can be coupled covalently to the Qb VLP surface via the lysine residues (Qb platform, Figure 2.1.3 A). By presenting antigens in highly repetitive order to the immune system B-cells can be directly activated and strong antibody responses can be induced even against small, low molecular weight (lmw) haptens [Bachmann et al., 1993; Jegerlehner et al., 2002; Lechner et al., 2002]. Based on this immunological principle VLP based drugs can be used for example for the therapeutical treatment of chronic diseases such as hypertension (e.g. angiotensin as antigen) [Ambuehl et al., 2007] and drug addiction like smoking (e.g. nicotine as hapten) [Maurer et al., 2006].

Furthermore, by packaging immunostimulatory nucleic acid sequences, so called cytosine-phosphate-guanosine motifs (CpGs), into antigen-decorated VLP cytotoxic T-cell induction in addition to the above described B-cell activation can be achieved (QbG10 platform, Figure 2.1.3 B) . The CpGs activate dendritic cells via stimulation of Toll-like receptor 9 consequently leading to a more pronounced activation of cytotoxic T-cells. This second feature can be used to treat diseases such as cancer and chronical viral infections [Storni et al., 2004].

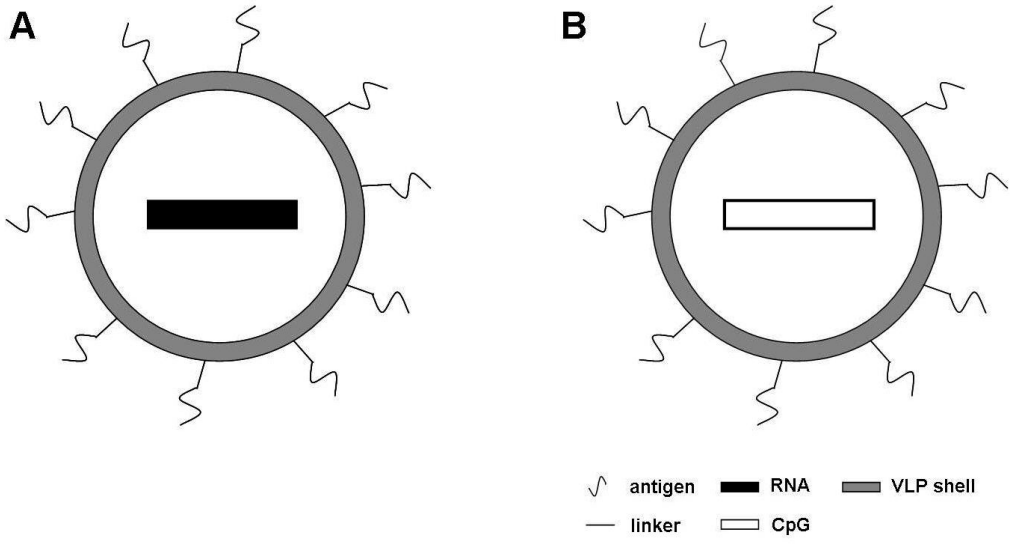


Figure 2.1.3 Scheme of Qb (A) and QbG10 (B) platform.

In this work four different types of VLP were used. Three were based on the above described Qb VLP platform, namely NicQb, AngQb and QbSAMSA, and one, QbG10p33, based on the VLP carrier vaccine platform QbG10.

NicQb

NicQb is a therapeutic vaccine for the treatment of nicotine addiction. Nicotine is coupled to the VLP of RNA bacteriophage Qb via a succinyl linker to the lysine side chains of the Qb coat proteins [Maurer et al., 2005]. Vaccination with NicQb leads to the induction of nicotine specific antibodies. The antibodies bind nicotine (from inhaled tobacco smoke) in the blood and inhibit its passage to the brain, as antibodies normally cannot pass the blood brain barrier. Phase II clinical trials with NicQb have demonstrated that this vaccine is efficacious for smoking cessation and relapse prevention [Maurer et al., 2006].

AngQb

AngQb is a therapeutic vaccine for the treatment of hypertension. It consists of chemically synthesized angiotensin II (identical to human form) which is chemically cross-linked onto the surface of Qb VLP. The drug is designed to instruct the patient's immune system to produce a specific anti-angiotensin II antibody response*. Angiotensin II is a potent vasoconstrictor and part of the renine-angiotensin-aldosteron system, the main regulator of blood pressure [Mutschler et al., 2001]. Vaccination with AngQb is anticipated to induce antibodies that bind angiotensin II and thus blood pressure should be down regulated. In a phase IIa clinical trial the efficacy of the drug was proved [Ambuehl et al., 2007].

QbG10p33

QbG10p33 is a model VLP which consists of a model peptide p33 covalently bound to Qb VLP packed with CpG oligonucleotides [Storni et al., 2004]. QbG10p33 was used as model VLP to investigate antibody response and T-cell induction.

QbSAMSA

QbSAMSA is a fluorescence labeled VLP. SAMSA fluorescein is covalently bound to the surface of Qb VLP. In this work the labeled VLP were used as model VLP for encapsulation into microcapsules.

* htttp://www.cytos.com

2.1.2 Fine chemicals

Significant chemicals used in this work are listed in Table 2.1.1. All other chemicals were of at least analytical grade.

Table 2.1.1 Significant fine chemicals.

Reagent	Producer	Article number
L-Histidine	Merck KGaA (Darmstadt, Germany)	1.04352
D-Mannitol	Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany)	33440
Mineral oil	Sigma-Aldrich Chemie GmbH (Steinheim, Germany)	M1180
Poly(vinyl alcohol) 80 % hydrolyzed	Sigma Aldrich Chemie GmbH (Steinheim, Germany)	360627
Polyethylene glycol 3,350	Sigma Aldrich Chemie GmbH (Steinheim, Germany)	88276
Polyethylene glycol 6,000	Clariant GmbH (Sulzbach, Germany)	107926
Polysorbate 20	Fluka Chemie GmbH (Buchs, Switzerland)	44112
Resomer® RG 502	Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim, Germany)	60640667
Resomer® RG 502 H	Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim, Germany)	60640802
Resomer® RG 503	Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim, Germany)	60640661
Sesame oil refined	Henry Lamotte GmbH (Bremen, Germany)	84260
α, α-Trehalose dihydrate	Ferro Pfanstiehl Lab., Inc. (Waukegan, IL, USA)	T-104-1-MC
Tristearin (Dynasan 118)	Sasol GmbH (Witten, Germany)	106582

2.2 Methods

2.2.1 Processing

Handling of VLP solutions

All VLP solutions were stored at -80 °C until use. Thawing was performed at 25 °C in agitated water baths. Before using the active pharmaceutical ingredient (API) bulk material VLP solutions were generally filtered through 0.22 µm polyvinylidene difluoride (PVDF) syringe-driven filter units.

Freeze-thawing (FT)

VLP solutions were filled into standard Eppendorf reaction tubes (Eppendorf AG, Hamburg, Germany). Samples were frozen by placing the tubes in a refrigerator at -80 °C. After a minimum of 3 hours the samples were thawed at ambient temperature. Freeze-thaw cycles were repeated for 5 times.

Freeze-drying (FD) of VLP formulations and microcapsule formulations

VLP formulations were prepared by mixing API bulk material with excipient stock solutions. After preparation, VLP formulations were sterile filtered through syringe-driven 0.22 µm PVDF filter units. Generally, aliquots of 600 µl of the final composition were transferred into 2R class I glass vials (Schott AG, Mainz, Germany) for freeze-drying. The samples were freeze-dried either in an EPSILON 2-6D pilot scale freeze-drier or an EPSILON 2-12D freeze-drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) (Figure 2.2.1) using three different protocols, A, B and C (Figure 2.2.2).

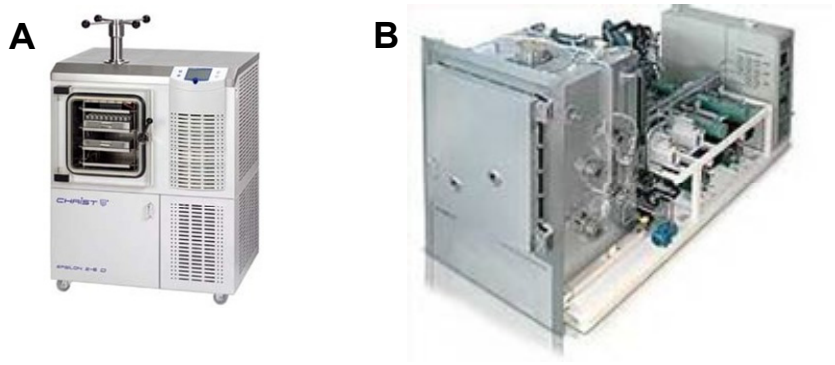


Figure 2.2.1 Freeze-driers: Epsilon 2-6D (A) and Epsilon 2-12D (B).

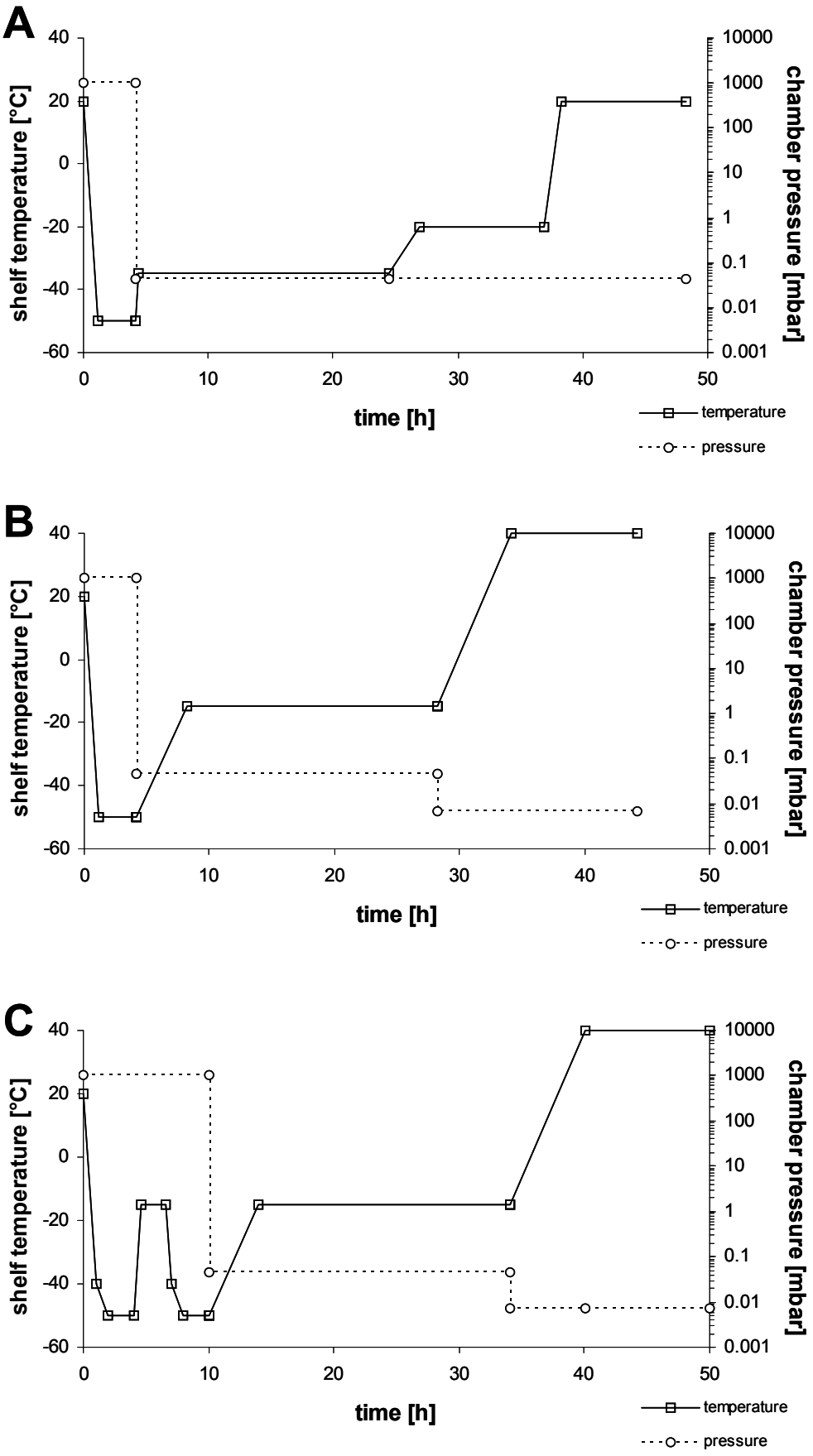


Figure 2.2.2 Freeze-drying cycles – VLP formulations.

Samples were frozen to -50 °C with a cooling rate of 1 °C / min if the freeze-drier EPSILON 2-12D was used. When using freeze-drier EPSILON 2-6D samples were first frozen to -40 °C at a freezing rate of 1 °C /min. Due to technical limitation the samples were then frozen to -50 °C at a cooling rate of 0.16 °C / min. Subsequently, the temperature was held at -50 °C for 3 hours. Protocol A comprises two primary drying steps, 20 hours at -35 °C and 10 hours at -20 °C, and a 10 hour secondary drying step at 20 °C at a pressure of 0.045 mbar. In protocol B, primary drying was conducted at -15 °C for 20 hours at 0.045 mbar and secondary drying at 40 °C for 10 hours at 0.007 mbar. Protocol C was alike protocol 2 with a 2 hour annealing step at -15 °C. After annealing the samples were again frozen to -40 °C with a cooling rate of 1 °C / min and to -50 °C at a freezing rate of 0.16 °C / min. The temperature was held at -50 °C for further 3 hours before primary drying. After freeze-drying the chamber was vented with nitrogen and the vials were stoppered with polydimethylsiloxane (PDMS) and ethylenetetrafluoroethylen (ETFE) coated lyophilization stoppers (West Pharmaceutical Services, Inc., Lionville, PA, USA) under vacuum at 800 mbar. The samples were rehydrated with highly purified water to a volume being equivalent to the volume prior to lyophilization.

Microcapsule formulations were freeze-dried with the EPSILON 2-6D pilot scale freeze-drier according to freeze-drying protocol D (Figure 2.2.3). Aliquots of about 0.5 mL were transferred into 2R class I glass vials (Schott AG, Mainz, Germany), frozen to -30 °C at a cooling rate of 1 °C / min and held at -30 °C for 1 hour. Primary drying was conducted at -30 °C and 0.100 mbar for 6 hours and secondary drying at 20 °C and 0.05 mbar for 10 hours. After freeze-drying the chamber was vented with nitrogen and the samples were stoppered with PDMS and ETFE coated lyophilization stoppers at 800 mbar.

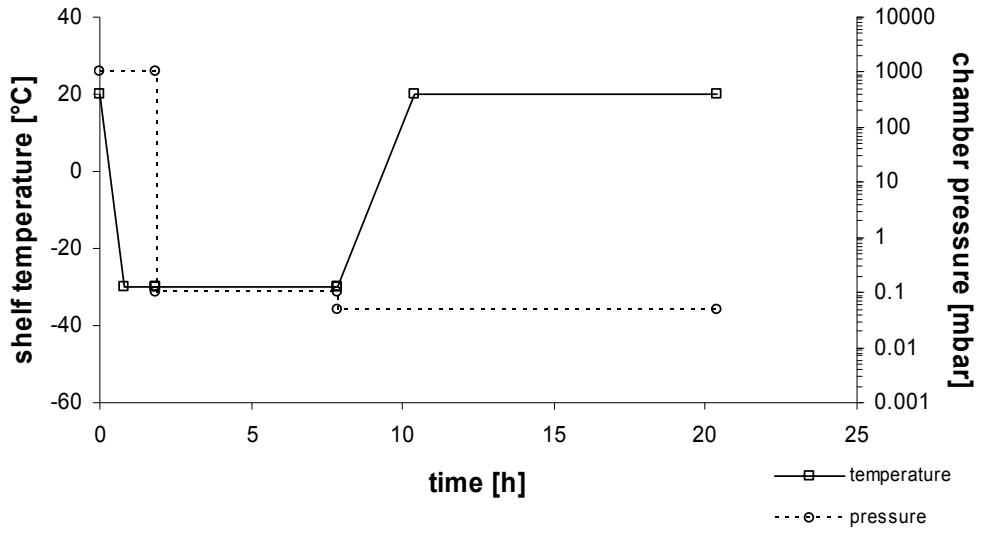


Figure 2.2.3 Freeze-drying cycle – microcapsule formulations.

Stability studies

Stability studies were carried out at controlled temperature levels. The conditions applied were 2-8 °C, 25 °C / 60 % relative humidity (RH) and 40 °C / 75 % RH. VLP lyophilizates were stored in 2R class I glass vials either open or sealed with PDMS and ETFE-coated lyophilization stoppers. VLP solutions and oily suspensions were stored either in LoBind Eppendorf reaction tubes or in 2R class I glass vials, open or sealed with the above mentioned stoppers.

In vivo study - NicQb lyophilizates
(performed by Cytos Biotechnology AG, Schlieren, Switzerland)

For bioactivity testing reconstituted lyophilizates and API bulk material were assessed. Groups of 10 female balb / c mice were immunized subcutaneously with a dose of 100 μg of vaccine. Mice were boosted after seven days with the same amount of drug. Sera were collected after another seven days and analyzed by Enzyme-linked Immunosorbent Assay (ELISA). ELISA analysis was carried out by measuring the specific anti-nicotine IgG titers of individual mice on RNase-nicotine conjugate. In brief, ELISA plates were coated overnight at 5 ± 3 °C with 4.25 µg / ml RNase-nicotine conjugate. After blocking, mouse serum dilutions were added to the plate and incubated for 2 hours at room temperature. Binding of NicQb-antibodies was detected by goat anti-mouse IgG-horseradish

peroxidase-conjugate after 1 hour incubation time. A pooled standard mouse serum was used to establish an anti-nicotine antibody standard curve. Data fitting of the standard curve was performed using a 4-parameter Marquardt fit. Titers of individual mice were determined from triplicate analysis of different dilutions. Individual measurements within the linear range of the ELISA curves were used for calculations.

Manufacture of implants

VLP loaded, PLGA or tristearin based implants with a total weight of each 25 mg were produced as follows: VLP lyophilizate (10 % of total mass), matrix materials PLGA or tristearin (80 - 86 % of total mass) and varying amount of further excipients (PEG and magnesium hydroxide, in sum 4 - 10 % of total mass) were ground in an agate mortar. Preparation was performed in a dry-nitrogen-purged glove box to prevent moisture uptake from the ambient atmosphere. 25 mg of this mixture were transferred into a compaction tool (3 mm diameter) and compressed for 15 seconds with a 5 ton hydraulic press (Maassen, Eningen, Germany) (Figure 2.2.4) at 2.0 kN.

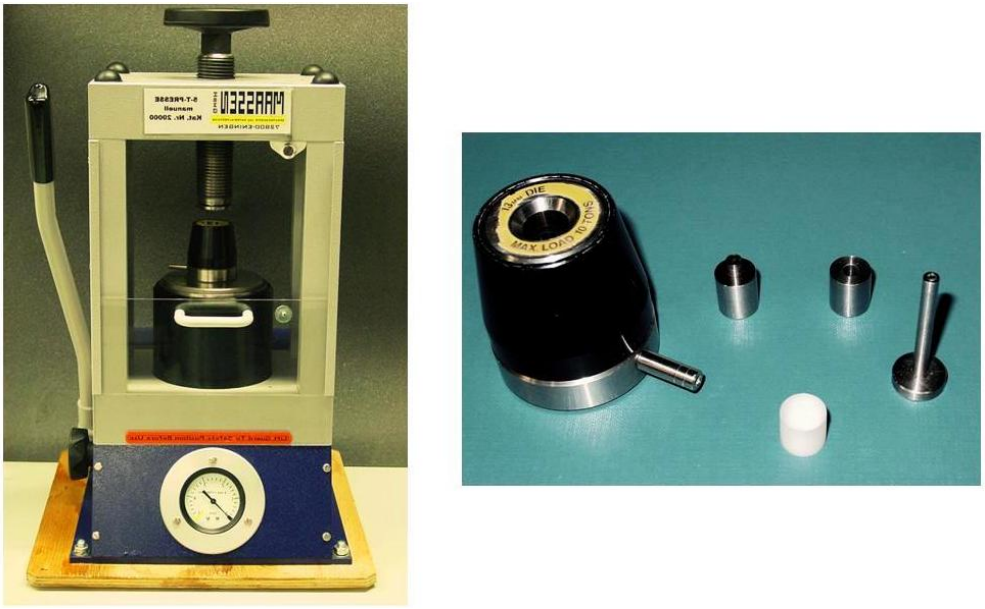


Figure 2.2.4 Hydraulic press and compacting equipment.

Release studies

The implants were placed into LoBind Eppendorf reaction tubes together with 1 mL release buffer. The samples were incubated at 37 °C at an agitation speed of 40 rpm in a B. Braun Biotech International Certomat® IS chamber (Melsungen, Germany). After defined time intervals, release media were exchanged with fresh release buffer. The Qb coat protein content in the release medium was determined via RP-HPLC. Therefore, the VLP were first disassembled with dithiothreitol (DTT) and guanidine hydrochloride (GuaHCl). Specific samples were analyzed via SE-HPLC for VLP integrity. The release buffer consisted of 20 mM sodium phosphate buffer, 0.01 % polysorbate 20 and 0.05 % sodium azide, pH 7.2.

In vivo study – QbG10p33 implants
(performed by Cytos Biotechnology AG, Schlieren, Switzerland)

For bioactivity testing different PLGA and tristearin implant formulations containing 300 µg VLP and placebo implants (negative control) were assessed. Additionally, liquid VLP formulations (reconstituted QbG10p33 lyophilizates) containing 50 µg VLP were applied either 1 x or 3 x (weekly application) as positive control. The different formulations were administered to groups of 3 - 5 female C57BL / 6 mice. The implants were placed under isofluorane anesthesia after surgical incision in the subcutaneous neck tissue. The liquid formulation was applied subcutaneously. Sera were collected after 1, 2, 3, 4, 6, 9 and 12 weeks. Samples were analyzed either by ELISA to measure the specific anti-p33 IgG titers or by fluorescence-activated cell sorting (FACS) to investigate the activation of p33-specific $CD8^+$ T cells.

ELISA measurements were performed by using a robotic liquid handling system (Hamilton Bonaduz AG, Bonaduz, Switzerland). In brief, Elisa plates were coated for 12 hours with 10 µg / ml p33-antigen coupled to RNase. Mouse serum dilutions were added to the plate and incubated for 2 hours. Binding of anti-p33-antibodies was detected by goat anti-mouse IgG-Fc-horseradish peroxidase-conjugate after 1 hour incubation time. ELISA titers from individual mouse sera were calculated using a 4-parameter Marquardt fit. The titer corresponds to the dilution needed to achieve half maximal optical density (OD).

For FACS analysis samples were incubated for 20 min at 4 °C with Strep-Tactin PE-labeled (IBA, Göttingen, Germany) p33-H-2^b tetrameric complexes and subsequently 30 min with anti-mouse CD8a APC Abs (BD PharMingen, San Diego, CA, USA). $CD8^+$ p33 specific T cells were acquired in a FACSCalibur device and analyzed by using CellQuest software (BD PharMingen, San Diego, CA, USA).

Manufacture of microcapsules

Oil-Based PLGA microcapsule formulations were prepared by a solvent extraction / solvent evaporation process introduced by Sanchez et al. [Sanchez et al., 1996]. The microcapsules consisted of VLP-loaded oily droplets coated with PLGA.

VLP lyophilizate was suspended in mineral oil using a SpeedMixer™ DAC 150 FVZ (Hauschild Engineering, Hamm, Germany). The resulting suspension was dispersed by vortexing in acetonitrile, ethyl acetate or mixtures of both solvents containing PLGA. Subsequently, the suspension was slowly added through a capillary with an inner diameter of 0.8 mm to 10 mL of a PVA solution (0.75 % w/v) under vigorous magnetic stirring. After stirring for 3 minutes additional 10 mL of the PVA solution were slowly added for extraction of the organic solvents. After stirring for further 10 minutes 30 mL water were added. Then, the emulsion was stirred for 30 minutes to enable evaporation of the organic solvents and hardening of the microcapsules (Figure 2.2.5). Afterwards, the microcapsules were collected by vacuum filtration and rinsed with hexane. The final microcapsule formulations were washed with water and lyophilized as described in “Freeze-drying (FD) of VLP formulations and microcapsule formulations”.

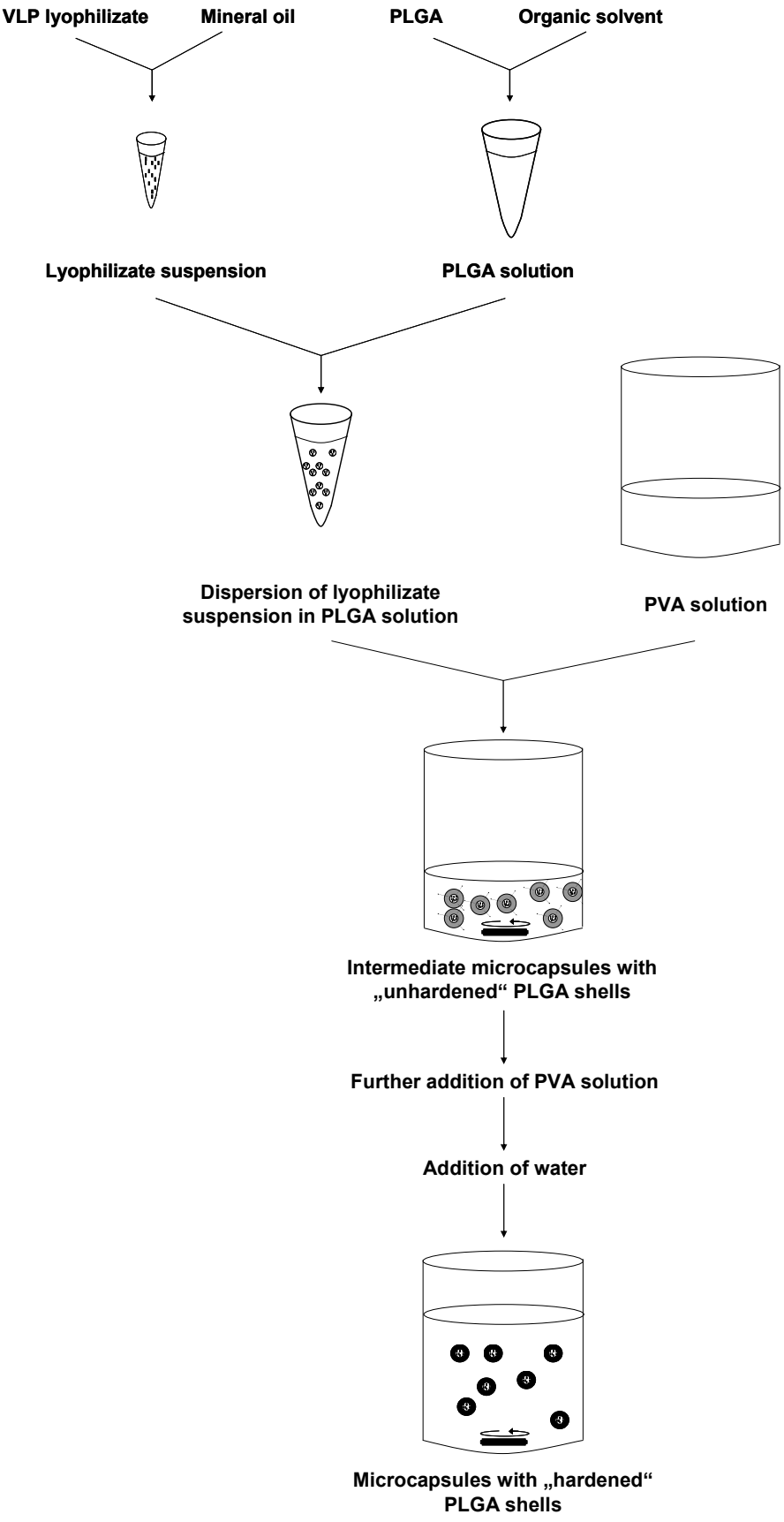


Figure 2.2.5 Schematic display of manufacture process of microcapsules.

2.2.2 Analytics

Asymmetrical flow field-flow fractionation (AF4)

AF4, a quasi chromatographic fractionation technique, is gaining more and more interest in protein and nanoparticle analytics. It is described to be capable of separating particles from a few nm up to several µm. By coupling AF4 to UV and multiangle laser light scattering (MALLS) detectors it became possible to quantify and determine the molecular weight of the specific fractions [Fraunhofer et al., 2004; Zillies, 2007].

AF4 measurements were performed using either the Wyatt standard separation channel (25 cm) or the short channel (18 cm) equipped with a 350 µm spacer, an Eclipse2 separation system (Wyatt Technology Europe GmbH, Dernbach, Germany), Agilent 1100 HPLC series isocratic pump, autosampler, degasser, UV detector (Agilent Technologies, Palo Alto, CA, USA) and the Wyatt DAWN EOS MALLS detector. Regenerated cellulose (MWCO of 10 kDa) was used as ultrafiltration membrane.

NicQb samples were analyzed by applying separation method A (Figure 2.2.6) and using the standard channel. The running buffer consisted of sodium phosphate buffer (pH 7.0) and 150 mM sodium chloride.

AngQb and QbG10p33 samples were analyzed by applying separation method B (Figure 2.2.6) and using the small channel. The running buffer used for AngQb analysis was composed of sodium phosphate buffer (pH 7.2) with 50 mM sodium chloride. For QbG10p33 samples sodium phosphate buffer (pH 7.2) was used.

The VLP concentration was determined at 260 nm via UV detection. The MALLS detector was used for the determination of the average molecular weight of the VLP fractions.

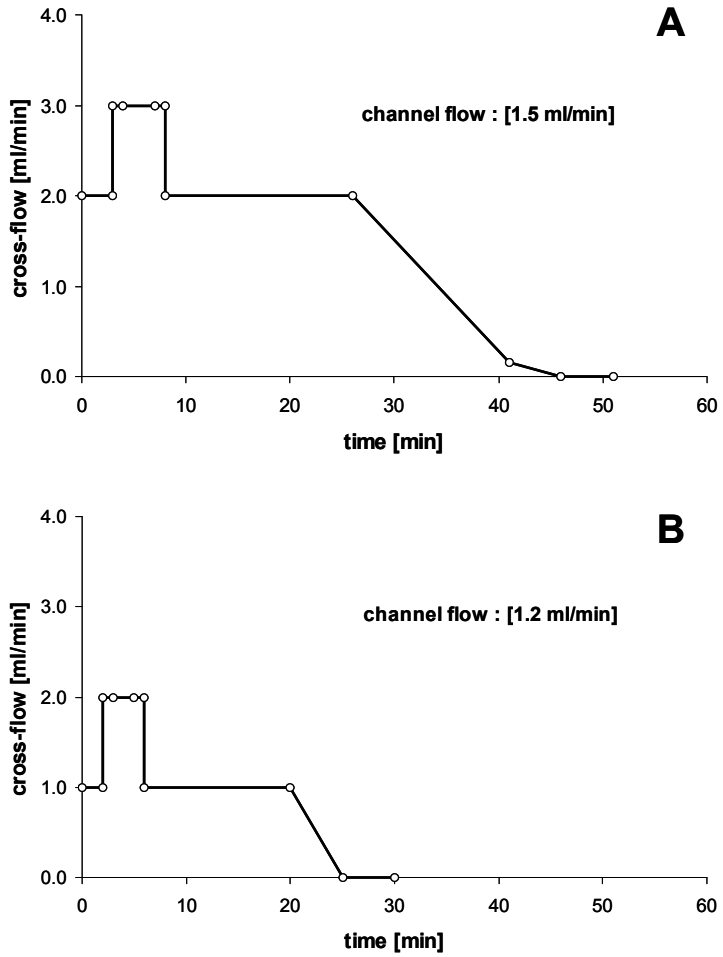


Figure 2.2.6: AF4 cross-flow profiles for NicQb (A), CYT006-AngQb and QbG10p33 (both B).

Size exclusion – high performance liquid chromatography (SE-HPLC) – VLP integrity

SE-HPLC is an analytical method typically applied for the investigation of soluble protein impurities such as degradation products and low molecular weight aggregates [Skoog et al., 1992; Rodriguez-Diaz et al., 2005]. In this context VLP degradation and aggregation was determined by SE-HPLC on a Summit HPLC System (Dionex GmbH, Idstein, Germany) using a TSKgel G5000PW$_{XL}$ SE-HPLC column (Tosoh Biosience GmbH, Stuttgart, Germany). The running buffer was composed of 20 mM sodium phosphate and 150 mM sodium chloride (pH 7.2). The analytics were performed at a flow rate of 0.8 mL / min with UV detection at 260 nm.

Dynamic light scattering (DLS)

DLS is a standard analytical tool applied for the investigation of VLP impurities [Brandau et al., 2003; Shi et al., 2005]. Measurements were performed using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The polydispersity index (PI) and the hydrodynamic diameter were determined by using a NNLS (Non-Negatively Constrained Least Squares) fitting algorithm. The size distributions by intensity and volume were calculated form the correlation function by using the multiple narrow mode of the Dispersion Technology Software version 4.00 (Malvern Instruments Ltd., Worcestershire, UK).

Determination of zeta potentials

Zeta potentials state electrostatic properties of particles in a dispersion medium [Leuenberger, 2002]. They were assessed with a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Malvern DTS 1060 disposable cells were filled with VLP solutions and analyzed at 25 °C via the automatic measurement mode.

Laser diffraction - particle size distribution

The size of the PLGA microcapsules was determined by laser diffraction using a Horiba LA-950 (Retsch Technology GmbH, Haan, Germany) particle size distribution analyzer equipped with a 10 mL fraction cell. Values of RI = 1.49 and absorption = 0.000 were used as optical model.

Light obscuration analysis – particulate matter

The particulate matter was analyzed with a PAMAS SVSS-C^{40} light blockage system (PAMAS GmbH, Rutesheim, Germany). Particles were counted due to their obscuration of light and were classified into 16 different size ranges from > 1 µm up to > 200 µm. Briefly, measurements were carried out as follows: The measurement cell was flushed with particle free water and rinsed with 0.3 mL sample. Subsequently, two independent measurements of 0.1 mL were performed, respectively. Particles larger than 1 and 10 µm were counted, and the mean values of the referring particle numbers per mL were displayed.

Transmission electron microscopy (TEM)
(performed by Prof. P. Wild at the Institute of Veterinary Anatomy and Virology, University of Zurich, Switzerland)

VLP samples were analyzed in a transmission electron microscope CM12 (Philips, Eindhoven, Netherlands) at 100 kV. Therefore, parlodion films mounted on 300 mesh / inch were carbon coated by electron gun evaporation at 10-5 mbar in a vacuum unit BAE 121 (BalTec Maschinenbau AG, Pfäfficon, Switzerland). The VLP were adsorbed onto these coated films and negatively stained with uranylacetate (Sigma Aldrich Chemie GmbH, Buchs, Switzerland). Images were taken using a CCD low scan camera (Gatan Inc., Pleasanton, CA, USA).

Reversed phase-HPLC (RP-HPLC) - determination of free nicotine derivatives
(performed by Cytos Biotechnology AG, Schlieren, Switzerland)

The free nicotine derivatives hydroxymethyl-nicotine and succinyl-hydroxy-methyl-nicotine were separated from NicQb by filtration at 14000 rcf in Nanosep 3K Omega spin filters from PALL Corporation (Dreieich, Germany). The filtrate was analyzed by RP-HPLC on a Summit HPLC System (Dionex GmbH, Idstein, Germany) using a Hypersil BDS-C18, 4.0 x 125 mm, 5 µm column (Agilent Technologies Deutschland GmbH, Böblingen, Germany). A flow rate of 1.0 mL / min with UV detection at 260 nm was applied. An elution gradient was used, applying acetonitrile and a sodium dihydrogen phosphate / triethylamine buffer (pH 7.0) as eluents. The concentration of the nicotine derivatives was calculated from the regression of a nicotine standard curve. The values for free nicotine derivatives were given as percentage of total nicotine.

RP-HPLC - determination of total nicotine
(performed by Cytos Biotechnology AG, Schlieren, Switzerland)

The nicotine moiety covalently linked to Qb VLP was quantitatively cleaved during 3 h incubation at 40 °C and pH > 11. Subsequently, proteins were precipitated with hydrochloric acid and removed by centrifugation. The concentration of the hydrolysis product hydroxymethyl-nicotine in the supernatant was determined as described above.

RP-HPLC – quantification of Qb coat proteins

For the quantification of Qb coat proteins in liquid samples VLPs were first disassembled by reducing disulfide bonds with DTT (50 mM) and disrupting non-covalent interactions with guanidine hydrochloride (2 M). Disassembled samples were centrifuged to remove particulate matter. The supernatant was analyzed by RP-HPLC on a Summit HPLC System (Dionex GmbH, Idstein, Germany) using a Jupiter C4, 300 A, 150 x 4.6 mm, 5 µm column (Phenomenex, Aschaffenburg, Germany). A flow rate of 1.0 mL / min with UV detection at 215 nm was applied. An elution gradient was used, introducing acetonitril (40 % v/v) and acetonitril (60 % v/v) as eluents under acidic conditions. The concentration of Qbeta coat protein was determined by external calibration with a VLP standard.

Bioanalyzer – RNA integrity
(performed by Cytos Biotechnology AG, Schlieren, Switzerland)

The integrity of RNA in Qb VLP was analyzed either by Bioanalyzer or by SE-HPLC. For sample preparation VLP formulations were first homogenized with TRI-Reagent® (Lucerna Chem AG, Luzern, Switzerland), a combination of phenol and guanidine thiocyanate in a monophase solution used to inhibit RNase activity. After incubation for 5 minutes at ambient temperature BCP Phase Separation Reagent (Lucerna Chem AG, Luzern, Switzerland) was added. Thereby, the homogenate is separated into aqueous and organic phases. Afterwards the mixture was centrifuged. RNA remains exclusively in the aqueous phase, DNA in the interphase and proteins in the organic phase. RNA was precipitated from the aqueous phase by addition of isopropanol, washed with ethanol and dissolved in diethyl pyro-carbonate (DEPC) - water. RNA was analyzed by using a 2100 Bioanalyzer (Agilent Technologies AG, Basel Switzerland) together with the RNA 6000 Nano assay (Agilent Technologies AG, Basel Switzerland). The Agilent 2100 Bioanalyzer uses a combination of microfluidics, capillary electrophoresis, and fluorescent dyes that bind to nucleic acid to simultaneously evaluate both RNA concentration and integrity. The RNA pattern of the samples was compared to a corresponding standard.

SE-HPLC – RNA integrity
(performed by Cytos Biotechnology AG, Schlieren, Switzerland)

The VLP samples were prepared as described in “Bioanalyzer – RNA integrity”. RNA degradation was determined by SE-HPLC on a Summit HPLC System (Dionex GmbH, Idstein, Germany) using a TSKgel G5000PW$_{XL}$ SE-HPLC column (Tosoh Biosience GmbH, Stuttgart, Germany). The running buffer was composed of 20 mM sodium phosphate and 150 mM sodium chloride (pH 7.2). The analytics were performed at a flow rate of 0.8 mL / min with UV detection at 260 nm. The retention time of the extracted RNA was determined relative to a tRNA standard analyzed in the same sequence.

Gel electrophoresis (LDS-PAGE) – VLP degradation
(performed by Cytos Biotechnology AG, Schlieren, Switzerland)

LDS-PAGE and SDS-PAGE (lithium / sodium dodecylsulfate polyacrylamide gel electrophoresis) is a standard tool to separate different fractions of a protein sample. After denaturing the protein with LDS / SDS, protein species are separated according to their mass on a polyacrylamide gel. In general, protein species with low molecular weight diffuse faster through the gel than those with larger molecular weight. LDS / SDS-PAGE can be carried out under non-reducing or reducing conditions (addition of DTT resulting in the break-up of covalent disulfide bonds) [Dunn, 2000; Wang et al., 2006].

This method was used for the determination of degradation products of Qb coat proteins. VLP samples and a Qb standard were incubated with DTT solution (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and NuPage® LDS sample buffer (Invitrogen Life Technologies Ltd, Paisley, UK). Thus, disassembling of VLP is enabled by the cleavage of disulfide bonds and non-covalent bonds. Invitrogen NuPage® 12% Bis-Tris gels and NuPage® MES LDS running buffer were used for separation of the samples in an Novex Xcell SureLock Mini-Cell (Invitrogen Life Technologies Ltd, Paisley, UK) connected to a BioRad Power Pac 300 power supply (Bio-Rad AG, Glattbrugg, Switzerland). Detection was carried out by silver staining with silver nitrate solution. SigmaMarker™ low range (6,500-66,000 Da) MW standard

(Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was run on each gel for estimation of molecular weights. Low molecular weight impurities and degradation products were detected. The intensity of bands of an apparent molecular weight smaller than the molecular weight of Qb coat protein monomer was compared to a Qb standard, applied at different concentrations to the same gel.

Osmolality

Osmolality measurements were conducted by using a Knauer Semimicro Osmometer Automatik (Knauer Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany). The osmolality of liquid formulations and reconstituted lyophilisates was determined via reduction of the freezing point of the solution in comparison to pure water.

Karl Fisher (KF) titration - determination of residual water content

Residual moisture contents of the lyophilizates were determined by coulometric Karl Fischer (KF) titration using an Aqua 40.00 titrator with a headspace module (Analytic Jena AG, Halle, Germany). In brief, samples were heated in 2R glass vials to 80 °C for at least 5 minutes. The vaporised water was transferred with nitrogen into the titration solution (Hydranal®-Coulomat AG, Sigma-Aldrich GmbH, Seelze, Germany), where its amount was determined.

X-Ray powder diffraction (XRD)

The morphology of lyophilizates was analyzed by XRD from 5-40 °2-θ, with steps of 0.05 °2-θ (2 s per step) on an XRD 3000TT X-ray diffractometer (Seiffert, Ahrenburg, Germany), equipped with a copper anode (40 kV, 30 mA, wavelength 154.17 pm).

Differential scanning calorimetry (DSC)

DSC is used to study the glass transition of the maximally freeze-concentrated solutions (Tg'), the glass transition (Tg) and / or melting temperature of lyophilizates, and the crystallization behavior of amorphous excipients [Nail et al., 2002; Hawe, 2006]. Samples were analyzed on a Differential Scanning Calorimeter 204

Phoenix (Netzsch-Geraetebau GmbH, Selb, Germany) in cold-sealed aluminum crucibles.

For the investigation of the Tg' of liquid samples heating and cooling rates of 10 °C / min were applied, in a range of -70 °C up to 20 °C. About 20 mg of the samples were analyzed. Tg' (point of inflection) of the compositions was determined during the heating scan.

For the investigation of dried samples 2-15 mg were used. Preparation was performed in a dry-nitrogen-purged glove box to prevent moisture uptake from the ambient atmosphere. In a first heating scan samples were heated from -20 to 90 °C at 10 °C / min, cooled to -20 °C at 10 °C / min and again heated in a second heating scan from -20 °C to 170 °C at 10 °C / min. Tg (point of inflection) of trehalose, melting temperature (peak) of mannitol and crystallization of amorphous mannitol were determined from the heating scans.

Light microscopy

Aliquots of 10 to 20 µL microcapsule formulations were pipetted on a glass object holder plate and were covered with a glass cover slide. The samples were assessed using a Nikon Labophot microscope (Nikon Instruments Inc., Melville, NY, USA), equipped with CFW 10x oculars and 4x, 10x, 20x and 40x objectives, respectively. Pictures were taken using a JVC TK-C1380 digital camera (JVC Deutschland GmbH, Friedberg, Deutschland). Microcapsule sizes were assessed by measuring the diameters of representative microcapsules by applying the calibrated JVC Digital Screen Measurement Comet software.

Scanning electron microscopy (SEM)

The morphology and structure of the freeze-dried microcapsules was analyzed with a Field Emission Scanning Electron Microscope Joel JSM-6500F (Joel Inc., Peabody, MA, USA). Therefore, microcapsules were first cut with a Mikrotom-Kryostat-HM500 microtome (MICROM Laborgeräte GmbH, Waldorf, Germany). Briefly, microcapsule samples were frozen on the sample holder pin of the microtome, cut, lyophilized and examined by SEM.

Samples were fixed with a double-sided adhesive carbon tape (Bal-Tec AG, Balzers, Liechtenstein) to a custom made brass stub. The fixed samples were sputtered with carbon (Bal-Tec AG, Balzers, Liechtenstein) under vacuum for approximately 2 minutes inside a MED020 sputtering device (Bal-Tec AG, Balzers, Liechtenstein). Subsequently, the sputtered samples were analyzed.

Confocal laser scanning microscopy (CLSM)

Aliquots of 10 to 20 µL of the FITC labeled VLP microcapsule formulations were pipetted on a glass object holder plate and were covered with a glass cover slide. The samples were assessed using a Zeiss LSM 510 Meta fluorescence confocal laser scanning microscope (Zeiss Microscope Systems, Jena, Germany) equipped with a C-Apochromat 40x / 1.2 W Korr objective. For excitation of the fluorophore the 488 nm line of an Ar laser was used. The CLSM pictures were analyzed with the Zeiss LSM Image Browser software (Zeiss Microscope Systems, Jena, Germany).

2.3 References

Ambuehl, P. M., Tissot, A. C., Fulurija, A., Maurer, P., Nussberger, J., Sabat, R., Nief, V., Schellekens, C., Sladko, K., Roubicek, K., Pfister, T., Rettenbacher, M., Volk, H. D., Wagner, F., Mueller, P., Jennings, G. T., and Bachmann, M. F.; A vaccine for hypertension based on virus-like particles: preclinical efficacy and phase I safety and immunogenicity; *Journal of Hypertension*, 2007, 25(1), 63-72

Bachmann, M. F., Rohrer, U. H., Kuendig, T. M., Buerki, K., Hengartner, H., and Zinkernagel, R. M.; The influence of antigen organization on B cell responsiveness; *Science*, 1993, 262(5138), 1448-1451

Brandau, D. T., Jones, L. S., Wiethoff, C. M., Rexroad, J., and Middaugh, C. R.; Thermal stability of vaccines; *Journal of Pharmaceutical Sciences*, 2003, 92(2), 218-231

Dunn, M. J.; Gel electrophoresis of peptides and proteins; *Drugs and the Pharmaceutical Sciences*, 2000, 101, 387-451

Fraunhofer, W., and Winter, G.; The use of asymmetrical flow field-flow fractionation in pharmaceutics and biopharmaceutics; *European Journal of Pharmaceutics and Biopharmaceutics*, 2004, 58(2), 369-383

Golmohammadi, R., Fridborg, K., Bundule, M., Valegaard, K., and Liljas, L.; The crystal structure of bacteriophage Q.beta. at 3.5 .ANG. resolution; *Structure (London)*, 1996, 4(5), 543-554

Hawe, A.; Studies on Stable Formulations for a Hydrophobic Cytokine, Dissertation, Ludwig-Maximilians-University Munich; 2006

Jegerlehner, A., Tissot, A., Lechner, F., Sebbel, P., Erdmann, I., Kundig, T., Bachi, T., Storni, T., Jennings, G., Pumpens, P., Renner, W. A., and Bachmann, M. F.; A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses; *Vaccine*, 2002, 20(25-26), 3104-3112

Kozlovska, T. M., Cielens, I., Dreilinna, D., Dislers, A., Baumanis, V., Ose, V., and Pumpens, P.; Recombinant RNA phage Q beta capsid particles synthesized and self-assembled in Escherichia coli; *Gene*, 1993, 137(1), 133-137

Lechner, F., Jegerlehner, A., Tissot, A. C., Maurer, P., Sebbel, P., Renner, W. A., Jennings, G. T., and Bachmann, M. F.; Virus-like particles as a modular system for novel vaccines; *Intervirology*, 2002, 45(4-6), 212-217

Leuenberger Hans; Physikalische Pharmazie; *Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart*, 2002

Maurer, P., and Bachmann, M. F.; Therapeutic vaccines for nicotine dependence; *Current Opinion in Molecular Therapeutics*, 2006, 8(1), 11-16

Maurer, P., Jennings, G. T., Willers, J., Rohner, F., Lindman, Y., Roubicek, K., Renner, W. A., Mueller, P., and Bachmann, M. F.; A therapeutic vaccine for nicotine dependence: Preclinical efficacy, and phase I safety and immunogenicity; *European Journal of Immunology*, 2005, 35(7), 2031-2040

Mutschler, E., Geisslinger, G., Kroemer, H. K., and Schäfer-Korting, M.; Arzneimittelwirkungen; *Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart*, 2001

Nail, S. L., Jiang, S., Chongprasert, S., and Knopp, S. A.; Fundamentals of freeze-drying; *Pharmaceutical Biotechnology*, 2002, 14, 281-360

Rodriguez-Diaz, R., Wehr, T., and Tuck, S.; Analytical Techniques for Biopharmaceutical Development; *Marcel Dekker,* 2005

Sanchez, A., Gupta, R. K., Alonso, M. J., Siber, G. R., and Langer, R.; Pulsed controlled-release system for potential use in vaccine delivery; *Journal of Pharmaceutical Sciences*, 1996, 85(6), 547-552

Shi, L., Sanyal, G., Ni, A., Luo, Z., Doshna, S., Wang, B., Graham, T. L., Wang, N., and Volkin, D. B.; Stabilization of human papillomavirus virus-like particles by non-ionic surfactants, *Journal of Pharmaceutical Sciences*, 2005, 94 (7), 1538-1551

Skoog Douglas A., and Leary James J.; Priniciples of Instrumental Analysis; *Saunders College Publishing*, 1992

Storni, T., Ruedl, C., Schwarz, K., Schwendener, R. A., Renner, W. A., and Bachmann, M. F.; Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects; *Journal of Immunology*, 2004, 172(3), 1777-1785

Wang, S., and Chen, W.; Analysis of plant peroxidase isoenzymes by lds-polyacrylamide gel electrophoresis (LDS-PAGE); *Zhiwu Shenglixue Tongxun*, 2006, 42(4), 713-716

Zillies, J.; Gelatin Nanoparticles for Targeted Oligonucleotide Delivery to Kupffer Cells - Analytics, Formulation Development, Practical Application; Dissertation, Ludwig-Maximilians-University Munich; 2007

3. AF4 AS NEW ANALYTICAL TOOL FOR THE INVESTIGATION OF THE PHYSICAL STABILITY OF VLP

3.1 Introduction

For the development of virus-like particle (VLP) based pharmaceutical products the application of reliable analytical tools is of great importance. For quality control purposes the methods need to be sufficiently sensitive to detect and quantify even small varieties between different API bulk materials, and varying formulations upon manufacture and storage. For the assessment of physical properties of viruses and virus-like particles three main techniques are established: (1) Transmission electron microscopy (TEM) [Vogt et al., 1999; Casini et al., 2004; Hansen et al., 2005], (2) Dynamic light scattering (DLS) often referred to as photon correlation spectroscopy (PCS) or quasi-elastic light scattering (QELS) [Santos et al., 1996; Brandau et al., 2003; Shi et al., 2005] and (3) Size exclusion chromatography (SE-HPLC) [Zlotnick et al., 2000; Rueda et al., 2000; Schmidt et al., 2000]. The utilization of TEM may be ascribed to the high resolution enabling accurate particle analysis. Inherent drawbacks of this technique are random sampling – instead of an overall sample analysis – and time consuming preparation and measurement procedures. DLS is described as a powerful, fast, non-destructive method for the determination of the average hydrodynamic radii, and size distributions of particles in their natural liquid environment. Thereby, increasing average sizes and polydispersity indices indicate aggregation of the particles [Griffin et al., 1993]. However, the size resolution is rather low and the obtained size distribution is rather inaccurate; the particles must differ in radius by about twofold to be resolved. Furthermore, the accurate quantification of different particle fractions is not feasible [Stock et al., 1985; Jiskoot et al., 2005]. SE-HPLC represents the gold standard for the analysis of the physical stability of proteins and is also used for the characterization of large biomolecules like virus-like particles. The drawbacks of SE-HPLC are its limited resolution capacity of especially high molecular weight molecules and the fact that only the analysis of soluble aggregates is possible [Litzen et al., 1993]. Consequently, for formulation development studies there is a strong need for a more sensitive

analytical tool that allows separation, characterization and quantification of different VLP fractions.

Field-flow fractionation (FFF) was introduced in 1966 as a new versatile technique for the separation and characterization of high-molecular-weight molecules and particles [Giddings, 1966]. Since then various FFF techniques like sedimentation FFF, thermal FFF, electrical FFF, flow FFF and asymmetrical flow FFF (AF4) have been developed as reviewed recently by Fraunhofer et al. [Fraunhofer et al., 2004] and Reschiglian et al. [Reschiglian et al., 2005]. Among these, AF4 is described as the most versatile and widely used. The theory and basic mechanisms of AF4 are discussed in detail elsewhere [Wahlund et al., 1987; Schimpf, 2000; Fraunhofer et al., 2004; Kowalkowski et al., 2006] and are thus only summarized in brief. In Figure 3.1.1 the principle of separation is illustrated.

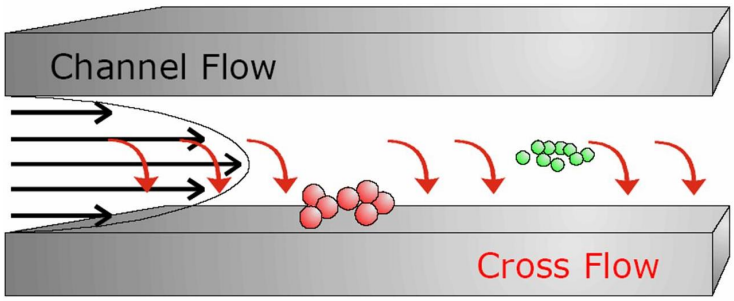


Figure 3.1.1: Separation principle of AF4.

A sample is injected into the hollow channel, which is at the downside ("accumulation wall") limited by an ultrafiltration membrane with a certain MWCO (open for solvent, but not for species to be analyzed). After a focusing step, samples are eluted by a parabolic channel flow. At the same time, a cross-flow perpendicular to the carrier flow is applied which "pushes" the dispersed particles in the direction of the accumulation wall, where the cross-flow exits the channel via the membrane. Thereby, analytes are partitioned into regions with different flow velocities in dependence of their diffusion properties. In essence, the larger a particle, the smaller its diffusion coefficient acting in opposite direction than the convection from the cross-flow. Thus, the larger a particle, the stronger it is influenced by the cross-flow and the longer it is retained in the channel. As a consequence, smaller particles elute faster than larger ones. Due to the open architecture of the separation channel

particles in the size range from several nanometers up to a few microns are accessible for separation by field-flow fractionation [Giddings, 1993].

During the last decades AF4 has gained more and more attention and was successfully applied for example for the analysis of the size and size distribution of monoclonal antibodies [Litzen et al., 1993], liposomes [Moon et al., 1993], lipid / DNA complexes [Lee et al., 2001], nanoparticles [Jores et al., 2004; Zillies, 2007], and viruses [Thielking et al., 1998]. Additionally, by coupling this technique with a multiangle laser light-scattering detector (MALLS) it became possible to obtain the molecular weight distributions of the fractionated species [Thielking et al., 1998; Wyatt, 1998; Fraunhofer et al., 2004].

In this context, it was the objective of the present work to develop an AF4 method as alternative tool for the analysis of VLP formulations and to investigate whether AF4 can provide a better insight into VLP compositions than DLS and SE-HPLC.

3.2 Results and Discussion

The VLP NicQb was used for the development of the AF4 method on an Eclipse2 AF4 system (Wyatt Technology Europe GmbH, Dernbach, Germany) equipped with the “standard channel (25 cm)” (see chapter 2). The separation efficiency of an AF4 method can be affected by manifold parameters like channel height, membrane material and molecular weight cut-off, eluent composition, focusing time, channel flow and cross-flow profile, and applied drug amount [Schimpf, 2000]. In preliminary experiments the following parameters were determined as fixed:

a) Spacer – 350 µm
b) Eluent – 20 mM sodium phosphate buffer (pH 7.0) with 150 mM sodium chloride
c) Ultrafiltration membrane molecular weight cut-off – 10 kDa
d) Channel flow – 1.5 mL / min
e) VLP amount – 20 µg

3.2.1 Selection of Membrane Material

First, the impact of the membrane material on the separation of VLP was analyzed. The proper selection of the membrane material is important because during intensive focusing and during separation at high initial cross-flow rates the analyte is accumulated at the membrane, and thus adsorption phenomena can occur [Schimpf, 2000]. In this context regenerated cellulose, cellulose triacetate and polyethersulfone membranes were assessed. The cross-flow was kept at 2.0 mL / min for 30 minutes and was subsequently reduced to 0 mL / min within 10 minutes. The fractograms, depicted in Figure 3.2.1, revealed that regenerated cellulose was the most suitable membrane material. The fractionation of apparently diverse VLP fractions was feasible and the recovery of NicQb, determined from the UV signal, was 96 %. Thus, it was assumed that almost no material was adsorbed onto the membrane. This might be related to the repulsion of the negatively charged NicQb (compare chapter 4) by the negatively charged cellulose residues. However, cellulose acetate, which is even more negatively charged, seemed not to be useful, as the

resolution of the distinctive peaks was worse and the recovery was clearly reduced (79 %) in comparison to regenerated cellulose. By using polyethersulfone two distinct VLP peaks with high resolution were obtained but a significant amount of NicQb was adsorbed to the membrane, the recovery was approximately 30 %. Hence, regenerated cellulose was determined as optimal membrane material.

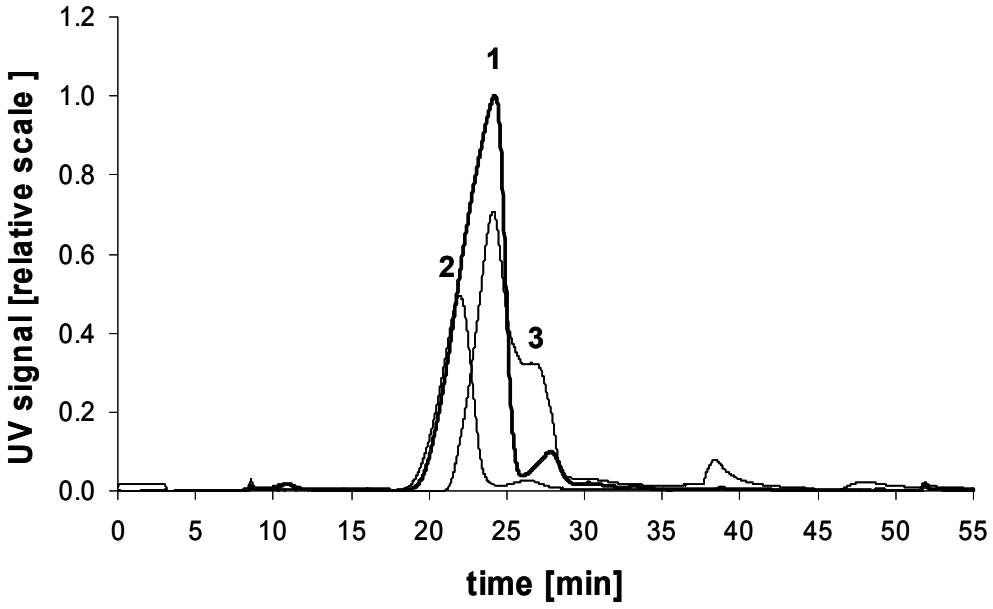


Figure 3.2.1 Effect of membrane material on the separation of VLP: regenerated cellulose (1), polyethersulfone (2), and cellulose triacetate (3); all 10 kDa cut-off.

3.2.2 Determination of Cross-Flow Profile

The initially applied cross-flow of 2 mL / min at a channel flow of 1.5 ml / min exhibited good separation properties for NicQb. However, in the next step the effect of various cross-flows in the range of 0.5 up to 3.0 mL / min on the separation of NicQb, the peak symmetry and width was investigated. The resulting fractograms are displayed in Figure 3.2.2. It was demonstrated that increasing cross-flows led on the one hand to improved separation of apparently different VLP fractions but on the other hand to prolonged separation time and broadening of the specific peaks. The recovery of NicQb was higher than 95 % for all samples indicating that adsorption of NicQb to the membrane was not an issue. Finally, a cross-flow of 2.0 mL / min was chosen taking into account separation capacity and the symmetry of the particular peaks.

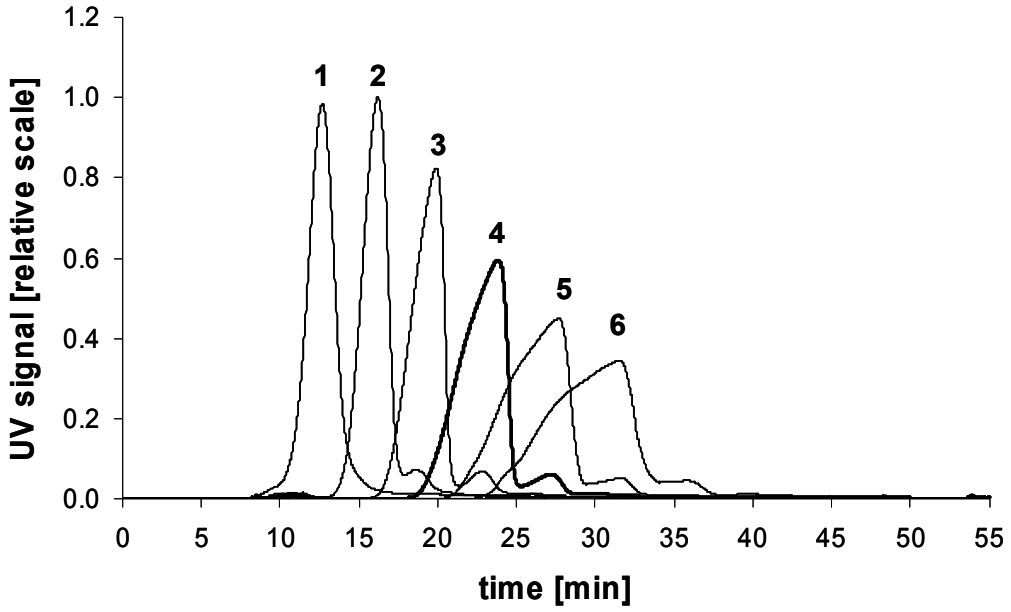


Figure 3.2.2 Effect of the varying cross-flow profiles on the separation of VLP: 0.5 mL / min (1), 1.0 mL / min (2), 1.5 mL / min (3), 2.0 mL / min (4), 2.5 mL / min (5), and 3.0 mL / min (6)

The main VLP peak was detected at about 23 minutes elution time. In order to further optimize the separation and to improve the elution of the "larger" VLP fractions the cross-flow profile was adjusted as described in the following. The cross-flow was reduced right after the elution of the main peak (26 min) from 2.0 mL / min to 0.15 mL / min within 15 minutes and kept at this low flow for further 5 minutes to enable the consecutive elution of larger VLP species. Subsequently, in a final step the cross-flow was completely deactivated to enable the elution of high molecular weight aggregates. The final cross-flow profile is illustrated in Figure 3.2.3.

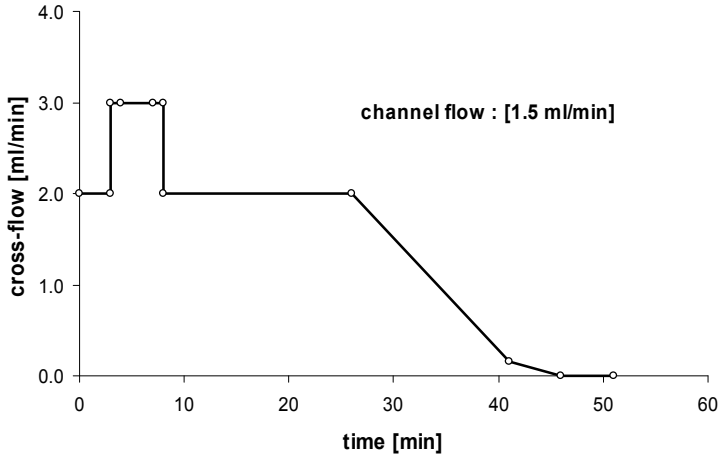


Figure 3.2.3 AF4 cross-flow profile method A.

In Figure 3.2.4 the resulting fractogram with the UV signal and the calculated molar masses of the specific VLP species is displayed. It could be shown that the applied AF4 separation protocol was capable to resolve VLP compositions in VLP fragments, monomers, dimers, and oligomers / aggregates. Furthermore, by coupling the AF4 to UV and MALLS detectors, quantification and determination of the MW of the specific fractions was possible. For VLP monomers a MW about 3.5 MDa was determined. As the recombinantly produced VLPs have a lower amount of host cell RNA (about 25 %) [Bachmann et al., 2006] as compared to the native bacteriophage Qb (50 % RNA content) the data obtained fit well with the data from literature appointing the MW of the native phage to 4.2 MDa [Hohn et al., 1970]. Furthermore, the MW of the VLP dimers and trimers could be assigned to 6.3 MDa and 9.8 MDa, respectively (Table 3.2.1). Due to the high sensitivity of MALLS on high molecular weight analytes, molar masses for higher-order oligomers and high molecular weight aggregates could be calculated up to values > 10^8 Da. The presence of VLP monomers, dimers and trimers was confirmed by TEM analysis (Figure 3.2.5).

Table 3.2.1 Molecular weights of specific NicQb fractions calculated from respective UV and MALLS signals (n = 3).

	VLP monomer	VLP dimer	VLP trimer
Molecular weight [MDa]	3.27 ± 0.02	6.27 ± 0.18	9.80 ± 0.82

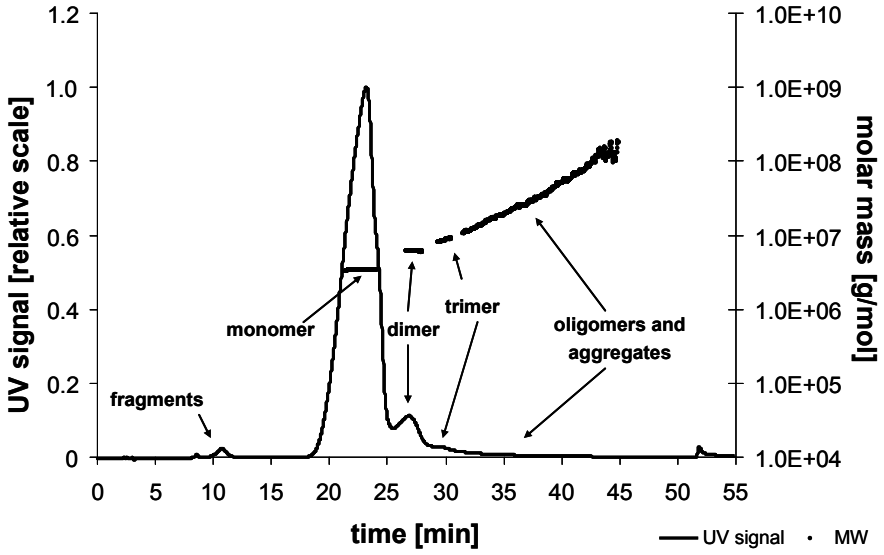


Figure 3.2.4 UV signal and molecular weights of specific NicQb fractions calculated from respective UV and MALLS data.

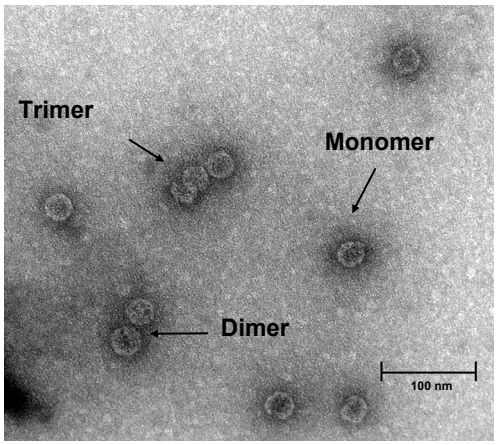


Figure 3.2.5 TEM picture of NicQb.

3.2.3 Repeatability

The intended use of AF4 as a reliable tool for the quality control of VLP formulations demands high repeatability of the method. It was therefore of interest to study the repeatability of the determination of the relative amount of the different VLP species, expressed as the percentage of the total peak areas. A NicQb bulk (20 µg / injection) was, according to the instructions of the ICH guideline Q2 (R1), subjected to six independent measurements by AF4. The results, presented in Table 3.2.2., prove the repeatability of the applied AF4 method. Even for the VLP fractions present at low amounts (fragments and oligomers / aggregates) a relative standard deviation of less than 7 % was determined.

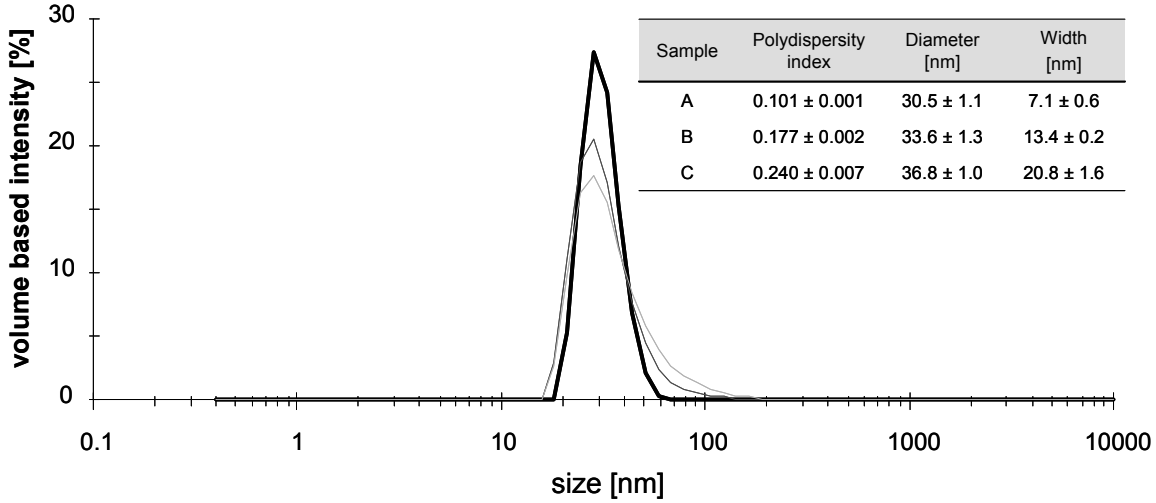
Table 3.2.2 Repeatability of the AF4 analysis of NicQb (20 µg / injection).

Injection	Fragments	Monomer	Dimer	Oligomers / Aggregates
	Relative peak areas [%]			
1	0.76	89.20	6.27	3.77
2	0.82	89.25	6.05	3.88
3	0.80	89.86	5.58	3.76
4	0.82	90.00	5.66	3.52
5	0.73	89.64	5.86	3.77
6	0.80	89.49	6.47	3.24
Average	0.79 ± 0.03	89.57 ± 0.32	5.98 ± 0.35	3.66 ± 0.23
SD_{rel} [%]	4.40	0.36	5.80	6.40

3.2.4 Comparison of AF4 to DLS and SE-HPLC

After successful development of an AF4 method for VLP it was essential to compare its efficiency to the commonly used DLS and SE-HPLC techniques. For a better illustration of the differences of these three techniques consciously a formulation which was due to its excipient composition unsuitable for the stabilization of NicQb during freeze-drying and storage was selected. In the following DLS, SE-HPLC and AF4 data obtained for the NicQb formulation A17 (compare chapter 4, page 103 to 105) prior to freeze-drying (A) and for dried samples stored for 6 weeks at 25 and 40 °C, respectively (B and C) are presented.

The DLS results are shown in Figure 3.2.6. For the unstressed NicQb sample A the mean size of the VLP was determined at 30 nm with a very narrow size distribution as indicated by the polydispersity index of 0.10 and the width of the peak. The stressed NicQb samples revealed for increasing storage temperatures increasing mean sizes of the VLP and increasing PIs and peak widths. PI values of particulate systems higher than 0.1 denote the presence of several species [Griffin et al., 1993], which was related in this case to the formation of VLP oligomers and aggregates. As expected, DLS was not capable to resolve the different VLP fractions.



Sample	Polydispersity index	Diameter [nm]	Width [nm]
A	0.101 ± 0.001	30.5 ± 1.1	7.1 ± 0.6
B	0.177 ± 0.002	33.6 ± 1.3	13.4 ± 0.2
C	0.240 ± 0.007	36.8 ± 1.0	20.8 ± 1.6

Figure 3.2.6 DLS size distribution by volume of NicQb sample A (black line), B (dark grey line), and C (light grey line).

The chromatograms obtained from the SE-HPLC analysis are depicted in Figure 3.2.7. It was obvious that the “stressed” samples B and C contained higher amounts of VLP oligomers and aggregates in comparison to the liquid formulation as for these samples a “shoulder” of the main peak appeared at a lower retention time.

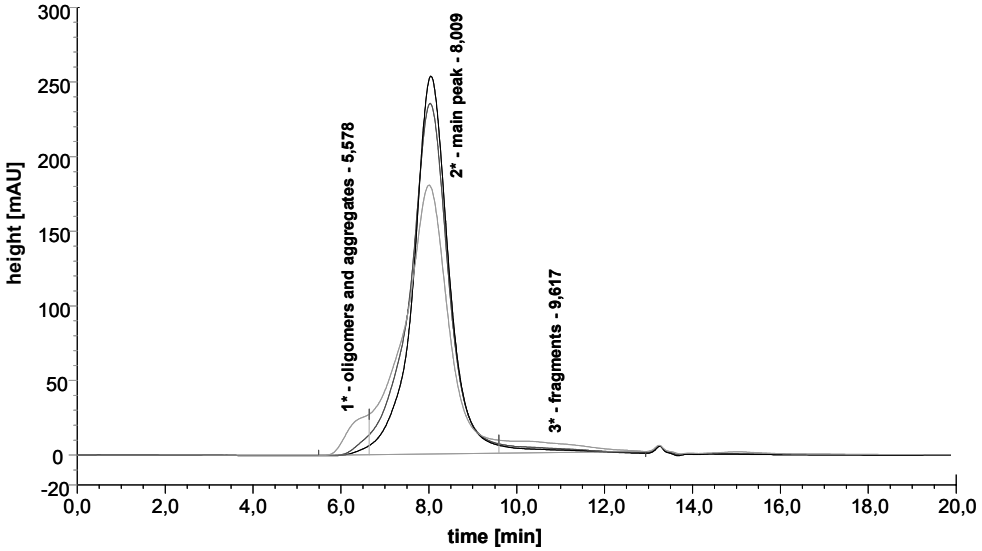


Figure 3.2.7 Characterization of NicQb samples A (black line), B (dark grey line), and C (light grey line) by means of SE-HPLC.

For a better understanding of the chromatogram SE-HPLC was coupled to a MALLS detector which enabled the determination of the molar mass distributions of the specific peaks (Figure 3.2.8).

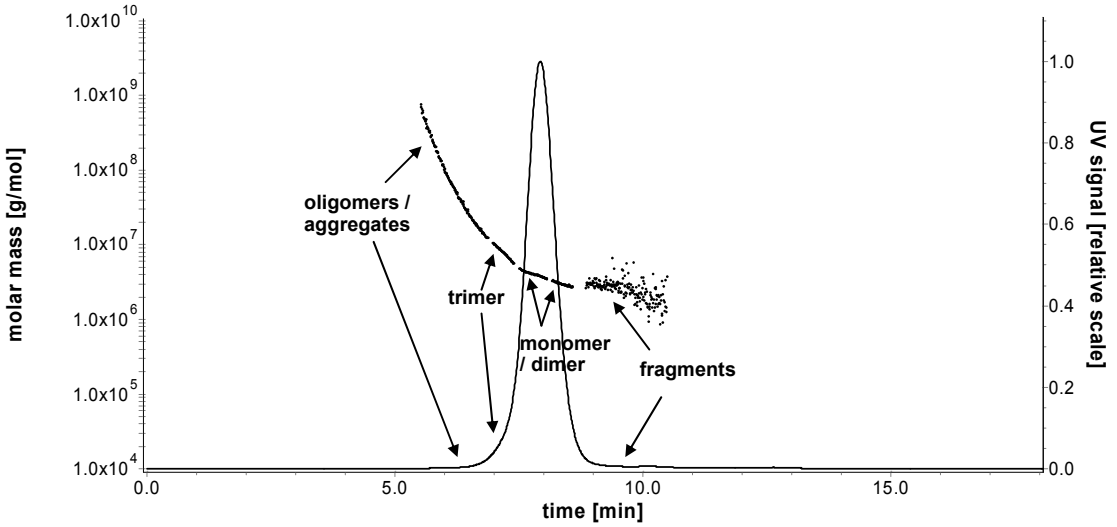


Figure 3.2.8 Interpretation of SE-HPLC analysis of NicQb by coupling SE-HPLC to MALLS.

The calculated molar masses indicated that the main peak not only consists of VLP monomer (~3.5 MDa) but additionally of VLP dimers (~7 MDa) and trimers (~10 MDa). The shoulder on the left side of the main peak comprises VLP oligomers and aggregates in a range of 10^7 up to 10^8 Da. The poor resolution of the different VLP fractions by SE-HPLC arises from the large size of the VLP which are already as monomers in the upper separation range of the used TSKgel G5000PW$_{XL}$ column (TSKgel PW Brochure*).

By contrast AF4 was capable of resolving the different VLP fractions into VLP fragments, monomer, dimers, trimers and larger oligomers and aggregates (Figure 3.2.9). The amounts of the specific VLP fractions could be easily determined. The relative amounts of the VLP species, expressed as percentage of the total peak areas are given in Table 3.2.3 and are compared to the results obtained by SE-HPLC.

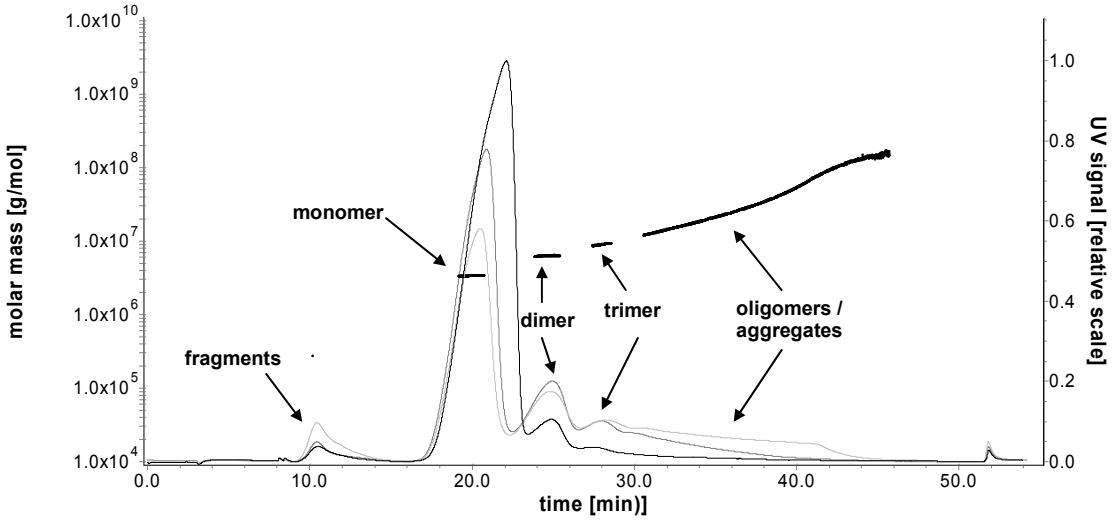


Figure 3.2.9 Characterization of NicQb samples A (black line), B (dark grey line), and C (light grey line) by AF4 coupled to MALLS.

* www. tosohbioscience.de

Table 3.2.3 Characterization of NicQb samples A, B and C via AF4 and SE-HPLC. As VLP monomers, dimers and trimers were not resolved by SE-HPLC they were summarized in the table.

	Sample	Fragments	Monomer	Dimer	Trimer	Oligomers / Aggregates
		Relative peak areas [%]				
AF4	A	1.37 ± 0.11	86.93 ± 0.16	5.98 ± 0.12	1.93 ± 0.03	3.79 ± 0.13
	B	2.92 ± 0.13	60.84 ± 0.27	16.25 ± 0.13	7.9 ± 0.04	12.09 ± 0.31
	C	6.11 ± 0.10	44.17 ± 0.80	16.16 ± 0.51	8.97 ± 0.42	24.60 ± 1.82
SE-HPLC	A	1.31 ± 0.08		98.54 ± 0.06		0.15 ± 0.02
	B	2.87 ± 0.03		95.49 ± 0.19		1.65 ± 0.22
	C	6.27 ± 0.14		88.09 ± 0.41		5.64 ± 0.27

Concerning the amount of VLP fragments both methods revealed the same values whereas the determined amounts of VLP oligomers (larger than VLP trimers) and aggregates varied significantly. SE-HPLC analysis revealed far lower amounts of VLP oligomers and aggregates which might be explained by: (1) Deprivation of insoluble high molecular weight aggregates from analysis due to the exclusion limit of the SE-HPLC column, (2) Inaccurate integration of the oligomer / aggregate peak due to the poor resolution, (3) Abrasion of VLP oligomers and aggregates due to the harsher conditions (high shear forces) connected with SE-HPLC in comparison to AF4 (no stationary phase), and / or (4) Generation of VLP aggregates during the focusing step of AF4 analysis. The deprivation of high molecular weight aggregates from SE-HPLC analysis could be excluded because no clear decrease of the total peak area could be observed. VLP aggregation induced during AF4 analysis seemed to be improbable due to the high reproducibility of the results obtained but further investigations in this context are necessary. Hence, it was assumed that either the shearing degradation by the stationary phase of VLP aggregates and / or the inaccurate integration of the VLP aggregate peak might have led to these different results.

It was found that AF4 exceeds by far the capabilities of DLS and SE-HPLC with regard to the analysis of the physical stability of VLP. AF4 can be used for the separation of VLP compositions and quantification of the single fractions, where DLS renders only trends. However, in terms of a fast progress in formulation development the utilization of DLS as a fast and easily applicable method seemed to be reasonable to delimitate initially large numbers of formulations to the most promising ones. SE-HPLC exhibited only a poor capability of analyzing the physical stability of VLP, but it was found that it is a reliable tool for the investigation of the chemical stability of VLP because of the proper determination of VLP fragments. However, it has to be mentioned that the currently used SE-HPLC method might be improved by using e.g. other column types, but a further optimization of the SE-HPLC method was beyond the scope of the present work. In summary it could be stated that AF4 is a very powerful tool for the accurate characterization of VLP formulations and overcomes the limitations of DLS and SE-HPLC.

3.2.5 Improvement of AF4 Method by Using New Channel Technology

In 2006 Wyatt Technology Europe GmbH introduced a new small AF4 separation channel (18 cm). It was described that, in comparison to the standard channel (25 cm), the application of the small channel improves resolution, enables shorter equilibration and analysis times, and reduces eluent volumes. At the same time increasing peak heights are obtainable which in turn might lead to lower detection limits, and thus, enable the reduction of the injected amount. Hence, it was investigated whether the before described AF4 method for VLP, developed for the standard AF4 channel (25 cm), could be optimized by using the new, shorter AF4 separation channel (18cm). For this purpose comparative AF4 measurements were performed. Qb VLP were analyzed either with the standard channel according to method A (Figure 3.2.3) or with the new channel by applying method B (Figure 3.2.10). VLP amounts in the range of 5 – 20 µg and 2.5 – 10 µg were applied for the standard and the shorter AF4 channel, respectively.

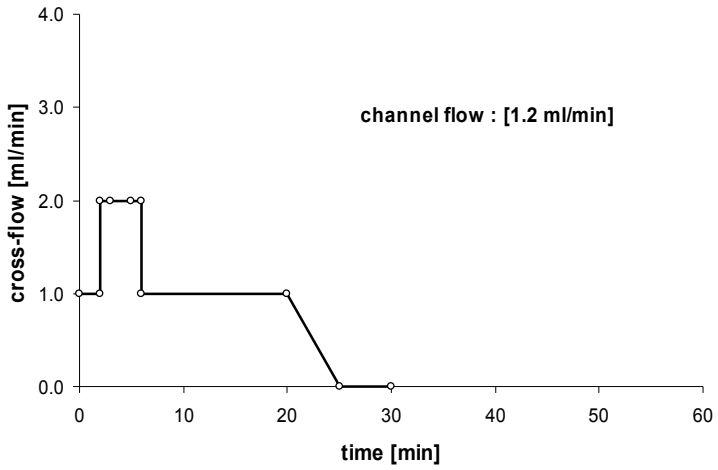


Figure 3.2.10 AF4 cross-flow profile method B.

The resulting AF4 fractograms are displayed in Figure 3.2.11. The figure illustrates the advantage of the small channel in comparison to the standard channel: at the same injected amounts sharper, higher peaks were obtained. Thus, the determination of the MW of the VLP monomer and dimer was feasible even at 2.5 µg injected VLP amount when using the small channel whereas for correct determination of the MW of the VLP dimer the injected amount needed to be higher than 10 µg when the standard channel was used.

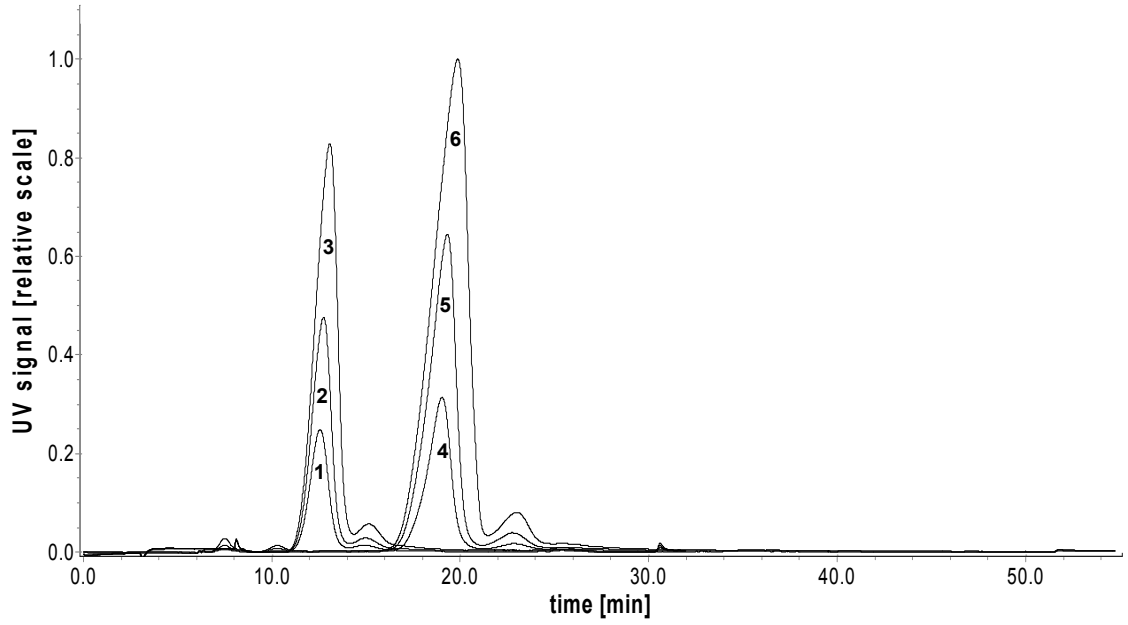


Figure 3.2.11 AF4 analysis of different amounts of NicQb by using Wyatt's new channel (18 cm) – 2.5 µg (1), 5 µg (2) and 10 µg (3) – in comparison to Wyatt's standard channel (25 cm) – 5 µg (4), 10 µg (5) and 20 µg (6).

Concerning the amounts of the different VLP species (Table 3.2.4) both methods revealed comparable values over the whole amount range tested. However, the lowest amount applied for the small channel (2.5 µg) was further reduced to one half of the lowest amount applied for the standard channel.

Table 3.2.4 AF4 analysis of NicQb by using Wyatt's standard channel (25 cm) in comparison to Wyatt's new channel (18 cm).

Channel	VLP amount [µg]	Fragments	Monomer	Dimer	Oligomers / Aggregates
		Relative peak areas [%]			
25 cm	20	0.80 ± 0.05	88.11 ± 0.03	6.56 ± 0.19	4.53 ± 0.18
	10	0.92 ± 0.03	87.67 ± 0.08	6.29 ± 0.19	5.12 ± 0.14
	5	0.84 ± 0.04	86.58 ± 1.01	6.84 ± 0.17	5.74 ± 0.80
18 cm	10	1.03 ± 0.15	87.42 ± 0.30	6.60 ± 0.51	4.95 ± 0.06
	5	1.02 ± 0.06	87.47 ± 0.30	6.47 ± 0.06	5.05 ± 0.18
	2.5	0.93 ± 0.06	87.65 ± 0.34	6.41 ± 0.01	5.01 ± 0.27

Furthermore, as indicated in Table 3.2.5 the analysis time and the eluent volume could be remarkably reduced by using Wyatt's small channel.

Table 3.2.5 Comparison between method A (standard channel) and B (small channel).

	Standard Channel 25 cm	Small Channel 18 cm
Time [min]	56	31
Eluent volume [mL]	159	70
Injection amount [µg]	10 - 20	2.5 - 10

Thus, it could be stated that the new channel technology was a clear improvement for VLP characterization as compared to the standard channel. It enables a clearly increased sample throughput and the accurate characterization of VLP compositions at remarkably low VLP concentrations.

3.3 Summary

The successful development of AF4 methods for the assessment of VLP compositions was presented. It was found that AF4 exceeds the separation capacity of DLS and SE-HPLC. AF4 enabled the proper separation of a VLP composition into VLP fragments, monomers, dimers, oligomers and aggregates. Furthermore, by coupling AF4 to UV and MALLS detectors accurate quantification of the specific fractions and the determination of the molecular weight distributions were feasible. Thus, AF4 seemed to be a valuable tool for the characterization of VLP compositions.

3.4 References

Bachmann, M., Maurer, P., Meijerink, E., Proba, K., and Schwarz, K.; Virus-like particle-antigen conjugates and their uses as vaccines; European Patent EP2006037787, 2006

Brandau, D. T., Jones, L. S., Wiethoff, C. M., Rexroad, J., and Middaugh, C. R.; Thermal stability of vaccines; *Journal of Pharmaceutical Sciences*, 2003, 92(2), 218-231

Casini, G. L., Graham, D., Heine, D., Garcea, R. L., and Wu, D. T.; In vitro papillomavirus capsid assembly analyzed by light scattering; *Virology*, 2004, 325(2), 320-327

Fraunhofer, W., and Winter, G.; The use of asymmetrical flow field-flow fractionation in pharmaceutics and biopharmaceutics; *European Journal of Pharmaceutics and Biopharmaceutics*, 2004, 58(2), 369-383

Fraunhofer, W., Winter, G., and Coester, C.; Asymmetrical Flow Field-Flow Fractionation and Multiangle Light Scattering for Analysis of Gelatin Nanoparticle Drug Carrier Systems; *Analytical Chemistry*, 2004, 76(7), 1909-1920

Giddings, C. J.; New separation concept based on a coupling of concentration and flow nonuniformities; *Separation Sciences*, 1966, 1(1), 123-125

Giddings, J. C.; Field-flow fractionation: analysis of macromolecular, colloidal, and particulate materials; *Science (Washington, DC, United States)*, 1993, 260(5113), 1456-1465

Griffin, W. G., and Griffin, M. C. A.; Time-dependent polydispersity of growing colloidal aggregates: Predictions from dynamic light scattering theory; *Journal of the Chemical Society, Faraday Transactions*, 1993, 89(15), 2879-2889

Hansen, R. K., Zhai, S., Skepper, J. N., Johnston, M. D., Alpar, H. O., and Slater, N. K. H.; Mechanisms of inactivation of HSV-2 during storage in frozen and lyophilized forms; *Biotechnology Progress*, 2005, 21(3), 911-917

Hohn, T., and Hohn, B.; Structure and assembly of simple RNA bacteriophages; *Advances in Virus Research*, 1970, 16, 43-98

Jiskoot, W., and Crommelin, J. A.; Methods for Structural Analysis of Protein Pharmaceuticals; *American Association of Pharmaceutical Scientists*, 2005

Jores, K., Mehnert, W., Drechsler, M., Bunjes, H., Johann, C., and Maeder, K.; Investigations on the structure of solid lipid nanoparticles (SLN) and oil-loaded solid lipid nanoparticles by photon correlation spectroscopy, field-flow fractionation and transmission electron microscopy; *Journal of Controlled Release*, 2004, 95(2), 217-227

Kowalkowski, T., Buszewski, B., Cantado, C., and Dondi, F.; Field-flow fractionation: theory, techniques, applications and the challenges; *Critical Reviews in Analytical Chemistry*, 2006, 36(2), 129-135

Lee, H., Williams, S. K. R., Allison, S. D., and Anchordoquy, T. J.; Analysis of self-assembled cationic lipid-DNA gene carrier complexes using flow field-flow fractionation and light scattering; *Analytical Chemistry*, 2001, 73(4), 837-843

Litzen, A., Walter, J. K., Krischollek, H., and Wahlund, K. G.; Separation and quantitation of monoclonal antibody aggregates by asymmetrical flow field-flow fractionation and comparison to gel permeation chromatography; *Analytical Biochemistry*, 1993, 212(2), 469-480

Moon, M. H., and Giddings, J. C.; Size distribution of liposomes by flow field-flow fractionation; *Journal of Pharmaceutical and Biomedical Analysis*, 1993, 11(10), 911-920

Reschiglian, P., Zattoni, A., Roda, B., Michelini, E., and Roda, A.; Field-flow fractionation and biotechnology; *Trends in Biotechnology*, 2005, 23(9), 475-483

Rueda, P., Fominaya, J., Langeveld, J. P. M., Bruschke, C., Vela, C., and Casal, J. I.; Effect of different baculovirus inactivation procedures on the integrity and immunogenicity of porcine parvovirus-like particles; *Vaccine*, 2000, 19(7-8), 726-734

Santos, N. C., and Castanho, M. A. R. B.; Teaching light scattering spectroscopy: the dimension and shape of tobacco mosaic virus; *Biophysical Journal*, 1996, 71(3), 1641-1650

Schimpf, M.; Field Flow Fractionation Handbook; *John Wiley & Sons, Inc. New York*, 2000

Schmidt, U., Rudolph, R., and Bohm, G.; Mechanism of assembly of recombinant murine polyomavirus-like particles; *Journal of Virology*, 2000, 74(4), 1658-1662

Shi, L., Sanyal, G., Ni, A., Luo, Z., Doshna, S., Wang, B., Graham, T. L., Wang, N., and Volkin, D. B.; Stabilization of human papillomavirus virus-like particles by non-ionic surfactants; *Journal of Pharmaceutical Sciences*, 2005, 94(7), 1538-1551

Stock, R. S., and Ray, W. H.; Interpretation of photon correlation spectroscopy data: a comparison of analysis methods; *Journal of Polymer Science, Polymer Physics Edition*, 1985, 23(7), 1393-1447

Thielking, H., and Kulicke, W. M.; Determination of the structural parameters of aqueous polymer solutions in the molecular, partially aggregated, and particulate states by means of FFFF/MALLS; *Journal of Microcolumn Separations*, 1998, 10(1), 51-56

Vogt, V. M., and Simon, M. N.; Mass determination of Rous sarcoma virus virions by scanning transmission electron microscopy; *Journal of Virology*, 1999, 73(8), 7050-7055

Wahlund, K. G., and Giddings, J. C.; Properties of an asymmetrical flow field-flow fractionation channel having one permeable wall; *Analytical Chemistry*, 1987, 59(9), 1332-1339

Wyatt, P. J.; Submicrometer particle sizing by multiangle light scattering following fractionation; *Journal of Colloid and Interface Science*, 1998, 197(1), 9-20

Zillies, J.; Gelatin Nanoparticles for Targeted Oligonucleotide Delivery to Kupffer Cells - Analytics, Formulation Development, Practical Application; Dissertation, Ludwig-Maximilians-University Munich, 2006

Zlotnick, A., Aldrich, R., Johnson, J. M., Ceres, P., and Young, M. J.; Mechanism of capsid assembly for an icosahedral plant virus; *Virology*, 2000, 277(2), 450-456

4 DEVELOPMENT OF A STABLE, FREEZE-DRIED FORMULATION FOR A VIRUS-LIKE PARTICLE BASED VACCINE AGAINST NICOTINE ADDICTION

4.1 Introduction

Tobacco misuse is the single leading preventable cause of death worldwide. The World Health Organization (WHO) estimates that there are 1.3 billion smokers worldwide and nearly 5 million tobacco-related deaths each year. Despite widespread knowledge of tobacco's dangerous health effects, smoking continues to pose a serious public health threat, as the number of smokers is increasing steadily[#]. According to the 2004 Surgeon General's Report* nearly 70 % of smokers want to stop smoking but less than 5 % who make a quitting attempt are successful. By 2006 smoking cessation products on the market included two types of medications: (1) Nicotine replacement therapy in the form of nicotine gums, inhalers, nasal sprays or transdermal patches, and (2) Treatment with the antidepressant bupropion, acting by attenuating withdrawal symptoms. However, clinical trials have shown that the long-term abstinence rates were only 6-10 % above placebo when using these medications[§]. Thus, there still is a high need for new therapies. In 2006 Chantix™ (Pfizer Inc.) with varenicline as a new chemical compound, acting by modulating receptor activity in the brain, was approved by the FDA. Even though varenicline exceeds commonly achieved abstinence rates, only 23 % of the smokers remained abstinent after 1 year [Maurer et al., 2007]. However, the sales of Chantix, 883 million dollars in 2007[§], clearly demonstrated the high economic value of new drugs for smoking cessation.

[#] http://www.wpro.who.int/media_centre/fact_sheets/fs_20060530.htm
* http://www.cdc.gov/tobacco/data_statistics/sgr/sgr_2004/index.htm
[&] http://www.nice.org.uk/Docref.asp?d=30634
[§] http://seekingalpha.com/article/61585-pfizer-s-chantix-poised-for-blockbuster-sales?source=feed

Vaccination against nicotine is an innovative approach followed by Nabi Pharmaceuticals (NicVAX), Celtic Pharma (TA-NIC), and Cytos Biotechnology AG (NicQb). Clinical trials with Pseudomonas aeruginosa exoprotein A (NicVAX), recombinant cholera toxin B (TA-NIC), and Qb VLP nicotine conjugates (NicQb) have been shown to induce strong nicotine-specific antibody responses leading to the prevention of relapses by sequestering nicotine in the blood from immunized smokers [Cerny, 2005; Maurer et al., 2006; Boyd, 2006; Maurer et al., 2007]. However, besides the proof of efficacy and safety of these vaccines the development of stable formulations is essential for their commercialization and might turn the scale in the nip-and-tuck race towards market entry. The aim of the present work was to develop stable formulations for the VLP based vaccine candidate NicQb from Cytos Biotechnology AG.

Many biopharmaceuticals, such as vaccines, proteins and peptides, are often not sufficiently stable in aqueous solutions to allow distribution and storage, particularly at room temperature. They are susceptible to chemical (hydrolysis of glycosilic and peptide bonds and linking sequences, oxidation, deamidation, disulfide exchange, racemisation, and beta elimination) and / or physical degradation (denaturation, aggregation, precipitation, and adsorption) in liquid formulations [Manning et al., 1989; Cleland et al., 1993; Brandau et al., 2003; Wang, 2005]. During shipping products can be subjected to further stresses that can lead to denaturation, e.g. agitation, exposure to high and / or low temperatures, and freezing [Arakawa et al., 2001]. Additionally, even if optimal formulation and shipping systems might be designed, damage during long-term storage may not be prevented sufficiently [Manning et al., 1989; Carpenter et al., 1997].

In order to achieve more robust formulations dried forms were often produced because in the dried state particularly chemical reactions are intended to be substantially retarded [Franks, 1998; Lai et al., 1999]. Common drying techniques are freeze-drying [Carpenter et al., 1997; Wang, 2000], vacuum-drying [Mattern et al., 1999; Sharma et al., 2004], spray-drying [Lee, 2002; Harris et al., 2004; Ameri et al., 2006] and spray-freeze-drying [Maa et al., 1999]. Among these freeze-drying is the most widely used technique for the preparation of biopharmaceuticals for parenteral administration. Currently 46 % of the marketed biopharmaceutical products are lyophilizates [Costantino et al., 2004]. It is a well established process to improve the

stability of particularly labile drugs such as proteins [Wang, 2000] and complex vehicles like virus vaccines [Scott et al., 1976; Adebayo et al., 1998; Sarkar et al., 2003; Zhai et al., 2004; Abdul-Fattah et al., 2007], viral vectors [Talsma et al., 1997; Evans et al., 2004; Cruz et al., 2006], liposomes [Engel et al., 1994; Zingel et al., 1996; Hinrichs et al., 2005] and lipid-DNA complexes [Allison et al., 2000; Molina et al., 2004].

A typical freeze-drying process consists of three main stages, i.e. freezing, primary drying and secondary drying. First the solution is frozen to a temperature below the critical temperature of the formulation and held for several hours at this temperature to allow complete solidification. The critical temperature of a formulation is the collapse temperature Tc, above which the intestinal water in the frozen matrix becomes significantly mobile, which in turn might lead to the loss of the macroscopic structure during freeze-drying. Tc has been considered to be about 2 °C higher than the Tg' of an amorphous system or to be equivalent to the eutectic temperature of a crystalline system. During primary drying ice is transferred from the product to the condenser by sublimation and crystallization onto the cold coils in the condenser. It starts when the chamber pressure is reduced to improve the rate of ice sublimation and the shelf temperature is raised to supply the heat removed by ice sublimation. The driving force is provided by the partial pressure difference of water at the subliming ice surface and at the condenser. Upon secondary drying the unfrozen, adsorbed water is removed from the product by desorption and subsequent condensation in order to reduce the residual moisture content to a level optimal for stability. Secondary drying is typically carried out at higher temperature so that desorption of water may occur at a practical rate [Pikal, 1994; Franks, 1998; Wang, 2000; Nail et al., 2002; Tang et al., 2004; Costantino et al., 2004].

However, during freeze-drying the drug is exposed to two distinct stresses which can cause significant damage to the drug, i.e. freezing and drying.

During freezing drug stability can be influenced by (1) Cold denaturation [Privalov, 1990] (2) Exposure to ice-water interfaces [Chang et al., 1996], (3) Salt and drug concentration effects [Pikal, 2004], (4) pH shifts due to selective crystallization of specific buffer species [Anchordoquy et al., 1996a; Sarciaux et al., 1999], (5) Mechanical damage by growing ice crystals [Zhai et al., 2004], and (6) Crystallization of protective excipients [Carpenter et al., 1989; Izutsu et al., 1993].

Upon drying the removal of stabilizing hydration shells can influence the stability of the drug [Prestrelski et al., 1993], In an aqueous solution proteins are fully hydrated. The removal of the hydration shell may disrupt the native state of a protein and cause denaturation and probably the loss of activity [Arakawa et al., 1991; Prestrelski et al., 1995].

Therefore, to ensure the stability of the drug during freeze-drying stabilizing excipients have to be employed [Wang, 2000; Rexroad et al., 2002; Carpenter et al., 2002; Tang et al., 2004].

Surfactants, like polysorbates, are added to prevent aggregation of the drug in the liquid formulation, during freezing and reconstitution. Surfactants drop the surface tension of formulations and might consequently reduce the driving force of protein adsorption and / or aggregation at ice-water interfaces formed during freezing [Chang et al., 1996; Jones et al., 2001; Shi et al., 2005].

Cryoprotectants like sugars (e.g. trehalose and sucrose), polymers (e.g. polyethylene glycol and human serum albumin), and amino acids are used to stabilize the active pharmaceutical ingredient in solution and upon freezing. They hinder protein molecules from unfolding by the preferential exclusion mechanism, which favours the native protein conformation exhibiting the minimum surface area [Arakawa et al., 1982; Arakawa et al., 1991; Carpenter et al., 1999].

Lyoprotectants, especially the disaccharides sucrose and trehalose, are utilized to stabilize the drug during drying [Wang, 2000]. The two main mechanisms proposed are the “vitrification“ and the “water-replacement” hypotheses. “Vitrification” ascribes to the formation of an amorphous glass during lyophilization leading to increased drug stability by slowing down conformational changes of biomolecules [Franks, 1994; Fox, 1995; Hancock et al., 1997; Crowe et al., 1998; Chang et al., 2005]. The “water replacement hypothesis” involves the formation of hydrogen bonds between the protein and excipients. The excipients serve as water substitutes and hinder protein unfolding and intra- or interprotein hydrogen bonding during dehydration [Carpenter et al., 1990; Arakawa et al., 1991; Crowe et al., 1993a; Crowe et al., 1993b; Allison et al., 1999].

Bulking agents are applied to achieve more elegant and stable cakes. In this role mannitol is often used because it crystallizes to a substantial degree during lyophilization and forms a mechanically strong cake. However, it is well-known that

crystalline excipients when used alone are not necessarily able to provide sufficient stability. Thus, in order to achieve elegant and stable products mixtures of mannitol with sucrose or trehalose are used [Johnson et al., 2002; Izutsu et al., 2002; Liao et al., 2005].

Upon long-term storage the stability of a labile biopharmaceutical can further be affected by several factors like storage temperature, glass transition temperature [Hancock et al., 1995], residual moisture content [Shalaev et al., 1996; Breen et al., 2001; Klibanov et al.i, 2004] and crystallization of amorphous excipients [Costantino et al., 1998; Lai et al., 1999].

In this chapter the development of stable NicQb lyophilizates meeting the requirements for large scale clinical study and commercial use is described. The influence of different pH values and various excipients like surfactants, polyols, sugars and salts on the stability of NicQb in liquid formulations, during freeze-thawing, freeze-drying, and finally upon storage of the dried product was investigated. Besides standard analytical tools like DLS, light obscuration, SE-HPLC, RP-HPLC and LDS-PAGE, the developed AF4 method described in chapter 3 is used for the analysis of VLP stability.

4.2 Results and Discussion

4.2.1 pH Stability Study - NicQb

The stability of vaccines in solutions is strongly influenced by solution conditions like pH, ionic strength, osmolality and the presence of excipients. Among these parameters formulation pH is described as one of the most critical variables concerning the chemical and physical stability of vaccines [Brandau et al., 2003]. As the drug has first to be formulated in a liquid prior to freeze-drying and has to be handled for several hours in the liquid state (especially in the context of large scale manufacture) the influence of the pH has to be properly investigated. Furthermore, the formulation pH can also affect the stability of dried products during long-term storage [Townsend et al., 1990; Costantino et al., 1994; Song et al., 2001].

Therefore, the stability of NicQb was investigated in dependence of the pH of a liquid formulation. NicQb solutions in a pH range of 4.6 up to 8.2 were manufactured by adjusting the pH of the bulk material either with 0.1 N NaOH or 0.1 N H_3PO_4 and subsequent dilution of the solutions to 1 mg / mL NicQb. The samples were stored at room temperature up to 14 days. After storage samples were analyzed via DLS, SE-HPLC and RP-HPLC at least in duplicate.

Chemical stability of NicQb

RP-HPLC analysis revealed that the content of free nicotine derivatives increased with increasing pH values (Figure 4.2.1). Up to a pH of 6.6 the amount of free nicotine derivatives was below 5 % even after storage for 14 days at ambient temperature. Between pH 6.6 and 8.2 the amount of cleaved nicotine increased markedly with increasing pH values upon storage. After 2 weeks, almost 50 % of total nicotine was cleaved at a pH of 8.2.

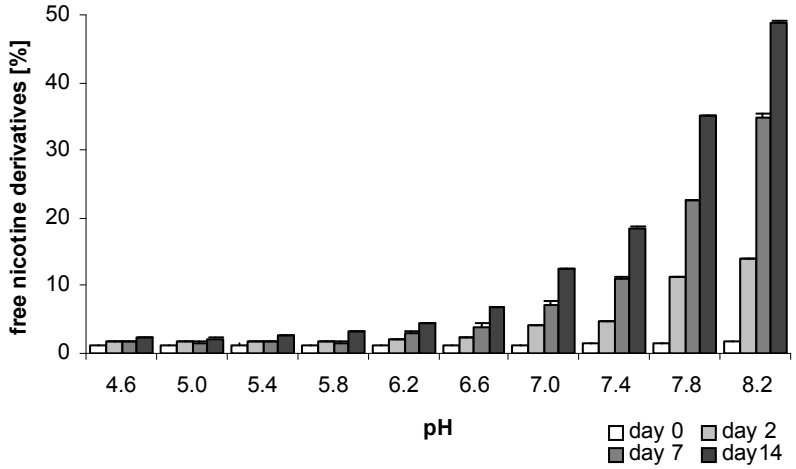


Figure 4.2.1 Amount of free nicotine derivatives as determined by RP-HPLC after manufacture and after 2, 7 and 14 days.

The data obtained from SE-HPLC analysis (Figure 4.2.2) revealed that with increasing pH values the amount of fragmentation products of NicQb increased clearly upon storage within 7 days at room temperature. Up to a pH of 7.0 the amount of fragmentation products increased only up to 2 % in comparison to the starting material whereas at higher pH values the amount of fragmentation products rose up to 15 % at a pH of 8.2.

Thus, addressing the stability of the esterbond between the VLP-linker and nicotine and the integrity of the VLP it could be stated that a pH value below 6.6 is desirable.

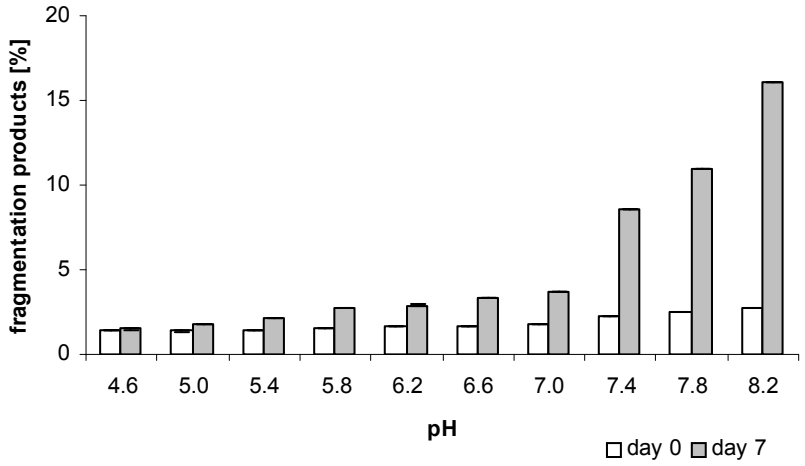


Figure 4.2.2 Amount of fragmentation products of NicQb as determined by SE-HPLC after manufacture and after 7 days.

Physical stability of NicQb

Concerning the physical stability of NicQb DLS measurements revealed that with increasing pH values the aggregation level of NicQb decreased (Figure 4.2.3). The polydispersity index (PI) decreased and the intensity of the main NicQb peak increased, indicating a decreasing polydisperse size distribution. Below a pH of 6.2 the intensity of the main NicQb peak was less than 95 %. At a pH of 4.6 not only a remarkable increase of the PI and decrease of the intensity of the main peak could be observed, but even a second peak at a size of several 100 nm appeared, proving the presence of VLP aggregates (Figure 4.2.4).

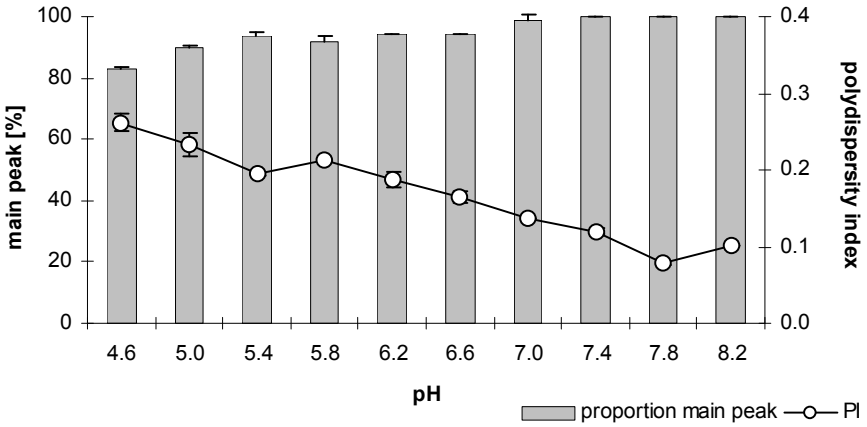


Figure 4.2.3 Proportion of main NicQb peak and PI as determined by DLS after 14 days storage at RT by applying the intensity conversion model.

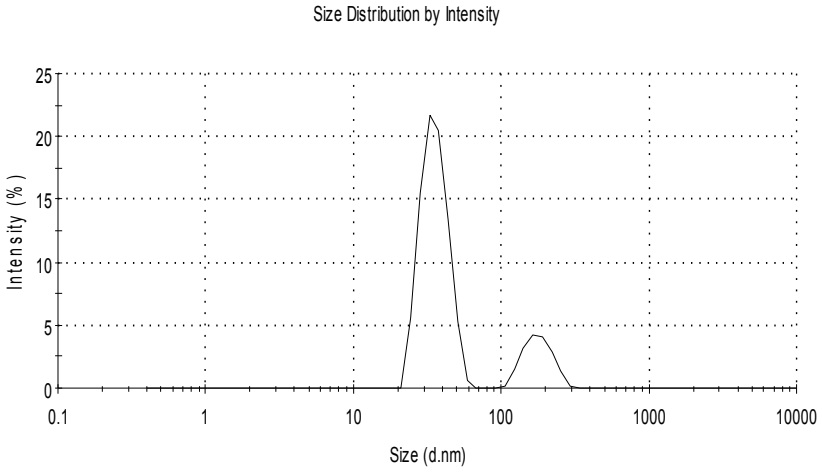


Figure 4.2.4 Size distribution as determined by DLS for NicQb sample at a pH of 4.6 stored for 14 days at RT.

Aggregation of dispersed particles can be influenced by the electrostatic properties of a formulation, like pH and salt concentration. If the zeta potential of charged particles such as protein complexes are near zero, particle aggregation can occur whereas at significant positive and negative zeta potential values particles might be stabilized by repulsive electrostatic forces [Jiskoot et al., 2005]. In order to find out whether the increasing amount of aggregated species of NicQb with decreasing pH values could be related to low absolute zeta potential values of the particles, NicQb solutions in a pH range of 3.4 to 7.2 were analyzed with the Malvern Zetasizer Nano ZS. The results are depicted in Figure 4.2.5.

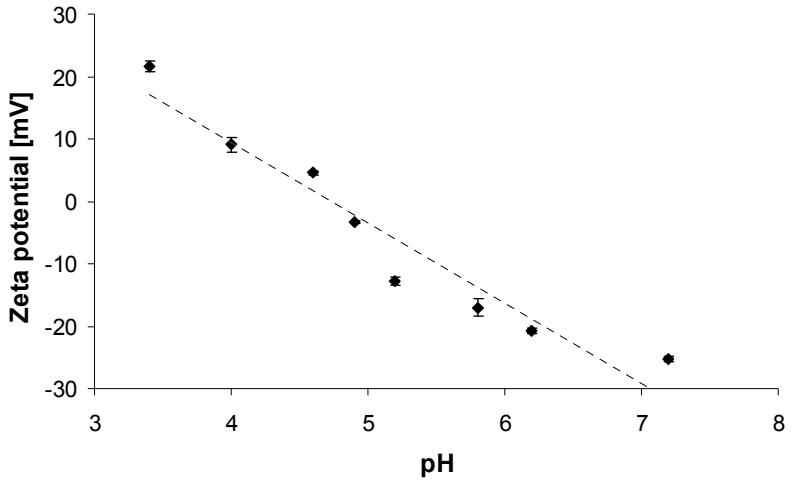


Figure 4.2.5 Zeta potential of NicQb in dependence of the pH.

It was found that the zeta potential of NicQb was 0 mV at a pH of 4.7, which marks the isoelectric point, and that in the pH range of 3 to 6 the absolute zeta potential values were below 20 mV which, as described above, can lead to particle aggregation.

Hence, the DLS and zeta potential measurements revealed that with respect to aggregation of NicQb the pH of the formulation should be higher than 6.2.

Summary

In summary two divergent results were observed. Concerning the chemical stability of NicQb pH values below 6.6 were favourable whereas with respect to the physical stability the pH of the solution should be higher than 6.2. Therefore, a compromise between the hydrolysis of the esterbond between VLP-linker and nicotine

and the degradation of the VLP on the one hand, and aggregation of the VLP at low pH values on the other hand had to be made. A pH range from 6.2 up to 6.6 appeared to be optimal to assure both chemical and physical stability of NicQb in a liquid formulation.

4.2.2 Freeze-Thaw (FT) Studies - NicQb

A freeze-drying process consists of two major steps: freezing of the drug solution, and drying of the frozen solid under vacuum. Each step generates different kinds of stresses, and depending on the specific properties of a drug its stability can be influenced by either one and / or both steps [Wang, 2000; Rexroad et al., 2002]. Therefore, the impact of each single step on the stability of NicQb and the effect of stabilizing excipients had to be scrutinized. Freeze-thawing is routinely used as a tool to determine the effects of various stresses connected to freezing on the stability of a drug [Jiang et al., 1998; Anchordoquy et al., 2001].

A wide variety of excipients have been employed to prevent freezing-induced denaturation, including surfactants, disaccharides and polymers. For example, polysorbates were described as potent cryoprotectants for viruses [Evans et al., 2004], virus-like particles [Shi et al., 2005] and proteins [Chang et al., 1996; Kreilgaard et al., 1998; Sarciaux et al., 1999; Jones et al., 2001]. The efficiency of trehalose as efficient cryo- and lyoprotectant was shown for several virus vaccines [Gupta et al., 1996; Worrall et al., 2001; Sarkar et al., 2003].

Thus, in order to study the effect of the freezing step on the stability of NicQb and the particular influence of the clinically approved excipients polysorbate 20 and trehalose on its stability during freeze-thawing was investigated. Additionally, the effect of sodium chloride, which is often used to adjust the tonicity of liquid formulations [Costantino et al., 2004] and is described to be able in some cases to stabilize biopharmaceuticals [Liu et al., 1991; Schwendeman et al., 1995], was analyzed.

1 mL of NicQb solutions with a drug concentration of 0.2 mg / mL, with 30, 60, 90 or 150 mM sodium chloride, 10 % trehalose, with or without addition of 0.005 % polysorbate 20 were filled into 1.5 mL Eppendorf Tubes (Eppendorf AG, Hamburg, Germany). Additionally, samples without trehalose, 0.005 % polysorbate 20 and 30 mM NaCl were prepared. With respect to the findings from the pH stability study

the samples were buffered with 20 mM sodium phosphate salts at a pH of 6.4. The samples were frozen by placing the tubes in a refrigerator at -80 °C. After a minimum of 3 hours the samples were thawed at ambient temperature. Freeze-thaw cycles were repeated 5 times. As aggregation of protein therapeutics induced by denaturation at ice-water interfaces is described as the major degradation pathway during freezing [Chang et al., 1996; Jones et al., 2001], the focus of this experiment was laid on the physical stability of NicQb. Thus, the samples were analyzed before and after freeze-thaw cycling via DLS and light obscuration at least in duplicate.

Effect of trehalose and polysorbate 20 on the stability of NicQb

In order to investigate the single effect of polysorbate 20 and trehalose on the stability of NicQb formulations with or without either one or both excipients was assessed after freeze-thawing. The results obtained by DLS and light obscuration are displayed in Figure 4.2.6 and Figure 4.2.7. It was found that trehalose had no clear stabilizing effect on NicQb during freeze-thawing whereas polysorbate 20 had a significant protective effect. For the formulations with polysorbate 20 neither a clear increase of the number of particles > 1 µm nor a clear increase of the polydispersity index and decrease of the main NicQb peak was detected. The protective effect of polysorbate 20 can be explained by either binding to hydrophobic regions of the drug thereby preventing interaction with hydrophobic surfaces [Bam et al., 1995] or by competing with the therapeutic for adsorption at liquid-surface interfaces [Shi et al., 2005].

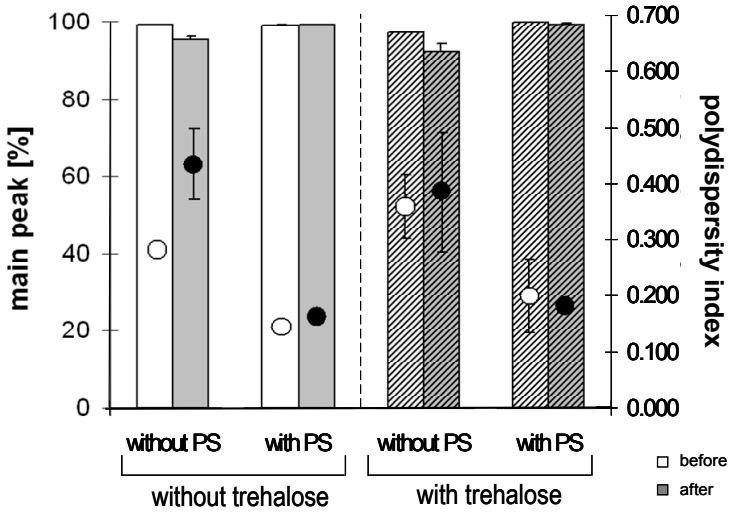


Figure 4.2.6 Polydispersity indices and proportions of main NicQb peak in dependence of trehalose (0 vs. 10 %) and polysorbate (0 vs. 0.005 %) addition before freeze-thawing and after 5 freeze-thaw cycles.

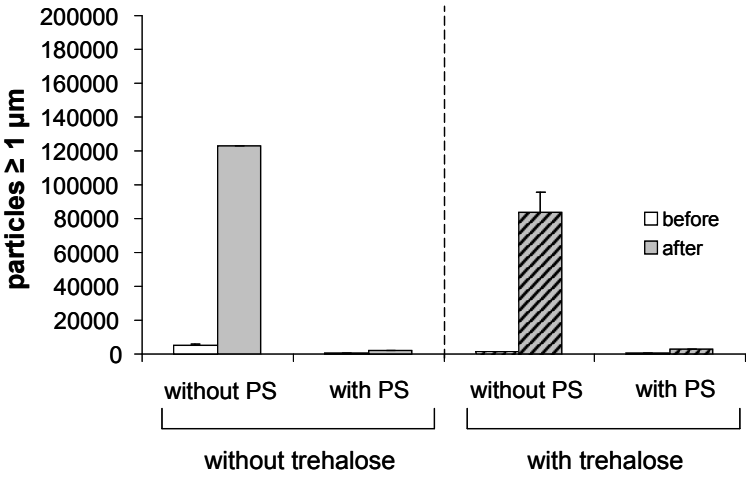


Figure 4.2.7 Number of particles > 1 µm in dependence of trehalose (0 vs. 10 %) and polysorbate (0 vs. 0.005 %) addition before freeze-thawing and after 5 freeze-thaw cycles.

Effect of sodium chloride and polysorbate 20 on the stability of NicQb

DLS data revealed that with increasing concentrations of sodium chloride in NicQb formulations without polysorbate 20 the proportion of the main NicQb peak decreased, indicating an increase of the aggregation level. Furthermore, the PI of these formulations was higher than 0.4, referring to a polymodal size distribution (Figure 4.2.8). Such a destabilizing effect of sodium chloride at higher salt concentrations on proteins and other biopharmaceuticals like for example hepatitis A virus has been reported in several cases [Volkin et al., 1996; Chi et al., 2003].

A proposed mechanism is the shielding of repulsive forces between similarly charged groups by sodium chloride leading to an increase in drug aggregation [Brange et al., 1997].

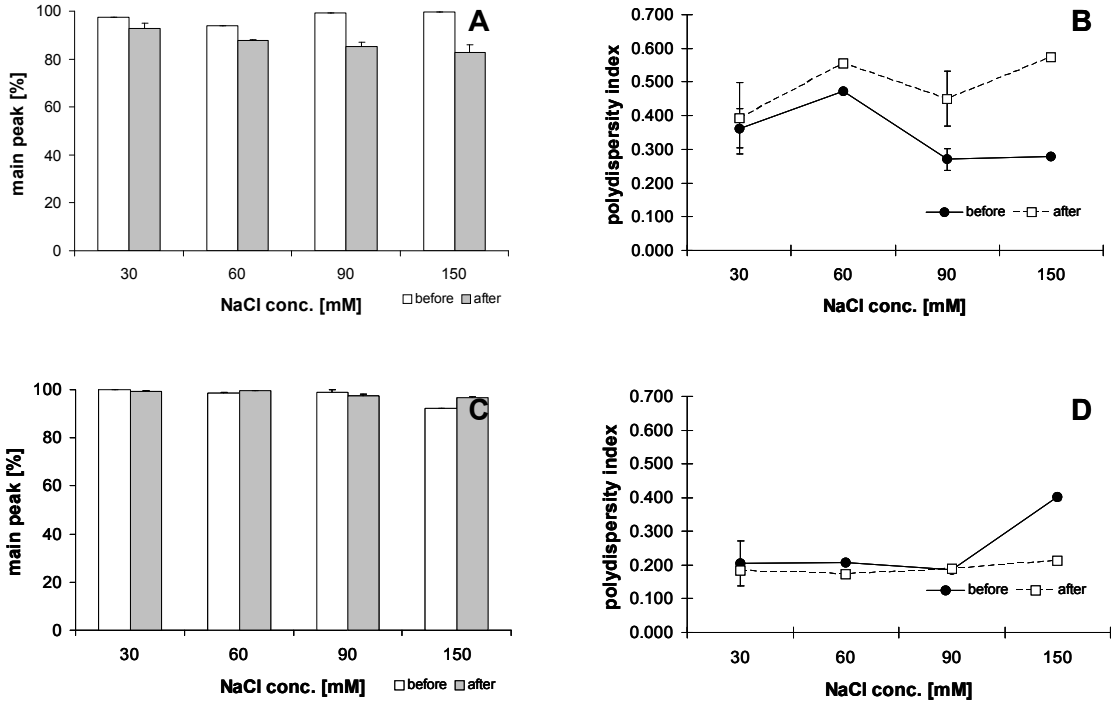


Figure 4.2.8 Polydispersity indices and proportions of main NicQb peak in dependence of sodium chloride concentration (30, 60, 90 and 150 mM), formulated without (A / B) or with (C / D) polysorbate 20 (0.005 %) before freeze-thawing and after 5 freeze-thaw cycles; samples contained 10 % trehalose.

In contrast, in the presence of polysorbate 20 aggregation of NicQb could be prevented, even at high sodium chloride concentrations. However, the best results rendered the NicQb formulation with the lowest amount of sodium chloride (30 mM), 0.005 % polysorbate 20 and 10 % trehalose with 99.9 % proportion of the main NicQb peak and a PI of 0.20 (Figure 4.2.8).

The findings of the DLS measurements were confirmed by data obtained by light obscuration. As illustrated in Figure 4.2.9 aggregation of NicQb upon freeze-thawing, indicated by increasing numbers of particles ≥ 1 µm, could be prevented by polysorbate 20 together with trehalose. However, the clear trend observed by DLS that increasing amounts of sodium chloride could be correlated to increasing aggregation levels was not supported by light obscuration measurements as the number of particles > 1 µm did not increase steadily.

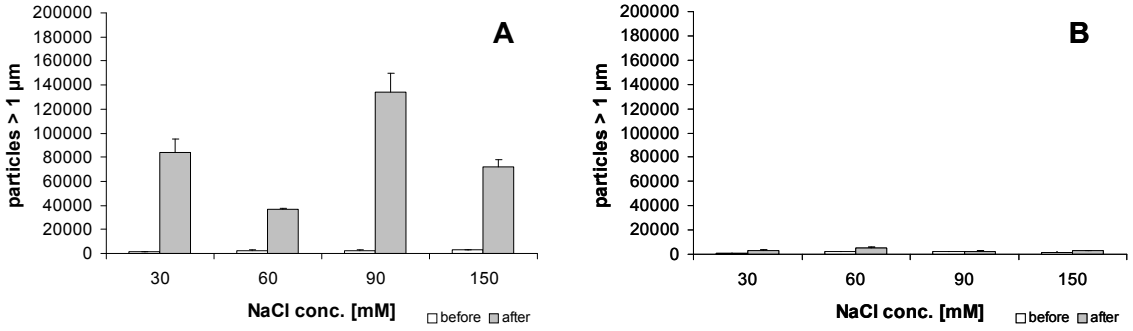


Figure 4.2.9 Number of particles > 1 µm in dependence of sodium chloride concentration (30, 60, 90 and 150 mM), formulated without (A) or with (B) polysorbate 20 (0.005 %) before freeze-thawing and after 5 freeze-thaw cycles; samples contained 10 % trehalose.

Summary

It can be stated that the addition of polysorbate 20 was very beneficial to prevent aggregation of NicQb during freeze-thawing and that the addition of high concentrations of sodium chloride should be avoided. Even though it was found that trehalose had no protective effect during freeze-thawing it might serve as lyoprotectant during the drying step.

4.2.3 Freeze-Drying (FD) Studies - NicQb

The effect of the freezing step on the stability of NicQb was investigated during freeze-thaw experiments. Now, the stability of NicQb throughout the whole FD process depending on formulation composition and process parameters had to be investigated. The focus was laid on the development of formulations which meet the following criteria: (1) The drug should be stable during manufacture and storage, ideally even at ambient temperature; (2) It should be composed of FDA approved excipients; (3) It should have an acceptable and reproducible appearance; and (4) Ideally the lyophilizate should be reconstitutable with water to a parenteral applicable isotonic solution. Concerning the final freeze-drying process the aim was to establish a rapid, economical and robust process with respect to drug stability and appearance of the resulting lyophilizate. Furthermore, the process parameters like cooling rate, temperature, and vacuum should be easily transferable to standard large-scale lyophilizers enabling the manufacture of larger batches in the future.

Selection of formulations

The lead concentration of NicQb was 1.0 mg / mL and the fill volume was determined to 0.6 mL / vial. Furthermore, trehalose was selected as lyoprotectant and hereby in a concentration of 10%.

Trehalose and sucrose are often used as lyoprotectants. The proposed stabilizing mechanisms were described in previous sections. Trehalose has been selected as lyoprotectant because of the following reasons:

(1) A freeze-concentrated trehalose solution has a Tg' about 3 °C higher than sucrose (trehalose -29.5 °C vs. sucrose -32°C [Levine et al., 1988]) enabling drying at higher temperatures resulting in shorter freeze-drying times [Tang et al., 2004];

(2) Dried amorphous trehalose has a higher glass transition temperature as compared to sucrose [Slade et al., 1995], and thus, a higher temperature of "zero" mobility [Yu, 2001] which can lead to an improved long-term stability [Green et al., 1989; Molina et al., 2004], especially at ambient temperature;

(3) Trehalose is less hygroscopic [De Giulio et al., 2005];

(4) Trehalose has a very low chemical reactivity [Roser, 1991; O'Brien, 1996];

(5) The glycosilic bond of trehalose is in comparison to sucrose far more stable [Moelwyn-Hughes, 1929; Higashiyama, 2002] which might lead to an improved long-term stability as the resulting decomposition product glucose has the propensity to degrade proteins via the Maillard reaction [Hageman, 1992].

A trehalose concentration of 10 % has been selected. This high concentration was chosen because high amounts of amorphous stabilizers protect complex particulate systems like viruses, lipid / DNA complexes and nanoparticles [Allison et al., 2000; Zhai et al., 2004; Zillies, 2007] better than lower amounts. However, at trehalose concentrations higher than 10 % the primary drying time could increase significantly [Hatley et al., 1996; Nail et al., 2002]. Furthermore, at a trehalose concentration of 10 % the resulting solution could already be isoosmotic [Cleland et al., 2001], and thus, the lyophilizate could easily be reconstituted with water to the initial volume to a parenterally applicable solution.

The advantage of the addition of polysorbate 20 as cryoprotectant was described in the previous paragraph. In this study the adequate concentration of polysorbate should be selected.

Another important factor is the selection of the pH and the buffer species. The pH stability study revealed that NicQb is stable only in a narrow pH range. Above a pH of 6.6 it is prone to chemical degradation and below a pH of 6.2 NicQb tends to aggregate. In contrast to the formulations investigated in the freeze-thaw stability study the pH was reduced from 6.4 to 6.2 with the intention to further increase the chemical stability of the drug (see pH stability study). This seemed to be reasonable as it was found from the freeze-thaw studies that the aggregation of the drug could be prevented by polysorbate 20.

Concerning the buffer type sodium phosphate, potassium phosphate and histidine / histidine HCl (all 20 mM) were tested. Even though sodium phosphate is one of the most commonly used buffer species for lyophilizates [Schwegman et al., 2005], there is still a lively discussion about a possible pH shift to more acidic pH values, caused by a more readily crystallization of Na_2HPO_4 than NaH_2PO_4 during the freezing step, which can lead to denaturation of proteins [Nema et al., 1993; Chang et al., 1996; Anchordoquy et al., 1996b]. Hence, as for potassium phosphate buffered systems a pH shift is not that distinct [Sarciaux et al., 1999] and histidine buffered formulations show no pH shift [Osterberg et al., 1999] these two buffer species were tested as alternatives to sodium phosphate.

Additionally, the effect of varying concentrations of sodium chloride (0 mM up to 150 mM) on the stability of NicQb upon the whole freeze-drying process was investigated. AF4 was introduced to monitor the effect of sodium chloride on the physical stability of NicQb more closely.

Finally, in view of clinical dose finding studies, the most promising formulation candidate should be assessed towards its capability to stabilize NicQb in the concentration range of 0.2 to 2.0 mg / mL.

Consequently, the NicQb formulations described in Table 4.2.1 were prepared and used for freeze-drying studies.

Table 4.2.1: Compositions of NicQb formulations.

Code	NicQb [mg]	Trehalose dihydrate [% (w/v)]	Polysorbate20 [% (w/v)]	Sodium chloride [mM]	Sodium phosphate [mM]	Potassium phosphate [mM]	Histidine [mM]	pH
A01	0.60	10	-	-	20	-	-	6.2
A02	0.60	10	0.0025	-	20	-	-	6.2
A03	0.60	10	0.005	-	20	-	-	6.2
A04	0.60	10	0.0075	-	20	-	-	6.2
A05	0.60	10	0.010	-	20	-	-	6.2
A06	0.60	10	0.005	-	-	20	-	6.2
A07	0.60	10	0.005	30	-	20	-	6.2
A08	0.60	10	0.005	60	-	20	-	6.2
A09	0.60	10	0.005	90	-	20	-	6.2
A10	0.60	10	0.005	150	-	20	-	6.2
A11	0.60	10	0.005	-	-	-	20	6.2
A12	0.12	10	0.005	-	20	-	-	6.2
A13	0.36	10	0.005	-	20	-	-	6.2
A14	1.2	10	0.005	-	20	-	-	6.2

Cycle development rationale

The aim was to develop a freeze-drying cycle possible for the lyophilization of all the formulations described in Table 4.2.1. As agreed with Cytos Biotechnology AG, the cycle was developed for a 0.6 mL fill in 2R class 1 glass vials. Therefore, freeze-drying cycle A (Table 4.2.2) was designed.

The formulations were cooled to - 50 °C at a cooling rate of 1 °C / min. This moderate cooling rate was applied because as described by Tang et al. [Tang et al., 2004] this seems to be a good compromise between reasonable supercooling resulting in moderate ice surface areas and uniform ice structure, and short times during which the drug is exposed to the freeze-concentrated solution. Higher supercooling achievable by freezing methods like liquid nitrogen freezing, loading vials onto precooled shelves, or ramped cooling on the shelves leads to the formation of small ice crystals and larger ice / liquid interfaces [Jiang et al., 1998]. This might be beneficial if the formation of larger ice crystals is crucial for the drug stability, e.g.

disrupture of especially complex, large particles like viruses [Zhai et al., 2004]. However, the large ice / water interface can lead to surface-induced denaturation [Chang et al., 1996]. Furthermore, applying such enhanced freezing methods can lead to heterogeneity between vials and is not practical because the cooling rate in common freeze-driers is limited to less than 2 °C / min [Costantino et al., 2004]. Slow freezing leads to smaller ice / liquid interfaces which reduces surface-induced degradation, but prolongs the time the drug exists in the freeze-concentrated liquid state which in turn can affect its stability [Heller et al., 1999; Bhatnagar et al., 2007].

Table 4.2.2 Freeze-drying protocol A.

Step	Time [min]	Temperature [°C]	Pressure [mbar]
Loading	00:00:00	20	1013
Freezing	00:01:10	-50	1013
	00:03:00	-50	1013
Primary drying	00:00:01	-50	0.045
	00:00:15	-35	0.045
	20:00:00	-35	0.045
	00:02:30	-20	0.045
	10:00:00	-20	0.045
Secondary drying	00:01:20	20	0.045
	10:00:00	20	0.045

The target freezing temperature was set to -50 °C in order to cool the formulations below their glass transition temperature, the temperature above which a product might loose its macroscopic structure due to mobilization of the intestinal water in the frozen matrix [Wang, 2000]. In order to determine the Tg' of the formulations DSC measurements were performed. The results obtained are summarized in Table 4.2.3. The lowest Tg' was -39 °C for formulation A10. The decrease of Tg' with increasing sodium chloride concentrations is related to the increase of the quantity of unfrozen water in the freeze-concentrate. The unfrozen water in turn acts as plasticizer and decreases Tg' [Her et al., 1995]. In order to provide a safety margin and to allow complete solidification of all formulations the target freezing temperature was determined to -50 °C and kept for 3 hours prior to primary drying.

Table 4.2.3: Glass transition temperatures of maximally freeze-concentrated solutions.

Code	Tg' [°C]	Code	Tg' [°C]
A01	-34.3	A08	-35.2
A02	-34.0	A09	-36.7
A03	-34.4	A10	-39.0
A04	-34.4	A11	-31.5
A05	-34.8	A12	-33.2
A06	-32.8	A13	-33.1
A07	-34.1	A14	-33.6

In a next step the chamber pressure and the shelf temperature to be applied during primary drying had to be defined. Current dogma requires that formulations are freeze-dried below Tg' to avoid macroscopical collapse [Pikal, 1990; Wang, 2000; Tang et al., 2004; Costantino et al., 2004]. Even though freeze-drying below Tg' demands a very low target product temperature and in consequence longer processes it was decided to follow this recommendation as the major goal was to establish a robust and safe freeze-drying cycle. Therefore, the target product temperature was determined to -40 °C (vapour pressure of ice = 0.128 mbar) in the initial phase of the freeze-drying process. The chamber pressure impacts both heat and mass transfer. According to Nail et al. it should be between one-fourth and one-half of the vapour pressure of ice at the target product temperature to allow a high sublimation rate, but high enough to enable sufficient and homogeneous heat transfer [Nail et al., 2002]. Therefore, the chamber pressure was set to 0.045 mbar which seemed to be a good compromise between a high sublimation rate and heat transfer provided by the conductivity of the vapour phase. The chamber pressure was controlled by a Pirani gauge. In order to provide the heat removed by sublimation the shelf temperature is normally set a few degrees above the target product temperature. As an excessive heat input may lead to an increase of the product temperature above its Tg' which might lead to product collapse [Adams et al., 1996] the shelf temperature was first set to -35 °C and was raised to -20 °C after 20 hours to accelerate primary drying. The endpoint of primary drying is reached when all the frozen water is removed and the rate of water sublimation is significantly reduced. Then, the product temperature shows a clear increase, followed by a plateau [Nail et al., 2002]. To monitor the

product temperature during freeze-drying thermocouples were placed in the centre of the vials. Figure 4.2.10 shows an exemplarily process documentation. It illustrates that during primary drying steps the product reaches or exceeds the shelf temperature indicating that primary drying was completed within the determined time frames. Furthermore, no macroscopical collapse of any of the tested formulations was observed.

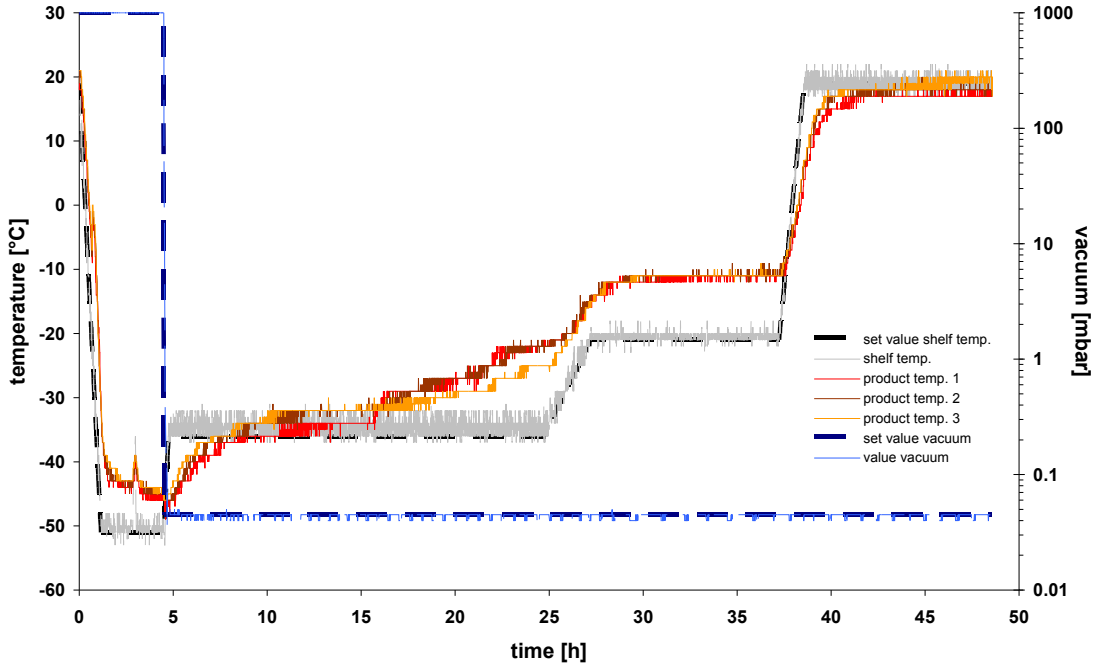


Figure 4.2.10 Example - process documentation.

Secondary drying is typically carried out at accelerated temperature to enable fast desorption of the remaining water [Franks, 1998]. However, the shelf temperature should be increased slowly, because early in secondary drying the amorphous product has high residual moisture content, and thus, low glass transition temperature, which make collapse possible [Tang et al., 2004]. Consequently, the temperature was increased from -20 °C to 20 °C at a heating rate of 0.5 °C / min. Then, the product was dried for further 10 hours at 20 °C and 0.05 mbar to reduce the residual moisture content.

After freeze-drying all formulations showed excellent appearance, exemplarily shown for formulation A03 in Figure 4.2.11, and were amorphous as indicated by XRD measurements (Figure 4.2.12). The lyophilizates could be easily reconstituted with

water within 5 seconds. All formulations were clear after reconstitution and contained no visible particles.

Figure 4.2.11 Appearance NicQb formulation A03 after freeze-drying.

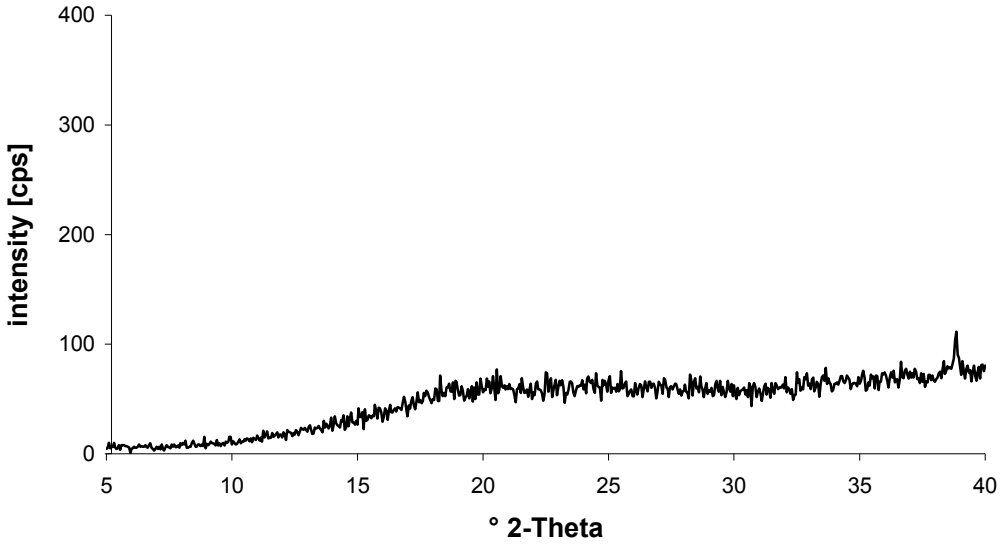


Figure 4.2.12 XRD of freeze-dried NicQb formulation A03.

Furthermore, it could be shown that by applying the developed process low residual moisture < 1.3 % (Table 4.2.4) could be achieved for all formulations tested. Low residual moisture contents are desirable, especially in view of long-term stability, because water can affect the drug stability in two ways, as reactant or as a plasticizer of the amorphous formulation [Shalaev et al., 1996].

Table 4.2.4: Residual moisture content after freeze-drying.

Code	RM [%]	Code	RM [%]
A01	1.3	A08	0.7
A02	0.9	A09	0.9
A03	1.1	A10	1.2
A04	1.1	A11	0.4
A05	1.2	A12	0.5
A06	0.3	A13	0.6
A07	0.6	A14	0.5

High glass transition temperature is considered to be one of the most important parameters for long-term stability of a labile drug. It has been suggested that the glass transition temperature should be 50 °C above the intended storage temperature to minimize molecular mobility to the point of zero mobility and achieve sufficient stability [Hancock et al., 1995; Breen et al., 2001]. It is described that the Tg of a formulation can be reduced by 10 °C by each percent of moisture content [Franks, 1994; Rossi et al., 1997] which might lead at high residual water contents to product collapse and degradation of the drug [Franks, 1998; Wang, 2000; Molina et al., 2004]. The glass transition temperatures determined for the formulations without sodium chloride were above 80 °C (Table 4.2.5), which is in good correlation with literature [Crowe et al., 1996; Hancock et al., 1997; Chen et al., 2000]. Exemplarily, the thermogram obtained for formulation A03, is shown in Figure 4.2.13. Hence, it was assumed that concerning the mobility of the dried formulations all formulations should be capable of stabilizing NicQb. Unfortunately, the formulations A07 – A10, formulations with increasing sodium chloride concentrations, could not be analyzed by DSC due to the limitation of lyophilized samples.

Table 4.2.5: Tg of NicQb formulations after freeze-drying (n=2).

Code	Tg [°C]	Code	Tg [°C]
A01	85.0	A08	ND
A02	82.6	A09	ND
A03	84.1	A10	ND
A04	82.9	A11	94.0
A05	81.1	A12	101.4
A06	89.4	A13	102.7
A07	ND	A14	100.6

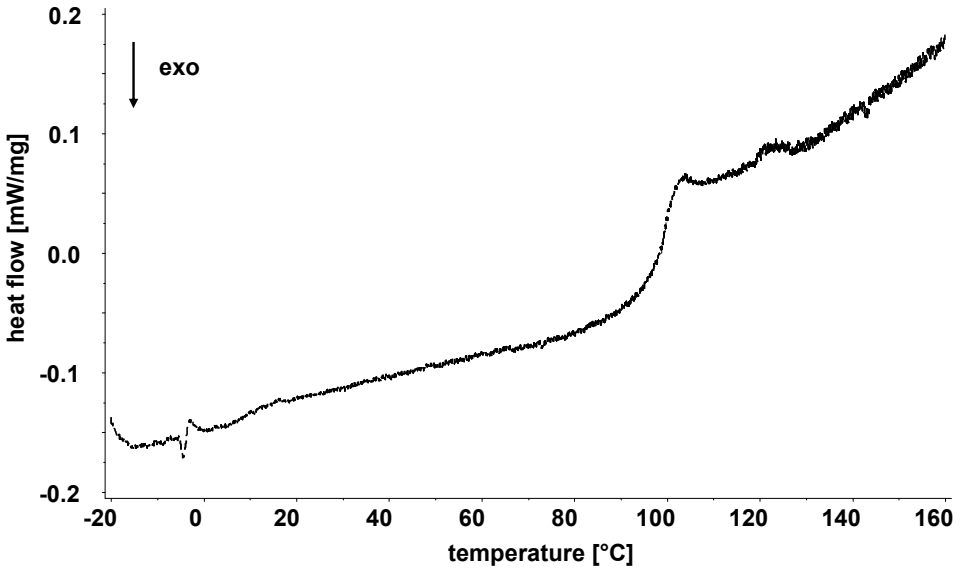


Figure 4.2.13 DSC heating scan of freeze-dried NicQb formulation A03.

In summary it could be stated that the developed freeze-drying process led to amorphous products with excellent appearance, low residual moisture levels and high glass transition temperatures. It could be concluded that this freeze-drying cycle is suitable for all the formulations tested. However, by employing more aggressive conditions it should be possible to achieve higher drying efficiency, but a further process optimization was not in the focus of this work because the current cycle already met the requirements of being robust, short and transferable to any pilot and production freeze-dryer.

In the following sections the effects of the different formulations (Table 4.2.1) on the stability of NicQb upon freeze-drying are described. Therefore, the samples were analyzed before and after freeze-drying by SE-HPLC, AF4 and DLS at least in duplicate. In the following paragraphs SE-HPLC and AF4 results are presented. DLS data are not shown as for all samples comparable results were obtained and it was shown that AF4 exceeds by far the capability of DLS with regard to the analysis of the physical stability of VLP (compare chapter 3).

Effect of varying polysorbate 20 concentrations on the stability of NicQb

In order to determine the minimum concentration of polysorbate 20 necessary to prevent NicQb aggregation during freeze-drying five NicQb formulations (1 mg / mL) with increasing polysorbate 20 concentrations from 0 up to 0.01 % were prepared. The formulations also contained 10 % trehalose dihydrate (w / v) as lyoprotectant and were buffered with 20 mM sodium phosphate (formulations A01 – A05, Table 4.2.1).

AF4 measurements revealed that aggregation of NicQb upon freeze-drying could be clearly influenced by polysorbate 20. Formulation A01, without polysorbate, showed a clear increase of the aggregation level from initially 3.9 % aggregates (the term aggregates includes all VLP species larger than VLP dimers) to 7.9 % after freeze-drying indicating that trehalose alone was not sufficient to avoid aggregation of NicQb upon freeze-drying. By contrast, if polysorbate was added in a concentration of 0.0025 % aggregation could be prevented. A further increase of the polysorbate 20 concentration showed no further positive effect on the stability of NicQb (Figure 4.2.14 A).

The data obtained from SE-HPLC analysis showed that with the exception of formulation A01, none of the formulations led to an increase (more than 1.0 %) of NicQb fragments (Figure 4.2.14 B). Thus, it seemed that polysorbate 20 not only prevented NicQb aggregation but also inhibited VLP degradation during lyophilization.

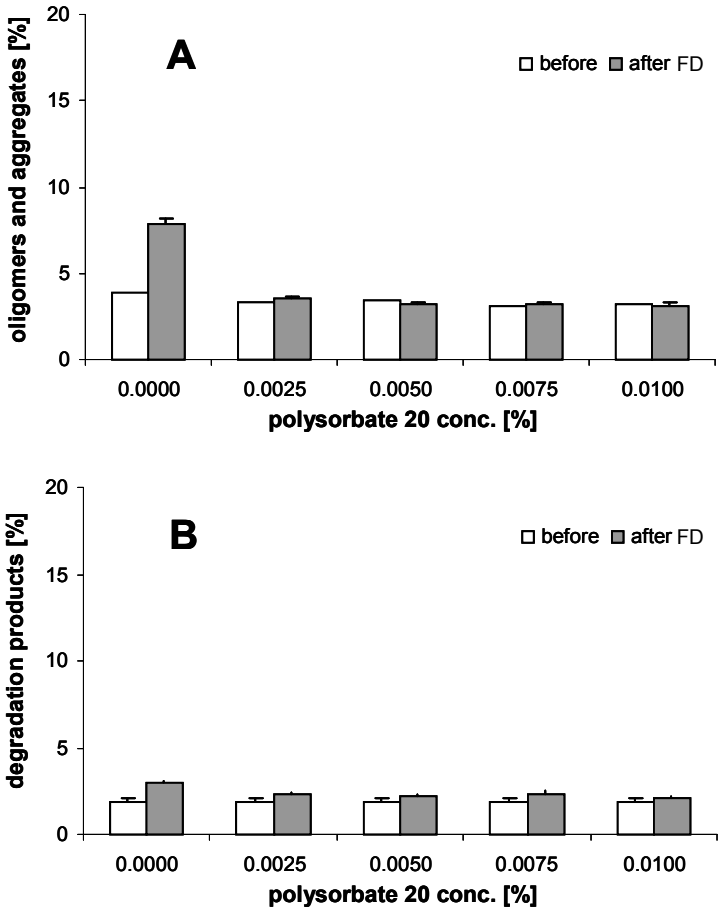


Figure 4.2.14 Stability of NicQb (1 mg / mL) in dependence of varying polysorbate concentrations. Aggregation level determined by AF4 (A). Amount of NicQb degradation products determined by SE-HPLC (B).

Consequently, it could be stated that the addition of polysorbate 20 was very beneficial to stabilize NicQb upon freeze-drying, especially with respect to the inhibition of NicQb aggregation. A concentration of 0.005 % seemed to be recommendable in order to provide a safety margin to the lowest effective concentration (0.0025 %).

Effect of different buffer systems on the stability of NicQb

In order to investigate the effect of different buffer types on the stability of NicQb upon freeze-drying formulations including sodium phosphate, potassium phosphate and histidine buffer (all 20 mM) were assessed (formulations A03, A06 and A11, Table 4.2.1). The pH was adjusted to 6.2. The NicQb concentration was 1 mg / mL.

Additionally, the formulations contained trehalose dihydrate 10 % (w / v) and 0.005 % polysorbate 20.

AF4 measurements revealed that in none of the formulations the aggregation level of NicQb was increased upon freeze-drying. The amount of aggregated species of NicQb in the histidine buffered formulations was already initially slightly higher than in the phosphate buffered formulations (Figure 4.2.15 A). The reason for this result is not clear because the different buffered NicQb solutions were all prepared from the same API batch.

The data obtained from SE-HPLC analysis showed that none of the formulations led to a significant increase of the fragmentation products. In comparison to the liquid formulation prior to freeze-drying the fragmentation level was increased for at most 1.0 %, again the histidine buffered solution showed the highest initial level of NicQb fragments (Figure 4.2.15 B).

In conclusion it could be stated that none of the tested buffer compositions had a negative effect on the stability of NicQb during freeze-drying, and thus, all buffer species tested were conceivable. As described above a pH drop in the sodium phosphate buffered formulation might occur due to a more readily crystallization of one buffer component during the freezing step [Pikal, 1994; Shalaev et al., 2002]. From the results obtained during the pH stability study it was assumed that a pH drop could lead to an increase of the aggregation level. However, as no significant alteration of NicQb purity was observed it was supposed that either crystallization of the buffer was prevented by the amorphous trehalose matrix, a mechanism already proposed by Chang et al. [Chang et al., 1992], and / or the low buffer concentration [Pikal, 1999], and thus, no pH change occurred, or aggregation of NicQb was inhibited by polysorbate 20 and trehalose and fragmentation did not happen in the short time the formulation was present in the liquid state. Due to the slightly higher levels of aggregated and fragmented VLP species in the histidine buffered liquid formulation prior to freeze-drying, as compared to the phosphate buffered formulations, histidine was not considered in further studies.

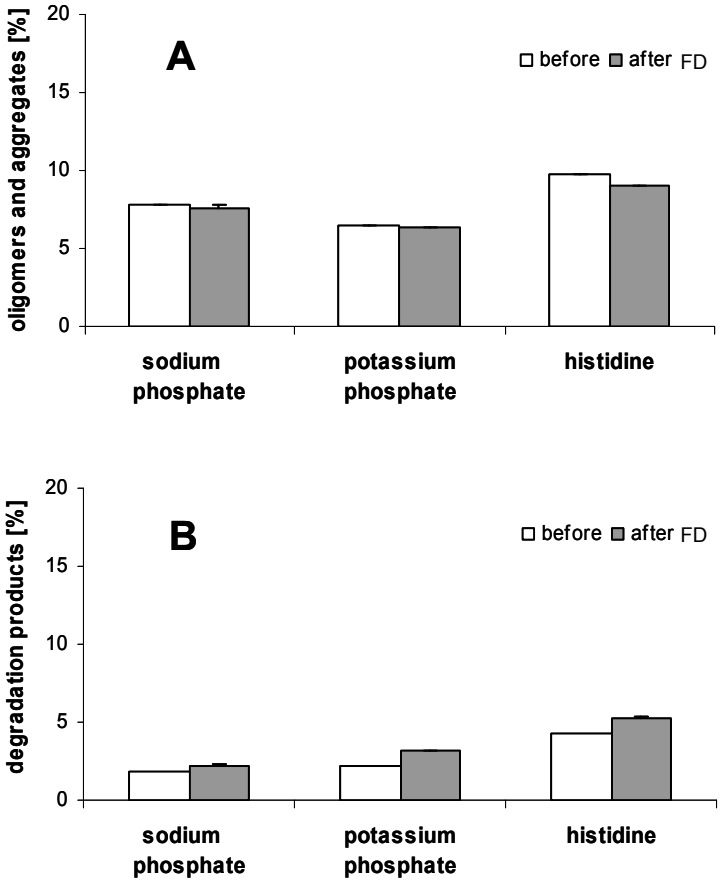


Figure 4.2.15 Stability of NicQb (1 mg / mL) in dependence of different buffer species. Aggregation level determined by AF4 (A). Amount of NicQb degradation products determined by SE-HPLC (B).

Effect of varying sodium chloride concentrations on the stability of NicQb

Even though the results obtained from the freeze-thaw experiments already indicated that sodium chloride leads to aggregation of NicQb during freezing, it was the intention of the current experiment to investigate the effect of sodium chloride in the concentration range of 0 up to 150 mM (formulations A06 – A10) on the stability of NicQb upon the whole freeze-drying process. This was interesting because at this time point an AF4 method, as a more sensitive tool for the analysis of VLP aggregates in comparison to DLS, was available. All formulations tested contained 1 mg / mL NicQb, 10 % trehalose dihydrate and 0.005 % polysorbate 20.

AF4 measurements revealed that in the presence of polysorbate 20 and trehalose up to a sodium chloride concentration of 60 mM no aggregation of NicQb occurred, whereas at sodium chloride concentrations higher than 90 mM the aggregation level of the NicQb formulations increased with increasing sodium chloride

concentrations. For formulation A10 (150 mM sodium chloride) the level of aggregates increased from initially 6.5 % prior to freeze-drying up to 10.3 % after freeze-drying (Figure 4.2.16 A).

Addressing the integrity of NicQb SE-HPLC analysis showed that sodium chloride in the concentrations tested had no negative effect as no increase of the fragmentation level could be observed (Figure 4.2.16 B).

It was concluded that sodium chloride has a negative effect on NicQb when added in concentrations higher than 90 mM, even in the presence of polysorbate 20. However, as no protective effect of sodium chloride was observed at lower concentrations and the addition of the salt is not necessary to adjust the tonicity of the formulations it was assumed that the addition of sodium chloride to lyophilized NicQb formulations should be prevented.

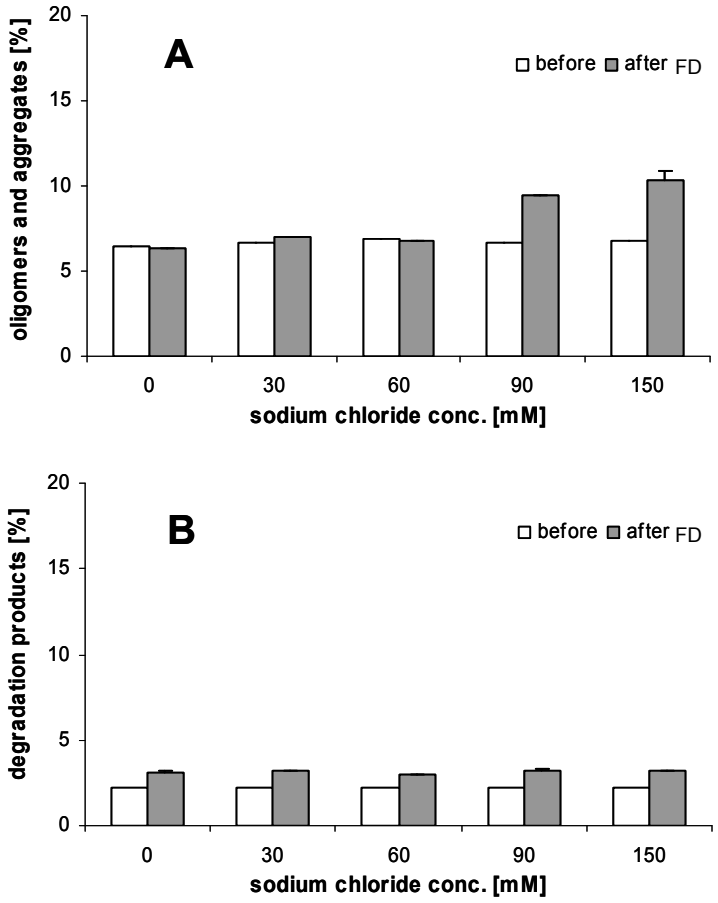


Figure 4.2.16 Stability of NicQb (1 mg / mL) in dependence of varying sodium chloride concentrations. Aggregation level determined by AF4 (A). Amount of NicQb degradation products determined by SE-HPLC (B).

Effect of varying trehalose / NicQb weight ratios on the stability of NicQb

From previous experiments it was found that an optimal formulation for NicQb at a concentration of 1 mg / mL should be composed of trehalose dihydrate (10 %) and polysorbate 20 (0.005 %), buffered with either sodium phosphate or potassium phosphate. Finally, with regard to clinical dose finding studies, formulations with different NicQb concentrations had to be prepared. Ideally, in respect of double blind trials, the lyophilizates with different NicQb amounts should have the same appearance and should be reconstitutable to isotonic, parenterally applicable solutions of the same final volume. Thus, four formulations with NicQb concentrations in the range of 0.2 to 2.0 mg / mL, 10 % trehalose dihydrate, 0.005 % polysorbate 20 and 20 mM sodium phosphate with a fill of 0.6 mL were prepared (formulations A12, A13, A03 and A14, Table 4.2.1). In this context the effect of different trehalose to NicQb ratios could be investigated. The resulting trehalose to NicQb weight ratios are displayed in Table 4.2.6.

Table 4.2.6 Trehalose / NicQb weight ratios of specific NicQb formulations.

Code	NicQb conc. [mg / mL]	Trehalose conc. [mg / mL]	Weight ratio Trehalose / NicQb
A14	2.0	90	45
A03	1.0	90	90
A13	0.6	90	150
A12	0.2	90	450

Based on the findings of Zillies [Zillies, 2007], Allison et al. [Allison et al., 2000], Zhai et al. [Zhai et al., 2004] and Cleland et al. [Cleland et al., 2001] who showed that increasing ratios of disaccharide to gelatine nanoparticle, lipid / DNA complex, herpes simplex virus 2 and monoclonal antibody ratios, respectively led to increasing drug stability upon freeze-drying it was not expected that in our case the increase of the trehalose to NicQb weight ratio has a negative influence on the drug stability. Furthermore, it was not supposed that the slight decrease of the excipient to drug ratio of the “lead” formulation A03 from 90 : 1 to 45 : 1 in the formulation A14 might have a great influence on drug stability.

AF4 and SE-HPLC measurements revealed that in all formulations tested NicQb remained stable upon freeze-drying; neither increase of the aggregation level nor an increase of fragments was observed (Figure 4.2.17).

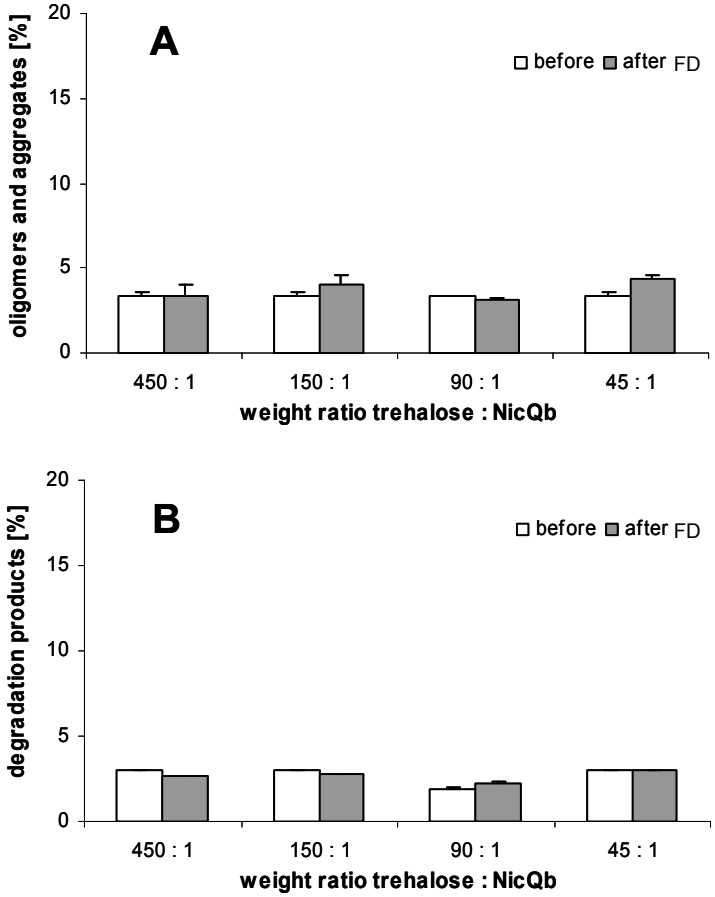


Figure 4.2.17 Stability of NicQb in dependence of varying drug concentrations. Aggregation level determined by AF4 (A). Amount of NicQb degradation products determined by SE-HPLC (B).

Consequently, it could be stated that in the range of 45 : 1 up to 450 : 1 trehalose / NicQb (w / w) in a solution also containing 0.005 % polysorbate 20, NicQb could be stabilized by trehalose upon freeze-drying. The preparation of lyophilizates with similar appearance, which could be easily reconstituted with 0.6 mL water to isotonic solutions, and NicQb concentrations of 0.2 mg / mL up to 2.0 mg / mL was feasible.

Conclusion

A robust freeze-drying cycle was developed that was successfully used to freeze-dry all formulations tested herein. Lyophilizates with elegant appearance and low residual moisture levels were achieved. Furthermore, from a technical point of view the freeze-drying cycle can easily be transferred to large-scale production freeze-dryers. It was found that a formulation composed of 10 % (w/v) trehalose dihydrate, 0.005 % (w/v) polysorbate 20 and either sodium phosphate or potassium phosphate as buffer agent was very beneficial to preserve the stability of NicQb in a concentration of 0.2 to 2.0 mg / mL during freeze-drying. Additionally, the lyophilizates could easily be reconstituted with water to parenterally applicable isotonic solutions.

4.2.4 Storage Stability Studies - NicQb Lyophilizates

In order to define a formulation capable of stabilizing NicQb upon storage four formulations (A03, A06, A14, and A15, Table 4.2.7) were assessed in a storage stability study for 15 weeks, at 2-8 °C, 25 °C / 60 % RH and 40°C / 75 % RH, respectively. Selected formulations, A03 and A15, were tested up to 25 weeks.

Table 4.2.7 Compositions of NicQb formulations assessed for long-term stability.

Code	NicQb [mg]	Trehalose dihydrate [% (w/v)]	Mannitol [% (w/v)]	Polysorbate20 [% (w/v)]	Sodium phosphate [mM]	Potassium phosphate [mM]	pH
A03	0.6	10	-	0.005	20	-	6.2
A06	0.6	10	-	0.005	-	20	6.2
A15	0.6	10	-	0.005	20	-	5.8
A16	0.6	1.1	4.4	0.005	20	-	6.2

A03 and A06 were the lead formulations designated from the freeze-drying experiments with either sodium phosphate or potassium phosphate as buffering agents. A15, a formulation similar to A03 except for a solution pH of 5.8, was included because of the higher chemical stability of the drug at lower pHs (refer to pH stability study). The aim was to investigate whether degradation of the VLP and the cleavage of nicotine upon storage are reduced at this pH. Based on the findings of the freeze-thaw and freeze-drying studies it was now in the focus to determine whether

the theoretically more pronounced aggregation of NicQb at the lower pH value might be prevented by polysorbate 20. These three formulations were freeze-dried according to protocol A (chapter 2). Additionally, formulation A16 composed of mannitol as bulking agent and trehalose as lyoprotectant in a weight ratio of 4:1 was included. The underlying rationale was that a combination of a crystalline bulking agent (mannitol) and a non-crystallizing disaccharide could offer on the one hand a robust crystalline matrix enabling shorter drying times (primary drying could be conducted at higher temperatures due to the eutectic point of mannitol at -1.5°C [Kim et al., 1998]), and on the other hand protects the drug by the amorphous disaccharide part [Johnson et al., 2002; Izutsu et al., 2002; Liao et al., 2005; Chatterjee et al., 2005]. Formulation A16 was freeze-dried according to protocol B (Table 4.2.8).

Table 4.2.8 Freeze-drying protocol B.

Step	Time [min]	Temperature [°C]	Pressure [mbar]
Loading	00:00:00	20	1013
Freezing	00:01:10	-50	1013
	00:03:00	-50	1013
Primary drying	00:00:01	-50	0.045
	04:00:00	-15	0.045
	20:00:00	-15	0.045
Secondary drying	00:00:01	-15	0.007
	06:00:00	40	0.007
	10:00:00	40	0.007

Primary drying was carried out at -15 °C according to Tang et al. [Tang et al., 2004] who proposed that combined formulations with both amorphous stabilizer and crystalline bulking agent can be dried more than 20 °C higher than the Tg' of the specific formulation without resulting in macrocollapse. The Tg' of formulation A016 was determined to -43.5 °C. Secondary drying was performed at 40 °C in order to prevent the possible formation of an unstable mannitol hydrate at lower drying temperature [Yu et al., 1999]. An annealing step typically applied to enable complete crystallization of mannitol [Izutsu et al., 1993; Searles et al., 2001; Ma et al., 2001; Lu et al., 2004] was not included because as proposed by Johnson et

al. mannitol crystallizes completely in formulations containing higher weight ratios of mannitol to disaccharide than 2 to 1 [Johnson et al., 2002].

The samples were analyzed at multiple time points, at least in duplicate, according to Table 4.2.9.

Table 4.2.9 Stability study plan.

CRITERIA		Test interval (weeks)			
		0	6	15	25 (option)
Characteristics	Test method	Liquid / LYO	2-8 °C / 25 °C / 40 °C	2-8 °C / 25 °C / 40 °C	2-8 °C / 25 °C / 40 °C
Appearance lyophilisate / reconstituted solution	Visual inspection	• / •	• / • / •	• / • / •	• / • / •
Reconstitution Time	Time	– / •	• / • / •	• / • / •	• / • / •
pH of reconstituted solution	pH meter	– / •	• / • / •	• / • / •	• / • / •
Residual moisture (RM)	KF titration	– / •	• / • / •	• / • / •	• / • / •
Tg (solid)	DSC	– / •	• / • / •	• / • / •	• / • / •
Morphology	XRD	– / •	– / – / –	– / – / •	– / – / –
Free nicotine	RP-HPLC	• / •	• / • / •	• / • / •	• / • / •
NicQb integrity	SE-HPLC	• / •	• / • / •	• / • / •	• / • / •
NicQb degradation	LDS-PAGE	• / •	• / • / •	• / • / •	• / • / •
RNA integrity	SE-HPLC	• / •	• / • / •	• / • / •	• / • / •
NicQb aggregation	AF4	• / •	• / • / •	• / • / •	• / • / •
NicQb aggregation	DLS	• / •	• / • / •	• / • / •	• / • / •
Particulate matter	Light obscuration	• / •	• / • / •	• / • / •	– / – / –

Complete data sets for all methods, formulations and storage conditions are presented in tables in the annex of this chapter. In the following paragraphs a selection of the most significant results is presented.

Characterization of the physical state of the lyophilizates

After freeze-drying all formulations showed excellent appearance. The trehalose based formulations A03, A06 and A15 resulted in stable cakes with only rare cracks and a marginal shrinkage. Upon storage, even at accelerated temperature none of these formulations showed significant alteration. The reason for the good macroscopical stability of the formulations A03, A06 and A15 is the high glass transition temperature of the mainly trehalose based matrix. Formulation A16 exhibited no shrinkage neither during freeze-drying nor upon storage which can be assigned to its mostly crystalline structure. Pictures of representative lyophilizates stored for 3 months at 40 °C are shown in Figure 4.2.18.

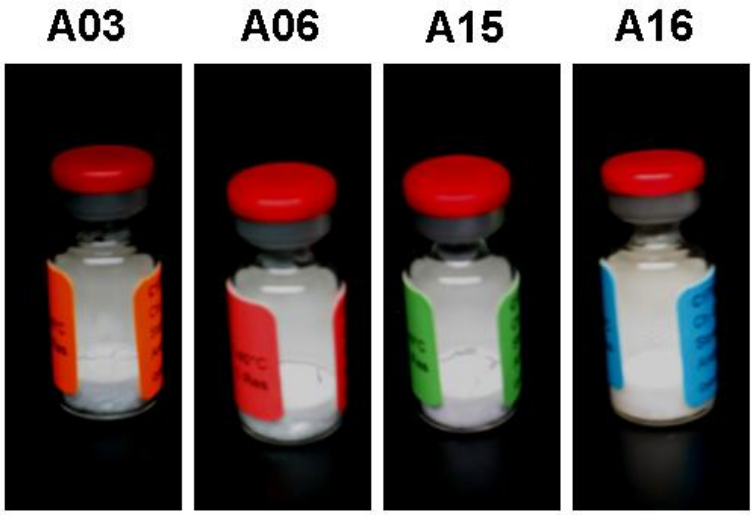


Figure 4.2.18 Appearance of NicQb lyophilizates after 15 weeks storage at 40 °C.

Residual moisture of all formulations was initially determined to be below 0.5 % (Figure 4.2.19). The mannitol / trehalose based formulation A16 exhibited the lowest initial water content of 0.15 % which could be related to the higher secondary drying temperature applied for this formulation (FD protocol B). Upon storage for 15 and 25 weeks at 2-8 °C and 25 °C / 60 % RH, respectively, only a negligible increase of the water content (increase of RM content < 0.3 %) was observed. Upon storage at 40 °C / 75 % RH significantly higher amounts of water were absorbed from the stoppers by all formulations, but even here the increase of the residual moisture level was below 1.0 % (Figure 4.2.19). According to Shalaev and Zografi low residual moisture content is important to minimize chemical degradation upon storage and to prevent plasticizing of the amorphous matrix [Shalaev et al., 1996].

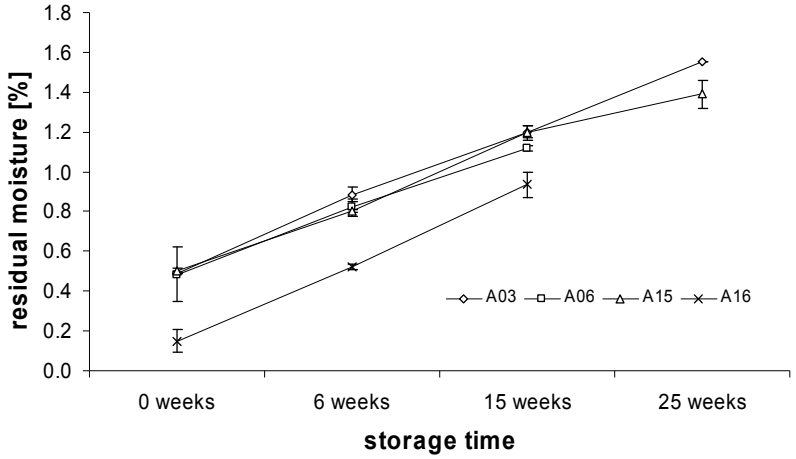


Figure 4.2.19 Residual moisture content of freeze-dried NicQb formulations right after the drying process and after 6, 15 and 25 weeks storage at 40 °C / 75 % RH.

The plasticizing effect of water was clearly observed by the Tgs of the amorphous trehalose based formulations A03, A06 and A15. Right after freeze-drying the glass transition temperatures were between 96 and 99 °C which is in good accordance to literature [Crowe et al., 1996; Hancock et al., 1997]. After storage at 2-8 °C and 25 °C / 60 % RH no clear changes of the Tgs could be detected. Upon storage at 40 °C / 75 % RH a decrease of the Tgs of 5-10 °C of the initial value was noted (Figure 4.2.20) which could be related to the increase of the water content. The decrease of the Tgs by increasing water contents is confirmed by literature; as described by Franks depression of Tg can amount to 10 degrees for each percent of moisture uptake by the lyophilizate [Franks et al., 1991].

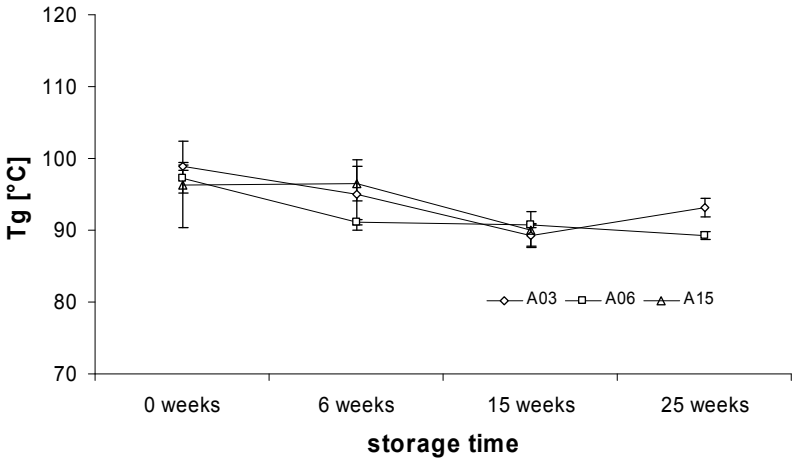


Figure 4.2.20 Glass transition temperatures of freeze-dried NicQb trehalose based formulations after the drying process and after 6, 15 and 25 weeks storage at 40 °C / 75 % RH.

As highlighted in the previous section high glass transition temperature of a formulation is considered to be one of the most important parameters for long-term stability of a labile drug. Following the recommendations of Hancock et al. the glass transition temperature should be 50 °C above the intended storage temperature to minimize molecular mobility to the point of zero mobility and achieve sufficient stability [Hancock et al., 1995]. The intended storage temperature was ambient temperature, so that the desired glass transition temperature was designated to 70 °C. The formulations A03, A06 and A15 met these requirements over the whole time span tested.

Additionally to the DSC runs XRD measurements revealed that the formulations A03, A06 and A15 were amorphous after freeze-drying and that the glassy state was maintained upon storage for 15 weeks, even at 40 °C, exemplarily shown for formulation A03 (Figure 4.2.21).

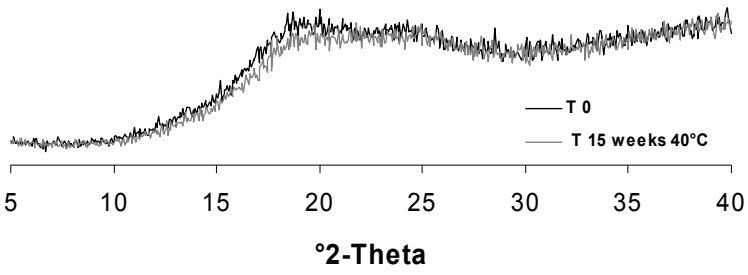


Figure 4.2.21 Physical state of NicQb formulation A03 determined by XRD after manufacture and after 15 weeks storage at 40 °C.

Concerning the physical state of the mannitol / trehalose based formulation A16 the XRD measurements revealed that after freeze-drying the lyophilizates were partially crystalline / partially amorphous and that the amorphous mannitol crystallized in an uncontrolled manner upon storage at 40 °C (Figure 4.2.22). The physical state was additionally analyzed by DSC. Amorphous mannitol is known to crystallize upon heating [Izutsu et al., 1994; Kim et al., 1998]. First DSC heating scans of the formulation stored for 15 weeks showed that mannitol crystallized completely upon storage at 40 °C because no exothermal event, indicating crystallization of amorphous mannitol could be observed. By contrast, the samples stored at 2-8 °C and 25 °C remained partially amorphous (Figure 4.2.23). Here, exothermal events comparable to

the starting material, indicating crystallization of mannitol, were observed. The melting point of mannitol, determined from the second heating scan was always around 156 °C; it was not affected by storage time or condition. According to Burger et al. this melting temperature corresponds to the δ-modification of mannitol [Burger et al., 2000]. This finding was confirmed by the XRD measurements (Figure 4.2.22), because specific peaks for the α- and β-modifications of mannitol at 17.3 °2-Theta and 14.7 °2-Theta, respectively, did not appear in the diffractogram [Hawe et al., 2006]. Furthermore, XRD measurements showed that mannitol hydrate was not built during freeze-drying as the specific peak of 17.9 °2-Theta did not appear in the diffractogram. These results indicate that mannitol, in contrast to the proposal of Johnson et al. [Johnson et al., 2002], did not completely crystallize during freeze-drying. An uncontrolled crystallization of mannitol upon storage is undesirable because it is proposed to affect the stability of labile drugs, as e.g, described for gelatin nanoparticles [Zillies, 2007] and monoclonal antibodies [Costantino et al., 1998].

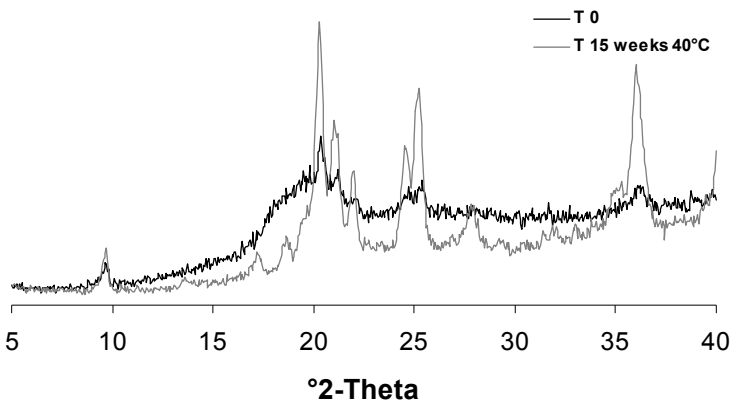


Figure 4.2.22 Physical state of NicQb formulation A16 determined by XRD after manufacture and after 15 weeks storage at 40 °C / 75 % RH.

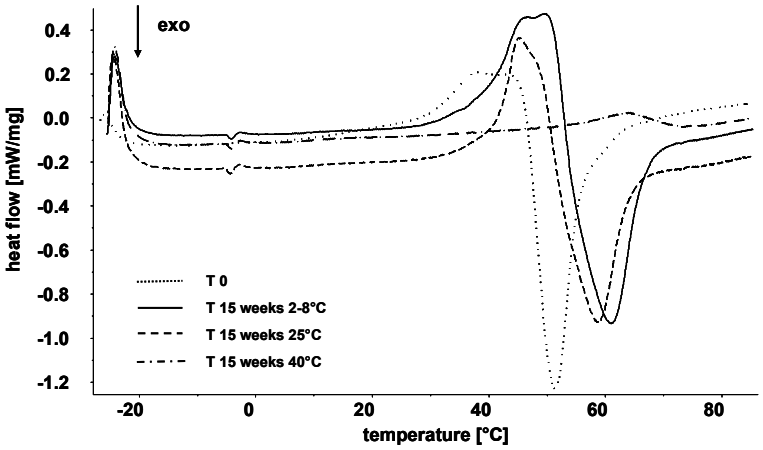


Figure 4.2.23 First DSC heating scans of NicQb formulation A16 after manufacture and after 15 weeks storage at 40 °C / 75 % RH.

Characterization of the reconstituted lyophilized formulations

The lyophilizates were reconstituted right after lyophilization and after storage with 0.6 mL highly purified water. All samples could easily be dissolved within 5 seconds. After reconstitution the appearance of all formulations was comparable and was not altered by storage temperature and time. The solutions were colourless and free of visible particles. Furthermore, the pH of the formulations remained stable upon the whole stability study. Additionally, the osmolality of reconstituted lyophilizates of all formulations was determined after manufacture. It was found that all formulations were isoosmotic (Table 4.2.10).

Table 4.2.10 Osmolality of reconstituted NicQb lyophilizates

Code	Osmolality [mosm/kg]
A03	318
A06	318
A15	314
A16	320

Stability of NicQb

The physical stability of NicQb was analyzed by AF4, DLS and light obscuration. In the following the results obtained for these three analytical methods are exemplarily shown for the samples before and after freeze-drying and after storage at

40 °C / 75 % RH. Complete data sets for all storage conditions are presented in Tables 4.5.1, 4.5.6 and 4.5.7 of the annex.

Concerning the aggregation of NicQb AF4 measurements (Figure 4.2.26) as well as DLS (Figure 4.2.25) and light blockage analysis (Figure 4.2.24) indicated that all formulations were capable of stabilizing the drug substance during freeze-drying. None of the performed analytical methods revealed any significant changes from the liquid formulations prior to freeze-drying to the lyophilized formulations. The preservation of the physical stability of NicQb during freeze-drying might be related to the prevention of VLP aggregation at interfaces by polysorbate 20 [Shi et al., 2005], and to the protection of the drug in the amorphous trehalose matrix by vitrification and water replacement [Hancock et al., 1997; Allison et al., 1999; Chang et al., 2005]. DLS measurements revealed no significant changes of the proportions of the main NicQb peak and the polydispersity indices (Figure 4.2.25 and Table 4.5.7 in the annex); furthermore, the determined average size of the VLP remained constant for all formulations and storage conditions (37 ± 1 nm). Additionally, light blockage measurements denoted no significant changes of NicQb stability upon storage for any of the assessed formulations, even at accelerated temperature (Figure 4.2.24 and Table 4.5.6 in the annex).

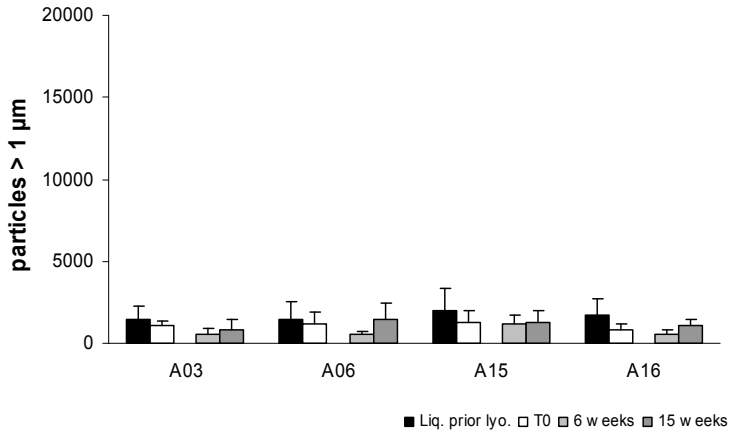


Figure 4.2.24 Number of particles > 1 µm, determined by light blockage, in NicQb formulations A03, A06, A15 and A16 prior to freeze-drying, right after freeze-drying and after storage for 6 and 15 weeks at 40 °C / 75 % RH.

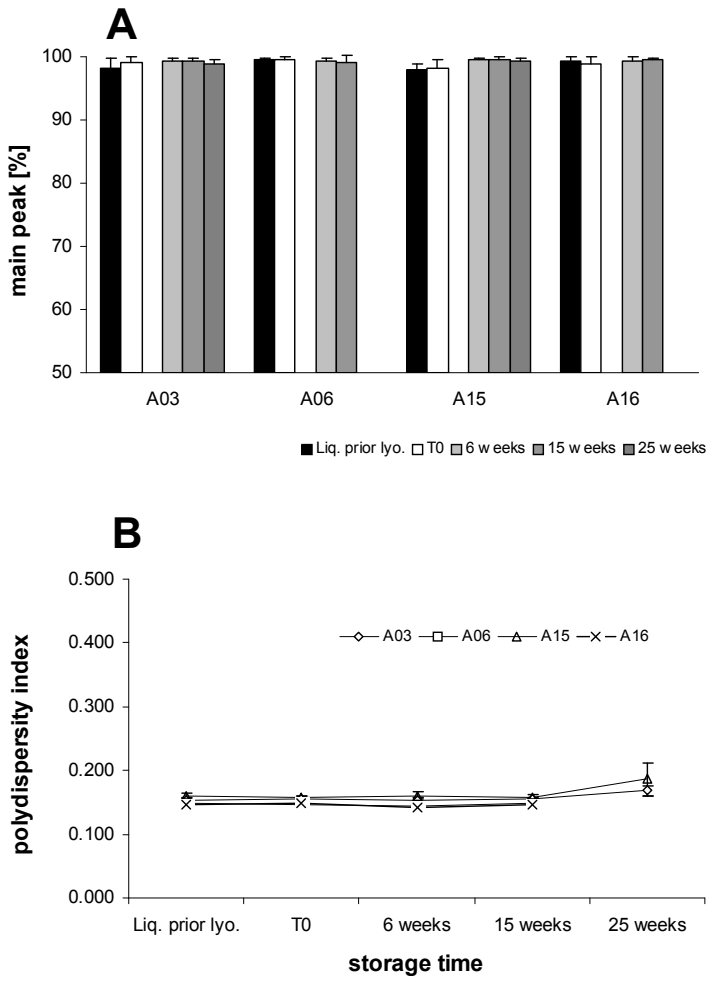


Figure 4.2.25 Proportions of main NicQb peak (A) and polydispersity indices of NicQb (B), determined by DLS, in NicQb formulations A03, A06, A15 and A16 prior to freeze-drying, right after freeze-drying and after storage for 6, 15 and 25 weeks at 40 °C / 75 % RH.

AF4 analysis, the most sensitive method for the determination of VLP aggregation (see chapter 3), indicated a slight increase (increase of aggregation level < 3 %) of NicQb aggregates in all formulations upon storage. This increase of the aggregation level was observed for all storage temperatures (Figure 4.2.26 and Table 4.5.1 in the annex). It was found that the theoretically more pronounced aggregation of the VLP at the lower pH value in formulation A15 was prevented by polysorbate 20. Hence, it seemed that NicQb could be stabilized in the amorphous trehalose matrix of formulations A03, A06 and A15 as well as in the partially crystalline (mannitol) / partially amorphous (trehalose) matrix of formulation A16. The crystallization of mannitol upon storage (see previous paragraph) did not affect the physical stability of NicQb, at least in the presence of amorphous trehalose.

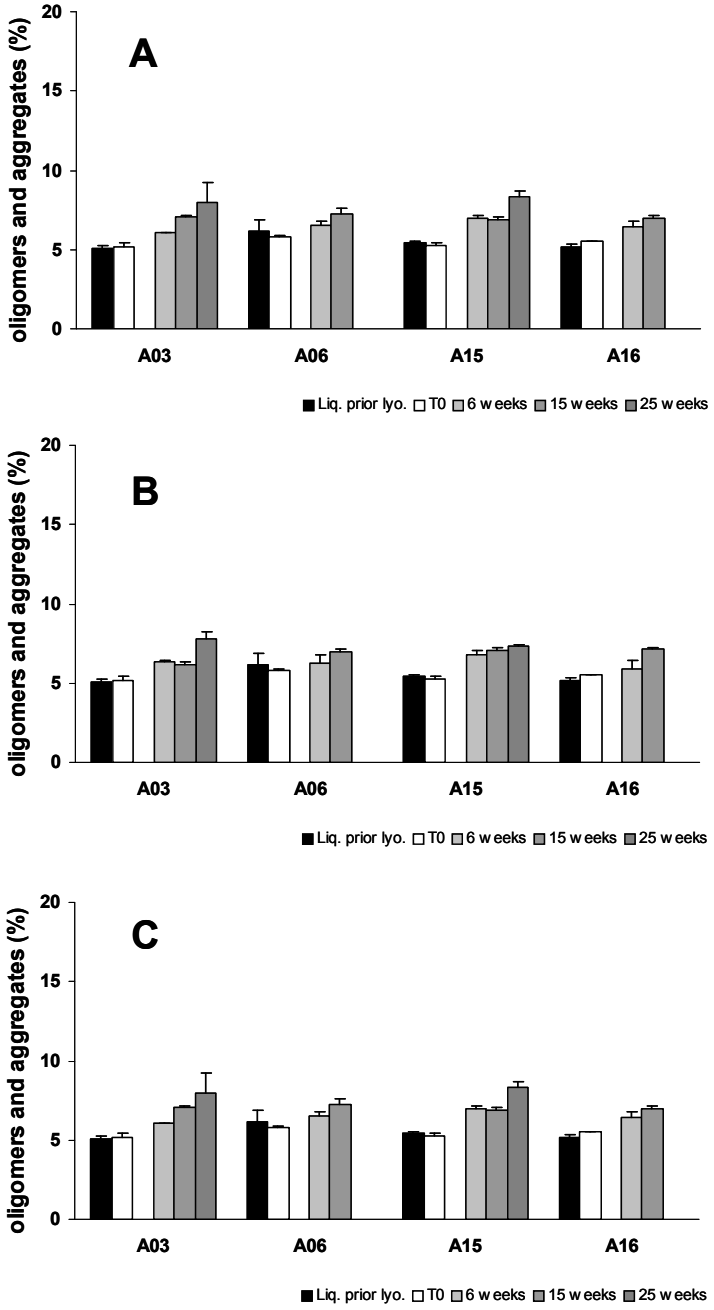


Figure 4.2.26 Amount of NicQb oligomers and aggregates, determined by AF4, in NicQb formulations A03, A06, A15 and A16 prior to freeze-drying, right after freeze-drying and after storage for 6, 15 and 25 weeks at 2-8 °C (A), 25 °C / 65 % RH (B) and 40 °C / 75 % RH (C).

The chemical stability of NicQb was investigated using SE-HPLC, RP-HPLC and LDS-PAGE. Chemical instability of NicQb results in the disassembly of the VLP into monomers or multimers of the Qb coat protein, degradation of the RNA incorporated in the VLP and / or the dissociation of the hapten nicotine and the Qb VLP. Furthermore, the single coat proteins could be hydrolyzed to smaller peptide chains.

SE-HPLC measurements revealed that the integrity of NicQb was preserved in all formulations during freeze-drying and subsequent storage at all temperatures tested. Only a slight increase of the amount of degradation products such as single Qb coat proteins or multimers of the Qb coat proteins and RNA form the inner core could be observed (Figure 4.2.27 and Table 4.5.2 in the annex). Thus, it appeared that all formulations were capable of stabilizing the VLP against disassembly even at accelerated storage temperature.

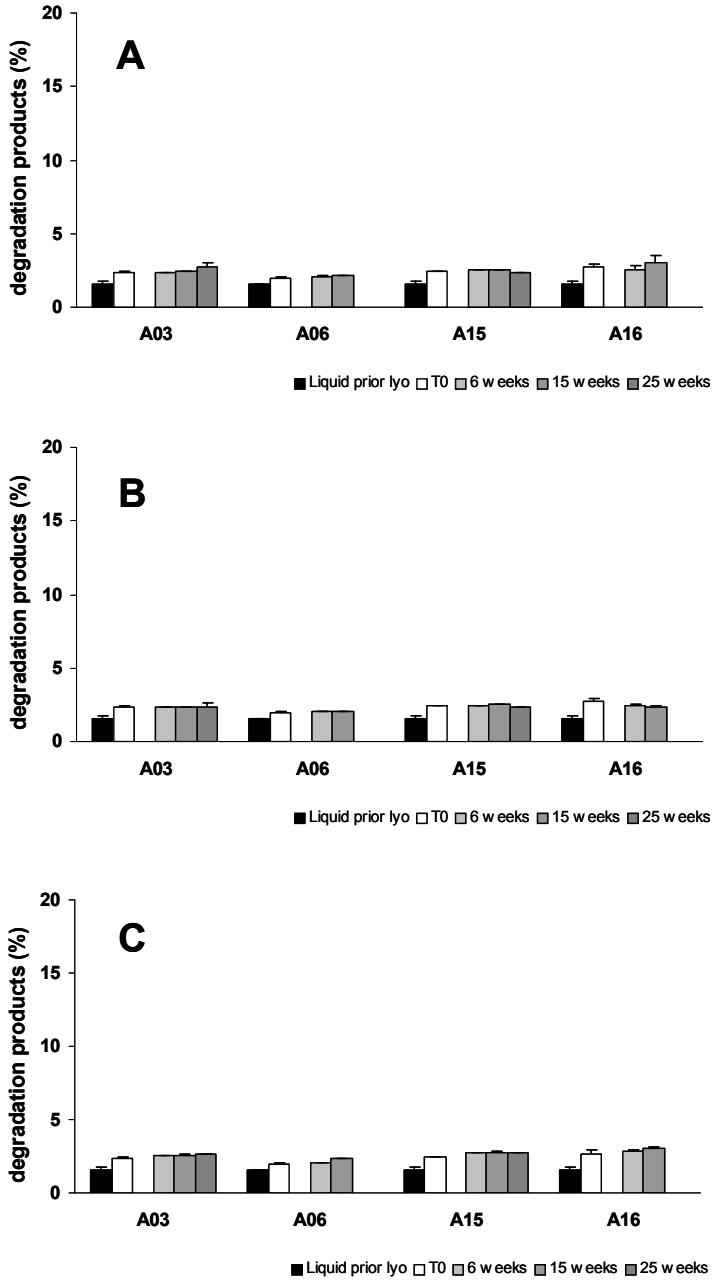


Figure 4.2.27 Amount of fragmentation products of NicQb, determined by SE-HPLC, in NicQb formulations A03, A06, A15 and A16 prior to freeze-drying, right after freeze-drying after storage for 6, 15 and 25 weeks at 2-8 °C (A), 25 °C / 65 % RH (B) and 40 °C / 75 % RH (C).

In order to assess the integrity of the incorporated RNA SE-HPLC analysis was carried out. This was important because as described by Vaughan et al. RNA might be prone to hydrolysis even in dried formulations [Vaughan et al., 2006]. The RNA was first extracted from the VLP and subsequently analyzed. Hydrolysis of the RNA would result in smaller RNA fragments that would lead to a shift of the RNA signal to higher retention times and / or the appearance of further RNA peaks. The SE-HPLC results revealed that the RNA remained stable in all formulations upon the whole time span tested, independent of the storage temperature. Neither shifts in the retention time of the three specific RNA peaks at 8.7, 10.4 and 10.9 min could be observed, nor could additional RNA peaks be detected. Exemplarily, the chromatograms obtained for formulation A03 after manufacture and after storage for 25 weeks at 40 °C / 75 % RH are depicted in Figure 4.2.28.

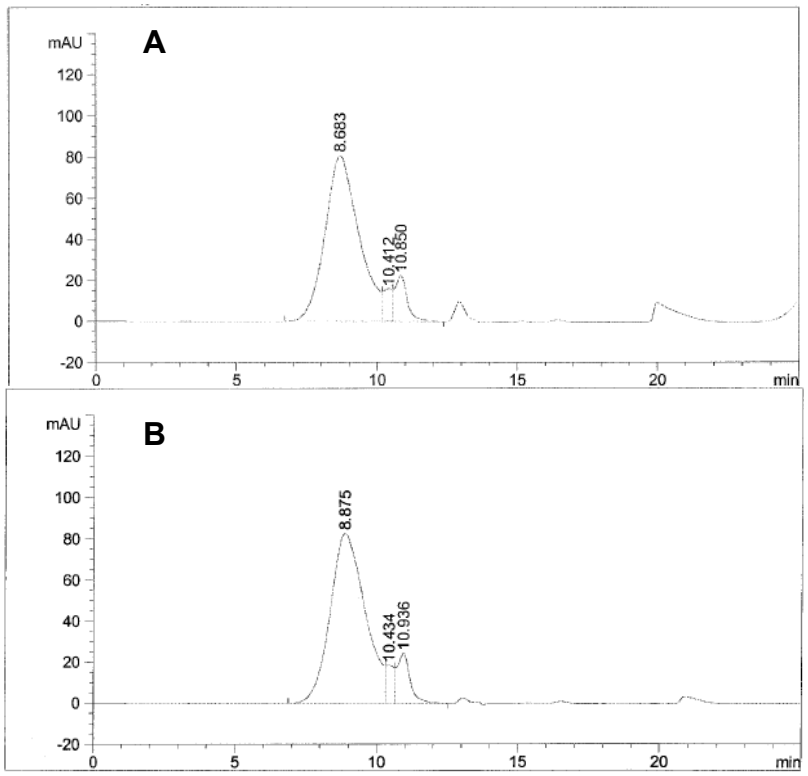


Figure 4.2.28 SE-HPLC analysis of RNA integrity of NicQb formulation A03 after freeze-drying and after storage for 25 weeks 40 °C / 75 % RH.

With respect to the stability of the esterbond linking nicotine to the VLP surface, RP-HPLC analysis revealed that it remained stable during freeze-drying in all formulations tested. Upon storage at 2-8 °C and 25 °C only a marginal increase (< 0.5 %) of the content of free nicotine derivatives was detected for all formulations

(Table 4.5.3 of the annex) which was obviously more pronounced after storage at 40 °C (Figure 4.2.29). The increasing hydrolysis of the esterbond could either be related to the increasing water content and / or decreased stability of the esterbond at higher temperatures, illustrated exemplarily in Figure 4.2.30 for formulation A03 after 25 weeks storage time. However, even for these samples the amount of free nicotine derivatives was below 1.5 % of total coupled nicotine after 25 weeks.

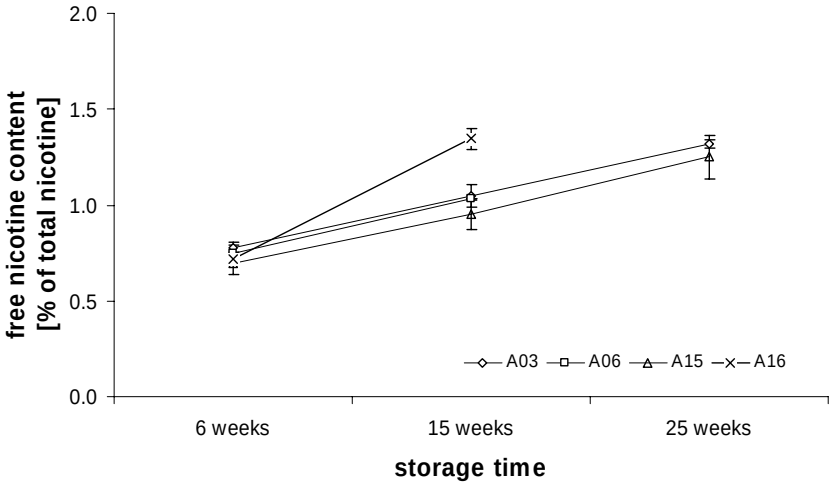


Figure 4.2.29 Amount of free nicotine derivatives, determined by RP-HPLC, in NicQb formulations A03, A06, A15 and A16 after storage for 6, 15 and 25 weeks at 40 °C / 75 % RH. Data for the liquid formulations and the lyophilized starting material are not shown, because the free nicotine content was below the limit of quantification (≤ 0.2 %).

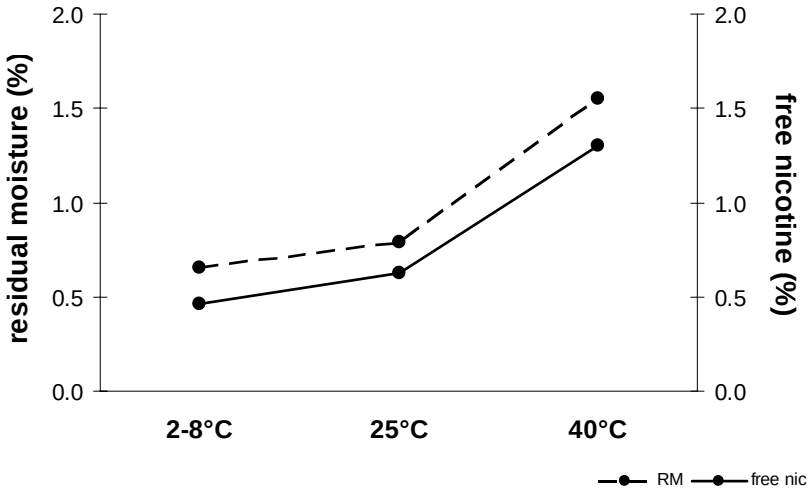


Figure 4.2.30 Amount of free nicotine derivatives, determined by RP-HPLC and moisture content, determined by KF titration, in NicQb formulations A03 stored at 40 °C / 75 % RH for 25 weeks.

LDS-PAGE was performed to analyze the stability of the Qb coat proteins. Degradation products of the Qb coat protein monomer appear as bands of an apparent molecular weight smaller than the molecular weight of the Qb coat protein monomer (14.1 kDa). All silver stained gels obtained for the formulations before freeze-drying, after freeze-drying and after storage, independent of storage conditions, showed two bands corresponding to an approximate molecular weight of 5-6 kDa and 11-13 kDa. After storage at 2-8 °C and 25 °C up to 15 weeks (A06 and A16) or 25 weeks (A03 and A15) an increase of these bands was not observed for any of the four formulations. After storage at 40 °C the intensity of these bands increased for all formulations, but quantification (comparison to standard dilutions) revealed that it was still less than 0.1 % of the intensity of the band corresponding to the Qb coat protein monomer. Exemplarily, an LDS-PAGE gel obtained for formulation A03 after storage for 25 weeks at 40 °C / 75 % RH is displayed in Figure 4.2.31.

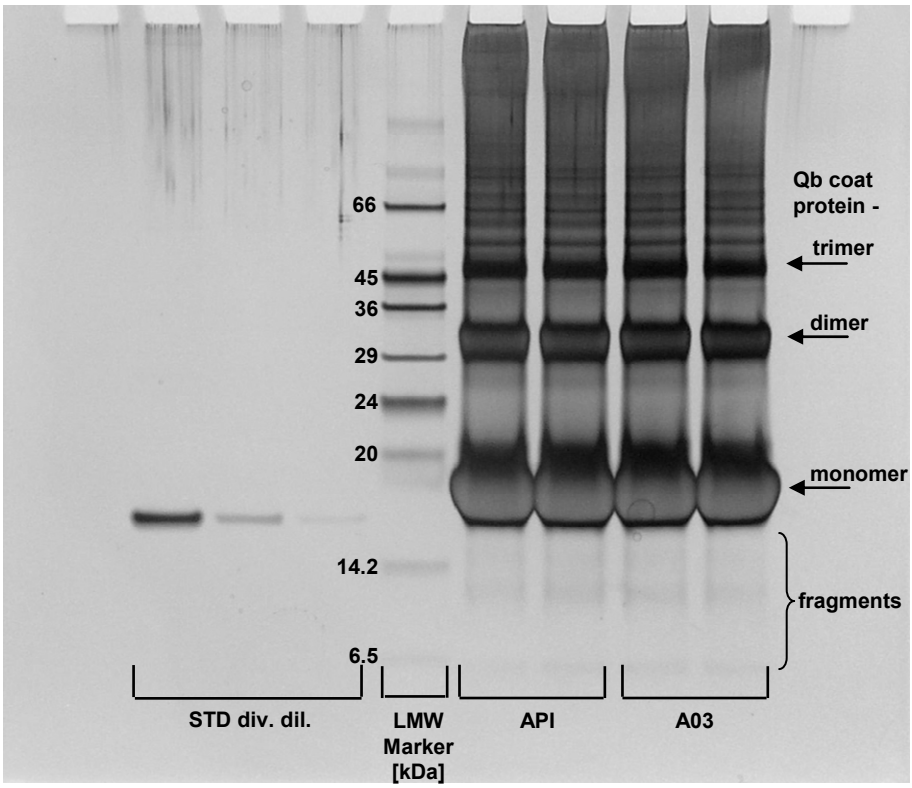


Figure 4.2.31 LDS-PAGE analysis of NicQb formulation A03 after 25 weeks storage at 40 °C / 75 % RH.

As the formulations A03 and A15, which differ only in the pH, showed almost similar results for the amounts of degradation products and free nicotine derivatives it

could be stated that for the dried formulation the pH effect is not that distinctive as for liquid formulations (compare pH stability study). The study revealed that lowering the pH in formulation A15 (pH 5.8) as compared to the lead formulation A03 (pH 6.2) led to no further improvement of the chemical stability of NicQb.

Conclusion

It was found that all formulations tested were capable of stabilizing NicQb during freeze-drying. Physical as well as chemical stability of NicQb was maintained throughout the performed freeze-drying processes. Stable lyophilizates with excellent appearance and moisture contents below 0.5 % were produced.

Upon storage over 15 weeks (formulations A06 and A16) and 25 weeks (formulations A03 and A15), respectively, at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH for all formulations no significant changes concerning the physical stability of NicQb could be observed. Concerning the chemical stability of NicQb it was found that all formulations were capable of preserving the integrity of the VLP, the incorporated RNA and the Qb coat proteins upon the whole time span, independent of the storage temperature. In view of the stability of the bond between nicotine and the Qb VLP only a marginal increase of the amount of nicotine derivatives, cleaved from the VLP surface, could be detected for the samples stored at 40 °C which could be traced back to the slight increase of the residual moisture content. However, the proportion of cleaved nicotine was for all formulations still below 1.5 %.

With respect to the morphology of the lyophilizates it was found that the trehalose based formulations remained amorphous upon storage at all temperatures whereas the mannitol / trehalose based formulation led to an uncontrolled crystallization of mannitol upon storage at 40 °C. Even though the stability of NicQb was not affected by this uncontrolled crystallization of mannitol such uncontrolled changes are undesirable. Therefore, the freeze-drying process applied for this formulation should be optimized so that mannitol crystallizes completely in a controlled manner during the process.

Finally, it was concluded that all formulations were suitable candidates for the stabilization of NicQb during freeze-drying and upon storage, even at ambient temperature.

4.2.5 Bioactivity Testing of NicQb Lyophilizates

The stability study revealed that the formulations A03, A06 and A15 were capable of stabilizing NicQb upon storage. Nevertheless, the retention of biological activity had to be verified. As the three formulations were comparable concerning the physical and chemical stability of NicQb, only formulation A03 was assessed for bioactivity. The bulk material (used as internal standard, stored at -80 °C), and lyophilized samples (frozen at -20 °C after manufacture) were compared to samples stored at 2-8 °C, 25 °C and 40 °C for 41 weeks. The respective antibody titers (dose of 100 µg) are shown in Figure 4.2.32.

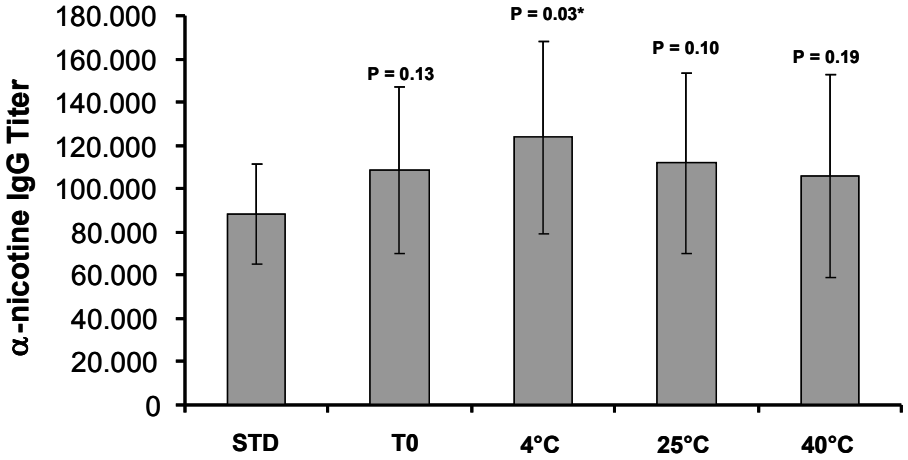


Figure 4.2.32 Bioassay analysis of NicQb formulation A03 upon immunisation of mice with 100 µg of formulated product stored at different temperatures for 41 weeks. P-values obtained by comparison with the bulk material (defined as internal standard STD) and formulated product stored at -20 °C (T0) using an unpaired two-tailed t-test with a confidence interval of 95 % are shown on top of each bar. Differences identified as significant are marked by asterisk (*).

Upon immunization with 100 µg NicQb none of the samples stored for 41 weeks, independent of storage temperature, showed loss of activity in comparison to the material right after manufacture (Figure 4.2.32). An unpaired two-tailed t-test (confidence interval 95 %) performed for the results of each preparation in comparison to the bulk material also showed no significant differences, except for A03 stored at 2-8 °C, with a p-value only slightly below 0.05. In this case an even higher antibody titer was determined for the lyophilisate as compared to the standard.

Finally, it could be concluded that even long-term storage of NicQb formulation A03 at accelerated temperatures did not result in lower antibody titers in comparison to the bulk material and the formulation right after freeze-drying.

4.2.6 Optimization of Freeze-Drying Process for Mannitol / Trehalose Based Formulation and Study of the Effect of a Pure Crystalline Formulation on the Stability of NicQb

Data obtained during the stability study for formulation A16 with mannitol as bulking agent and trehalose as lyoprotectant revealed that this composition was capable of stabilizing NicQb during freeze-drying and storage at 2-8 °C and 25 °C over 15 weeks. However, upon storage at 40 °C uncontrolled crystallization of the partially amorphous mannitol was observed. Thus, the aim of this experiment was to optimize the freeze-drying process to ensure controlled, complete mannitol crystallization during the freeze-drying process. This can be achieved by applying an annealing step, a thermal treatment step in which samples are maintained at a specific subfreezing temperature for a period of time [Izutsu et al., 1993; Lueckel et al., 1998a; Searles et al., 2001; Lu et al., 2004]. Hence, formulation A16 was freeze-dried according to two freeze-drying protocols: without (FD protocol B, chapter 2) and with (FD protocol C, chapter 2) a 2 hour annealing step at -15 °C. Additionally, for a better understanding of the effect of an amorphous matrix in comparison to a crystalline or partially crystalline matrix on the stability of NicQb a further formulation (A17), composed of 1 mg / mL NicQb, 5 % mannitol, 0.005 % polysorbate 20 and 20 mM sodium phosphate (pH 6.2) was freeze-dried according to freeze-drying protocol C (chapter 2). The stability of the drug and the physical state of all formulations were investigated right after manufacture and after storage for 6 weeks at 2-8 °C, 25 °C and 40 °C.

Characterization of the physical state of the lyophilizates

After freeze-drying all formulations showed excellent appearance which was not altered upon storage. The residual moisture content of all formulations was initially below 0.5 % and increased only slightly upon storage. Even after storage for 6 weeks at 40 °C the water content was still below 0.8 % (Figure 4.2.33).

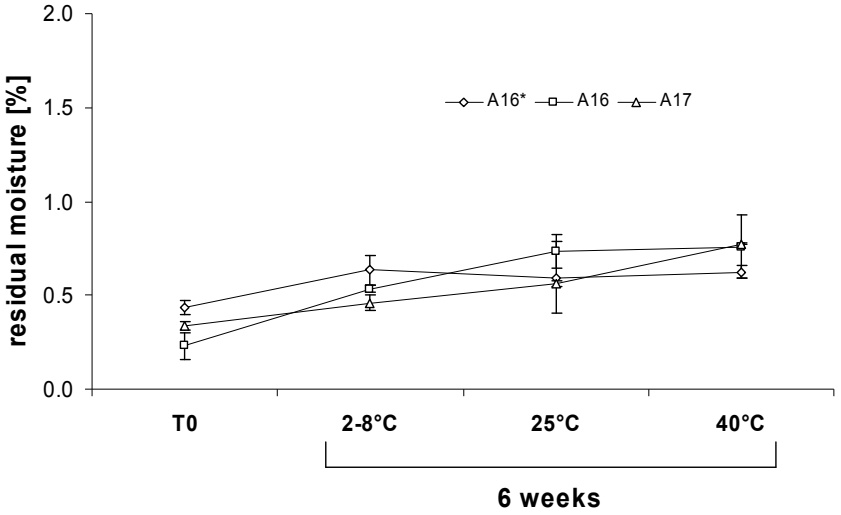


Figure 4.2.33 Residual moisture, determined by KF titration, in NicQb formulations A16 prepared by applying FD protocol B (marked with an asterisk) and by applying FD protocol C (without asterisk) and A17 (freeze-dried by using FD protocol C) right after freeze-drying and after storage for 6 weeks.

All dried formulations showed a typical XRD peak pattern for crystalline mannitol. For formulation A17 peaks of all mannitol modifications (α-, β- and δ-mannitol) were detected, whereas both A16 formulations, independent of implementation of an annealing step, showed solely the typical peaks for δ-mannitol [Kim et al., 1998; Hawe et al., 2006] (Figure 4.2.34). The formation of δ-modification, which is described to be less stable than the α- and β- modifications [Yu, 2003], might be provoked by the cosolute trehalose [Kim et al., 1998].

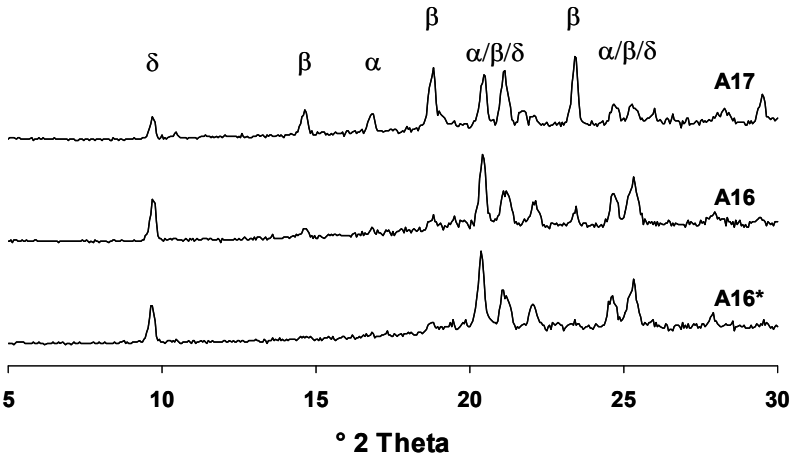


Figure 4.2.34 XRD diffractograms of NicQb formulations A16 prepared by applying FD protocol B (marked with an asterisk) and by applying FD protocol C (without asterisk) and A17 (freeze-dried by using FD protocol C) right after freeze-drying.

The DSC measurements revealed that mannitol in formulation A16 was only partially crystalline when freeze-dried without annealing whereas it was completely crystalline when an annealing step was applied. The first DSC heating scan of the non-annealed material showed an exothermal event which could be referred to the crystallization of amorphous mannitol [Kim et al., 1998]. This crystallization peak of mannitol was not detected for the lyophilizates of the formulations A16 and A17 which were produced according to freeze-drying protocol C including an annealing step (Figure 4.2.35).

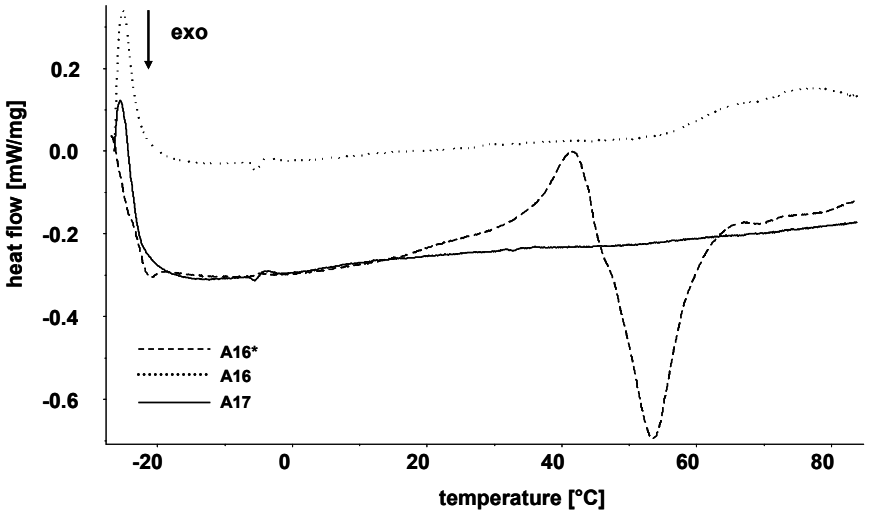


Figure 4.2.35 First heating DSC scan of NicQb formulations A16 prepared by applying FD protocol B (marked with an asterisk) and by applying FD protocol C (without asterisk) and A17 (freeze-dried by using FD protocol C) right after freeze-drying.

XRD measurements performed after 6 weeks storage showed no changes in the peak pattern, independent of storage temperature, for any of the formulations tested (data not shown). By contrast, DSC measurement revealed that, according to the findings from the former stability study, the partially amorphous mannitol in formulation A16 produced with FD protocol B crystallized completely upon storage at 40 °C. However, formulations A16 and A17 produced with FD protocol C exhibited no changes in the thermal behavior. Thus, it was concluded that applying an annealing step was necessary to achieve complete mannitol crystallization during freeze-drying and to prevent uncontrolled crystallization of mannitol upon storage.

Stability of NicQb

Concerning the physical stability of NicQb it was found that independent from applying an annealing step formulation A16 was capable of stabilizing the drug during freeze-drying and upon storage for 6 weeks at 2-8 °C, 25 °C and 40 °C. AF4 results revealed only a slight increase of the aggregation level during freeze-drying and upon storage (Figure 4.2.36). The preservation of the physical stability of NicQb by formulation A16 might be achieved by the prevention of direct interactions of the drug within the amorphous disaccharide portion as proposed for example by Chatterjee et al. and Johnson et al. [Johnson et al., 2002; Chatterjee et al., 2005]. By contrast, for formulation A17 an increase of the amount of aggregates was observed which was even more pronounced after storage at 25 °C and 40 °C, respectively (Figure 4.2.36). This observation can be explained by the findings of Izutsu et al. and Lueckel et al. who described that potentially protective interactions of mannitol with a labile drug are lost by crystallization of mannitol upon freeze-drying. Furthermore, it is described that crystallization of mannitol can also change the degree of freeze-concentration which in turn can lead to drug aggregation upon processing and storage [Izutsu et al., 1994; Lueckel et al., 1998b].

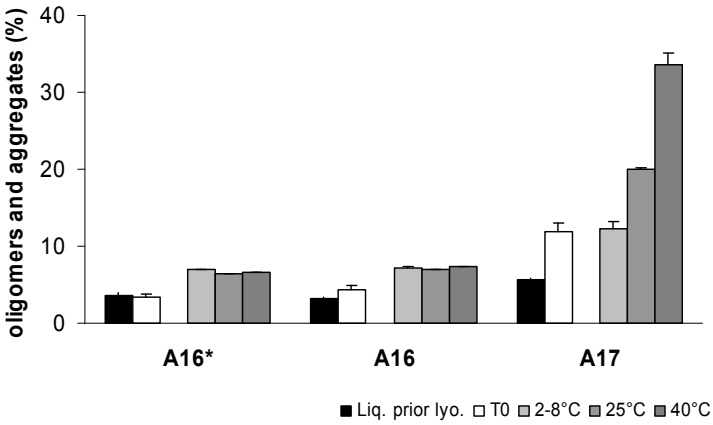


Figure 4.2.36 Amount of NicQb oligomers and aggregates, determined by AF4, in NicQb formulations A16 prepared by applying FD protocol B (marked with an asterisk) and by applying FD protocol C (without asterisk) and A17 (freeze dried by using FD protocol C) right after freeze-drying and after storage for 6 weeks.

With respect to the chemical stability of NicQb SE-HPLC and RP-HPLC measurements indicated that the stability of NicQb was preserved during freeze-drying and upon storage by formulation A16, independent of the applied FD

protocol, whereas for formulation an increase of NicQb fragments and of the amount of free nicotine derivatives was detected after storage at 40 °C (Figure 4.2.37 and Figure 4.2.38). Interestingly, the increase of the free nicotine derivatives in formulation A17 seemed not to be solely dependent on the residual water content as all formulations revealed comparable amount of residual moisture. It appeared that the instability of the bond between nicotine and the Qb VLP is closely related to the aggregation NicQb. One explanation might be an autocatalytic hydrolysis of the esterbond between nicotine and the succinyl linker enabled by the accumulation of NicQb, and thus, possible direct chemical interactions.

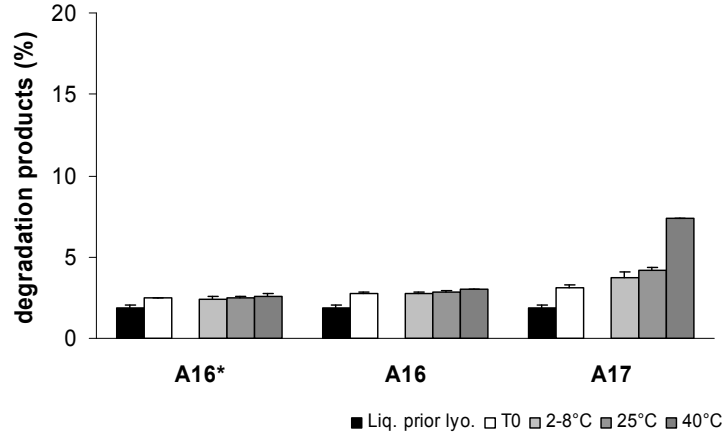


Figure 4.2.37 Amount of NicQb fragments, determined by SE-HPLC, in NicQb formulations A16 prepared by applying FD protocol B (marked with an asterisk) and by applying FD protocol C (without asterisk) and A17 (freeze-dried by using FD protocol C) right after freeze-drying and after storage for 6 weeks.

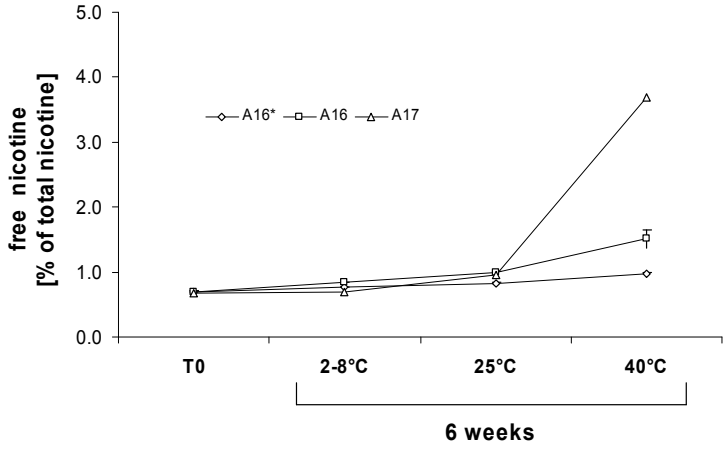


Figure 4.2.38 Amount of free nicotine derivatives, determined by RP-HPLC, in NicQb formulations A16 prepared by applying FD protocol B (marked with an asterisk) and by applying FD protocol C (without asterisk) and A17 (freeze-dried by using FD protocol C) right after freeze-drying and after storage for 6 weeks.

The integrity of the RNA incorporated in the VLP, determined by Bioanalyzer, remained stable for all formulations and storage condition, all samples showed the same RNA pattern. Furthermore, LDS-PAGE analysis revealed that the integrity of the Qb coat proteins was not affected by any of the formulations independent of the storage conditions, similar Qb coat protein band profiles were obtained for all samples.

Conclusion

It can be stated that a completely crystalline matrix was not capable of stabilizing NicQb during freeze-drying and upon storage. This observation is in accordance to the findings of Izutsu et al. and Chang et al. who proposed that the implementation of an amorphous lyoprotectant is indispensable for the protection of a labile drug [Izutsu et al., 1994; Chang et al., 1996]. On the other hand the combination of crystalline mannitol as bulking agent, leading to physically remarkably stable lyophilizates with excellent appearance, and amorphous trehalose, which stabilizes the drug, seemed to be a further promising formulation for NicQb. The performance of an annealing step led to complete crystallization of mannitol during the freeze-drying process, and thus the initially observed, unwanted uncontrolled crystallization of mannitol upon storage could be resolved.

4.3 Summary

During the present work the development of stable NicQb lyophilizates meeting the requirements for large scale clinical study and commercial use was demonstrated. Due to the well structured study setup from pH, freeze-thaw, freeze-drying and finally long-term stability studies a very fast progress towards a commercializable product was feasible.

A grid of parameters critical for stabilizing the VLP based vaccine NicQb during freeze-drying and storage at 2-8 °C and ambient temperature was determined. It was found that an amorphous disaccharide matrix was suitable for the stabilization of NicQb. The addition of polysorbate 20 was very beneficial for preventing aggregation of the VLP. Furthermore, it was shown that combined formulations of a crystalline bulking agent together with an amorphous disaccharide can also be applied for the manufacture of stable freeze-dried NicQb formulations. The freeze-dried formulations showed all excellent appearance, were composed of approved excipients, and could easily be reconstituted to parenterally applicable isotonic liquids.

Finally, full retention of biological activity of VLP lyophilizates, even after long-term storage, was demonstrated.

Furthermore, robust, short freeze-drying processes which can easily be transferred to large-scale production freeze-dryers were developed.

.

4.4 References

Adams, G. D. J., and Ramsay, J. R.; Optimizing the Lyophilization Cycle and the Consequences of Collapse on the Pharmaceutical Acceptability of Erwinia L-Asparaginase; *Journal of Pharmaceutical Sciences*, 1996, 85(12), 1301-1305

Adebayo, A. A., Sim-Brandenburg, J. W., Emmel, H., Olaleye, D. O., and Niedrig, M.; Stability of 17D yellow fever virus vaccine using different stabilizers; *Biologicals*, 1998, 26(4), 309-316

Allison, S. D., Molina, M., and Anchordoquy, T. J.; Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis; *Biochimica et Biophysica Acta, Biomembranes*, 2000, 1468(1-2), 127-138

Allison, S. D., and Anchordoquy, T. J.; Mechanisms of protection of cationic lipid-DNA complexes during lyophilization; *Journal of Pharmaceutical Sciences*, 2000, 89(5), 682-691

Allison, S. D., Chang, B., Randolph, T. W., and Carpenter, J. F.; Hydrogen Bonding between Sugar and Protein Is Responsible for Inhibition of Dehydration-Induced Protein Unfolding; *Archives of Biochemistry and Biophysics*, 1999, 365(2), 289-298

Ameri, M., and Maa, Y. F.; Spray drying of biopharmaceuticals: stability and process considerations; *Drying Technology*, 2006, 24(6), 763-768

Anchordoquy, T. J., and Carpenter, J. F.; Polymers protect lactate dehydrogenase during freeze-drying by inhibiting dissociation in the frozen state; *Archives of Biochemistry and Biophysics*, 1996a, 332(2), 231-238

Anchordoquy, T. J., Izutsu, K. I., Randolph, T. W., and Carpenter, J. F.; Maintenance of Quaternary Structure in the Frozen State Stabilizes Lactate Dehydrogenase during Freeze-Drying; *Archives of Biochemistry and Biophysics*, 2001, 390(1), 35-41

Arakawa, T., Kita, Y., and Carpenter, J. F.; Protein--solvent interactions in pharmaceutical formulations; *Pharmaceutical Research*, 1991, 8(3), 285-291

Arakawa, T., Prestrelski, S. J., Kenney, W. C., and Carpenter, J. F.; Factors affecting short-term and long-term stabilities of proteins; *Advanced Drug Delivery Reviews*, 2001, 46(1-3), 307-326

Arakawa, T., and Timasheff, S. N.; Preferential interactions of proteins with salts in concentrated solutions; *Biochemistry*, 1982, 21(25), 6545-6552

Bam, N. B., Randolph, T. W., and Cleland, J. L.; Stability of protein formulations: investigation of surfactant effects by a novel EPR spectroscopic technique; *Pharmaceutical Research*, 1995, 12(1), 2-11

Abdul-Fattah, A. M., Truong-Le, V., Yee, L., Pan, E., Ao, Y., Kalonia, D. S., and Pikal, M. J.; Drying-Induced Variations in Physico-Chemical Properties of Amorphous Pharmaceuticals and Their Impact on Stability II: Stability of a Vaccine; *Pharmaceutical Research*, 2007, 24(4), 715-727

Bhatnagar, B. S., Bogner, R. H., and Pikal, M. J.; Protein Stability During Freezing: Separation of Stresses and Mechanisms of Protein Stabilization; *Pharmaceutical Development and Technology*, 2007, 12(5), 505-523

Boyd, B.; NicVAX: Aid to smoking cessation nicotine vaccine; *Drugs of the Future*, 2006, 31(3), 203-205

Brandau, D. T., Jones, L. S., Wiethoff, C. M., Rexroad, J., and Middaugh, C. R.; Thermal stability of vaccines; *Journal of Pharmaceutical Sciences*, 2003, 92(2), 218-231

Brange, J., Andersen, L., Laursen, E. D., Meyn, G., and Rasmussen, E.; Toward Understanding Insulin Fibrillation; *Journal of Pharmaceutical Sciences*, 1997, 86(5), 517-525

Breen, E. D., Curley, J. G., Overcashier, D. E., Hsu, C. C., and Shire, S. J.; Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation; *Pharmaceutical Research*, 2001, 18(9), 1345-1353

Burger, A., Henck, J. O., Hetz, S., Rollinger, J. M., Weissnicht, A. A., and Stottner, H.; Energy/temperature diagram and compression behavior of the polymorphs of D-mannitol; *Journal of Pharmaceutical Sciences*, 2000, 89(4), 457-468

Carpenter, J. F., and Crowe, J. H.; An infrared spectroscopic study of the interactions of carbohydrates with dried proteins; *Biochemistry*, 1989, 28(9), 3916-3922

Carpenter, J. F., Crowe, J. H., and Arakawa, T.; Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states; *Journal of Dairy Science*, 1990, 73(12), 3627-3636

Carpenter, J. F., Izutsu, K. I., and Randolph, T. W.; Freezing- and drying-induced perturbations of protein structure and mechanisms of protein protection by stabilizing additives; *Drugs and the Pharmaceutical Sciences*, 1999, 96, 123-160

Carpenter, J. F., Manning, M. C., and Editors.; Rational Design of Stable Protein Formulations: Theory and Practice. *Kluwer Academic / Plenum Publishers, New York*, 2002

Carpenter, J. F., Pikal, M. J., Chang, B. S., and Randolph, T. W.; Rational design of stable lyophilized protein formulations: some practical advice; *Pharmaceutical Research*, 1997, 14(8), 969-975

Cerny, T.; Anti-nicotine vaccination: where are we?; *Recent Results in Cancer Research*, 2005, 166, 167-175

Chang, B. S., Kendrick, B. S., and Carpenter, J. F.; Surface-Induced Denaturation of Proteins during Freezing and Its Inhibition by Surfactants; *Journal of Pharmaceutical Sciences*, 1996, 85(12), 1325-1330

Chang, B. S., and Randall, C. S.; Use of subambient thermal analysis to optimize protein lyophilization; *Cryobiology*, 1992, 29(5), 632-656

Chang, B. S., Reeder, G., and Carpenter, J. F.; Development of a stable freeze-dried formulation of recombinant human interleukin-1 receptor antagonist; *Pharmaceutical Research*, 1996, 13(2), 243-249

Chang, L., Shepherd, D., Sun, J., Ouellette, D., Grant, K. L., Tang, X., and Pikal, M. J.; Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix?; *Journal of Pharmaceutical Sciences*, 2005, 94(7), 1427-1444

Chatterjee, K., Shalaev, E. Y., and Suryanarayanan, R.; Partially crystalline systems in lyophilization: II. Withstanding collapse at high primary drying temperatures and impact on protein activity recovery; *Journal of Pharmaceutical Sciences*, 2005, 94(4), 809-820

Chen, T., Fowler, A., and Toner, M.; Literature Review: Supplemented Phase Diagram of the Trehalose-Water Binary Mixture; *Cryobiology*, 2000, 40(3), 277-282

Chi, E. Y., Krishnan, S., Randolph, T. W., and Carpenter, J. F.; Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation; *Pharmaceutical Research*, 2003, 20(9), 1325-1336

Cleland, J. L., Lam, X., Kendrick, B., Yang, J., Yang, T. H., Overcashier, D., Brooks, D., Hsu, C., and Carpenter, J. F.; A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody; *Journal of Pharmaceutical Sciences*, 2001, 90(3), 310-321

Cleland, J. L., Powell, M. F., and Shire, S. J.; The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation; *Critical Reviews in Therapeutic Drug Carrier Systems*, 1993, 10(4), 307-377

Costantino, H. R., Andya, J. D., Nguyen, P. A., Dasovich, N., Sweeney, T. D., Shire, S. J., Hsu, C. C., and Maa, Y. F.; Effect of Mannitol Crystallization on the Stability and Aerosol Performance of a Spray-Dried Pharmaceutical Protein, Recombinant Humanized Anti-IgE Monoclonal Antibody; *Journal of Pharmaceutical Sciences*, 1998, 87(11), 1406-1411

Costantino, H. R., Langer, R., and Klibanov, A. M.; Solid-Phase Aggregation of Proteins under Pharmaceutically Relevant Conditions; *Journal of Pharmaceutical Sciences*, 1994, 83(12), 1662-1669

Costantino, H. R., Pikal, M. J., and Editors.; Lyophilization of Biopharmaceuticals. *American Association of Pharmaceutical Scientists*, 2004

Crowe, J. H., Carpenter, J. F., and Crowe, L. M.; The role of vitrification in anhydrobiosis; *Annual Review of Physiology*, 1998, 60, 73-103

Crowe, J. H., Crowe, L. M., and Carpenter, J. F.; Preserving dry biomaterials: The water replacement hypothesis. Part 2; *BioPharm (Duluth, MN, United States)*, 1993a, 6(4), 40-43

Crowe, J. H., Crowe, L. M., and Carpenter, J. F.; Preserving dry biomaterials: the water replacement hypothesis. Part 1; *BioPharm (Duluth, MN, United States)*, 1993b, 6(3), 28-9, 32

Crowe, L. M., Reid, D. S., and Crowe, J. H.; Is trehalose special for preserving dry biomaterials?; *Biophysical Journal*, 1996, 71(4), 2087-2093

Cruz, P. E., Silva, A. C., Roldao, A., Carmo, M., Carrondo, M. J. T., and Alves, P. M.; Screening of novel excipients for improving the stability of retroviral and adenoviral vectors; *Biotechnology Progress*, 2006, 22(2), 568-576

De Giulio, B., Orlando, P., Barba, G., Coppola, R., De Rosa, M., Sada, A., De Prisco, P. P., and Nazzaro, F.; Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying; *World Journal of Microbiology & Biotechnology*, 2005, 21(5), 739-746

Engel, A., Bendas, G., Wilhelm, F., Mannova, M., Ausborn, M., and Nuhn, P.; Freeze drying of liposomes with free and membrane-bound cryoprotectants - the background of protection and damaging processes; *International Journal of Pharmaceutics*, 1994, 107(2), 99-110

Evans, R. K., Nawrocki, D. K., Isopi, L. A., Williams, D. M., Casimiro, D. R., Chin, S., Chen, M., Zhu, D. M., Shiver, J. W., and Volkin, D. B.; Development of stable liquid formulations for adenovirus-based vaccines; *Journal of Pharmaceutical Sciences*, 2004, 93(10), 2458-2475

Fox, K. C.; Putting proteins under glass; *Science (Washington, D.C.)*, 1995, 267(5206), 1922-1923

Franks, F.; Freeze-drying of bioproducts: putting principles into practice; *European Journal of Pharmaceutics and Biopharmaceutics*, 1998, 45(3), 221-229

Franks, F.; Long-term stabilization of biologicals; *Biotechnology (N Y)*, 1994, 12(3), 253-256

Franks, F., Hatley, R. H. M., and Mathias, S. F.; Materials science and the production of shelf-stable biologicals; *BioPharm (Duluth, MN, United States)*, 1991, 4(9), 38- 55

Green, J. L., and Angell, C. A.; Phase relations and vitrification in saccharide-water solutions and the trehalose anomaly; *Journal of Physical Chemistry*, 1989, 93(8), 2880-2882

Gupta, C. K., Leszczynski, J., Gupta, R. K., and Siber, G. R.; Stabilization of respiratory syncytial virus (RSV) against thermal inactivation and freeze-thaw cycles for development and control of RSV vaccines and immune globulin; *Vaccine*, 1996, 14(15), 1417-1420

Hageman, M. J.; Water sorption and solid-state stability of proteins; *Pharmaceutical Biotechnology*, 1992, 2, 273-309

Hancock, B. C., Shamblin, S. L., and Zografi, G.; Molecular mobility of amorphous pharmaceutical solids below their glass transition temperatures; *Pharmaceutical Research*, 1995, 12(6), 799-806

Hancock, B. C., and Zografi, G.; Characteristics and Significance of the Amorphous State in Pharmaceutical Systems; *Journal of Pharmaceutical Sciences*, 1997, 86(1), 1-12

Harris, R. J., Shire, S. J., and Winter, C.; Commercial manufacturing scale formulation and analytical characterization of therapeutic recombinant antibodies; *Drug Development Research*, 2004, 61(3), 137-154

Hatley, R. H. M., Franks, F., Brown, S., Sandhu, G., and Gray, M.; Stabilization of a pharmaceutical drug substance by freeze-drying: A case study; *Drug Stability*, 1996, 1(2), 73-85

Hawe, A., and Friess, W.; Physico-chemical lyophilization behavior of mannitol, human serum albumin formulations; *European Journal of Pharmaceutical Sciences*, 2006, 28(3), 224-232

Heller, M. C., Carpenter, J. F., and Randolph, T. W.; Protein formulation and lyophilization cycle design: Prevention of damage due to freeze-concentration induced phase separation; *Biotechnology and Bioengineering*, 1999, 63(2), 166-174

Her, L. M., Deras, M., and Nail, S. L.; Electrolyte-induced changes in glass transition temperatures of freeze-concentrated solutes; *Pharmaceutical Research*, 1995, 12(5), 768-772

Higashiyama, T.; Novel functions and applications of trehalose; *Pure and Applied Chemistry*, 2002, 74(7), 1263-1269

Hinrichs, W. L. J., Sanders, N. N., De Smedt, S. C., Demeester, J., and Frijlink, H. W.; Inulin is a promising cryo- and lyoprotectant for PEGylated lipoplexes; *Journal of Controlled Release*, 2005, 103(2), 465-479

Izutsu, K., and Kojima, S.; Excipient crystallinity and its protein-structure-stabilizing effect during freeze-drying; *Journal of Pharmacy and Pharmacology*, 2002, 54(8), 1033-1039

Izutsu, K., Yoshioka, S., and Terao, T.; Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying; *Chemical & Pharmaceutical Bulletin*, 1994, 42(1), 5-8

Izutsu, K., Yoshioka, S., and Terao, T.; Decreased protein-stabilizing effects of cryoprotectants due to crystallization; *Pharmaceutical Research*, 1993, 10(8), 1232-1237

Jiang, S., and Nail, S. L.; Effect of process conditions on recovery of protein activity after freezing and freeze-drying; *European Journal of Pharmaceutics and Biopharmaceutics*, 1998, 45(3), 249-257

Jiskoot, W., and Crommelin, J. A.; Methods for Structural Analysis of Protein *Pharmaceuticals. American Association of Pharmaceutical Scientists*, 2005

Johnson, R. E., Kirchhoff, C. F., and Gaud, H. T.; Mannitol-sucrose mixtures-versatile formulations for protein lyophilization; *Journal of Pharmaceutical Sciences*, 2002, 91(4), 914-922

Jones, L. S., Randolph, T. W., Kohnert, U., Papadimitriou, A., Winter, G., Hagmann, M. L., Manning, M. C., and Carpenter, J. F.; The effects of Tween 20 and sucrose on the stability of anti-L-selectin during lyophilization and reconstitution; *Journal of Pharmaceutical Sciences*, 2001, 90(10), 1466-1477

Kim, A. I., Akers, M. J., and Nail, S. L.; The Physical State of Mannitol after Freeze-Drying: Effects of Mannitol Concentration, Freezing Rate, and a Noncrystallizing Cosolute; *Journal of Pharmaceutical Sciences*, 1998, 87(8), 931-935

Klibanov, A. M., and Schefiliti, J. A.; On the relationship between conformation and stability in solid pharmaceutical protein formulations; *Biotechnology Letters*, 2004, 26(14), 1103-1106

Kreilgaard, L., Jones, L. S., Randolph, T. W., Frokjaer, S., Flink, J. M., Manning, M. C., and Carpenter, J. F.; Effect of Tween 20 on Freeze-Thawing- and Agitation-Induced Aggregation of Recombinant Human Factor XIII; *Journal of Pharmaceutical Sciences*, 1998, 87(12), 1597-1603

Lai, M. C., and Topp, E. M.; Solid-State Chemical Stability of Proteins and Peptides; *Journal of Pharmaceutical Sciences*, 1999, 88(5), 489-500

Lee, G.; Spray-drying of proteins; *Pharmaceutical Biotechnology*, 2002, 13, 135-158

Levine, H., and Slade, L.; Principles of "cryostabilization" technology from structure/property relationships of carbohydrate/water systems - a review; *Cryo-Letters*, 1988, 9(1), 21-63

Liao, X., Krishnamurthy, R., and Suryanarayanan, R.; Influence of the Active Pharmaceutical Ingredient Concentration on the Physical State of Mannitol-Implications in Freeze-Drying; *Pharmaceutical Research*, 2005, 22(11), 1978-1985

Liu, W. R., Langer, R., and Klibanov, A. M.; Moisture-induced aggregation of lyophilized proteins in the solid state; *Biotechnology and Bioengineering*, 1991, 37(2), 177-184

Lu, X., and Pikal, M. J.; Freeze-drying of mannitol-trehalose-sodium chloride-based formulations: The impact of annealing on dry layer resistance to mass transfer and cake structure; *Pharmaceutical Development and Technology*, 2004, 9(1), 85-95

Lueckel, B., Bodmer, D., Helk, B., and Leuenberger, H.; Formulations of sugars with amino acids or mannitol-influence of concentration ratio on the properties of the freeze-concentrate and the lyophilizate; *Pharmaceutical Development and Technology*, 1998a, 3(3), 325-336

Lueckel, B., Helk, B., Bodmer, D., and Leuenberger, H.; Effects of formulation and process variables on the aggregation of freeze-dried interleukin-6 (IL-6) after lyophilization and on storage; *Pharmaceutical Development and Technology*, 1998b, 3(3), 337-346

Ma, X., Wang, D. Q., Bouffard, R., and MacKenzie, A.; Characterization of murine monoclonal antibody to tumor necrosis factor (TNF-MAb) formulation for freeze-drying cycle development; *Pharmaceutical Research*, 2001, 18(2), 196-202

Maa, Y. F., Nguyen, P. A., Sweeney, T., Shire, S. J., and Hsu, C. C.; Protein inhalation powders: spray drying vs spray freeze drying; *Pharmaceutical Research*, 1999, 16(2), 249-254

Manning, M. C., Patel, K., and Borchardt, R. T.; Stability of protein pharmaceuticals; *Pharmaceutical Research*, 1989, 6(11), 903-918

Mattern, M., Winter, G., Kohnert, U., and Lee, G.; Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems; *Pharmaceutical Development and Technology*, 1999, 4(2), 199-208

Maurer, P., and Bachmann, M. F.; Vaccination against nicotine: An emerging therapy for tobacco dependence; *Expert Opinion on Investigational Drugs*, 2007, 16(11), 1775-1783

Maurer, P., and Bachmann, M. F.; Therapeutic vaccines for nicotine dependence; *Current Opinion in Molecular Therapeutics*, 2006, 8(1), 11-16

Moelwyn-Hughes, E. A.; The kinetics of the hydrolysis of certain glucosides. II. Trehalose, a-methylglucoside and tetramethyl-a-methylglucoside; *Transactions of the Faraday Society*, 1929, 25, 81-92

Molina, M., Armstrong, T. K., Zhang, Y., Patel, M. M., Lentz, Y. K., and Anchordoquy, T. J.; The stability of lyophilized lipid/DNA complexes during prolonged storage; *Journal of Pharmaceutical Sciences*, 2004, 93(9), 2259-2273

Nail, S. L., Akers, M. J., and Editors; Development and Manufacture of Protein Pharmaceuticals. *Kluwer Academic / Plenum Publishers, New York*, 2002

Nail, S. L., Jiang, S., Chongprasert, S., and Knopp, S. A.; Fundamentals of freeze-drying; *Pharmaceutical Biotechnology*, 2002, 14,281-360

Nema, S., and Avis, K. E.; Freeze-thaw studies of a model protein, lactate dehydrogenase, in the presence of cryoprotectants; *Journal of Parenteral Science and Technology*, 1993, 47(2), 76-83

O'Brien, J.; Stability of trehalose, sucrose and glucose to nonenzymic browning in model systems; *Journal of Food Science*, 1996, 61(4), 679-682

Osterberg, T., and Wadsten, T.; Physical state of L-histidine after freeze-drying and long-term storage; *European Journal of Pharmaceutical Sciences*, 1999, 8(4), 301-308

Pikal, M. J.; Freeze-drying of proteins part II: formulation selection; *BioPharm (Duluth, MN, United States)*, 1990, 3(9), 26-30

Pikal, M. J.; Mechanisms of protein stabilization during freeze-drying and storage: the relative importance of thermodynamic stabilization and glassy state relaxation dynamics; *Drugs and the Pharmaceutical Sciences*, 2004, 137, 63-107

Pikal, M. J.; Freeze-drying of proteins: process, formulation, and stability; *ACS Symposium Series*, 1994, 567, 120-133

Pikal, M. J.; Mechanisms of protein stabilization during freeze-drying and storage: the relative importance of thermodynamic stabilization and glassy state relaxation dynamics; *Drugs and the Pharmaceutical Sciences*, 1999, 96, 161-198

Prestrelski, S., Tedeschi, N., Arakawa, T., and Carpenter, J. F.; Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers; *Biophysical Journal*, 1993, 65(2), 661-671

Prestrelski, S. J., Pikal, K. A., and Arakawa, T.; Optimization of lyophilization conditions for recombinant human interleukin-2 by dried-state conformational analysis using Fourier-transform infrared spectroscopy; *Pharmaceutical Research*, 1995, 12(9), 1250-1259

Privalov, P. L.; Cold denaturation of proteins; *Critical Reviews in Biochemistry and Molecular Biology*, 1990, 25(4), 281-305

Rexroad, J., Wiethoff, C. M., Jones, L. S., and Middaugh, C. R.; Lyophilization and the thermostability of vaccines; *Cell Preservation Technology*, 2002, 1(2), 91-104

Roser, B.; Trehalose drying: a novel replacement for freeze-drying; *BioPharm (Duluth, MN, United States)*, 1991, 4(8), 47-53

Rossi, S., Buera, M. P., Moreno, S., and Chirife, J.; Stabilization of the Restriction Enzyme EcoRI Dried with Trehalose and Other Selected Glass-Forming Solutes; *Biotechnology Progress*, 1997, 13(5), 609-616

Sarciaux, J. M., Mansour, S., Hageman, M. J., and Nail, S. L.; Effects of Buffer Composition and Processing Conditions on Aggregation of Bovine IgG during Freeze-Drying; *Journal of Pharmaceutical Sciences*, 1999, 88(12), 1354-1361

Sarkar, J., Sreenivasa, B. P., Singh, R. P., Dhar, P., and Bandyopadhyay, S. K.; Comparative efficacy of various chemical stabilizers on the thermostability of a live-attenuated peste des petits ruminants (PPR) vaccine; *Vaccine*, 2003, 21(32), 4728-4735

Schwegman, J. J., Hardwick, L. M., and Akers, M. J.; Practical formulation and process development of freeze-dried products; *Pharmaceutical Development and Technology*, 2005, 10(2), 151-173

Schwendeman, S. P., Costantino, H. R., Gupta, R. K., Siber, G. R., Klibanov, A. M., and Langer, R.; Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation; *Proceedings of the National Academy of Sciences of the United States of America*, 1995, 92(24), 11234-11238

Scott, E. M., and Woodside, W.; Stability of pseudorabies virus during freeze-drying and storage: effect of suspending media; *Journal of Clinical Microbiology*, 1976, 4(1), 1-5

Searles, J. A., Carpenter, J. F., and Randolph, T. W.; Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine Tg' in pharmaceutical lyophilization; *Journal of Pharmaceutical Sciences*, 2001, 90(7), 872-887

Shalaev, E. Y., Johnson-Elton, T. D., Chang, L., and Pikal, M. J.; Thermophysical properties of pharmaceutically compatible buffers at sub-zero temperatures: implications for freeze-drying; *Pharmaceutical Research*, 2002, 19(2), 195-201

Shalaev, E. Y., and Zografi, G.; How Does Residual Water Affect the Solid-State Degradation of Drugs in the Amorphous State?; *Journal of Pharmaceutical Sciences*, 1996, 85(11), 1137-1141

Sharma, V. K., and Kalonia, D. S.; Effect of vacuum drying on protein-mannitol interactions: the physical state of mannitol and protein structure in the dried state; *AAPS PharmSciTech*, 2004, 5(1), 58-69

Shi, L., Sanyal, G., Ni, A., Luo, Z., Doshna, S., Wang, B., Graham, T. L., Wang, N., and Volkin, D. B.; Stabilization of human papillomavirus virus-like particles by non-ionic surfactants; *Journal of Pharmaceutical Sciences*, 2005, 94(7), 1538-1551

Slade, L., and Harry Levine; Water and the Glass Transition - Dependence of the Glass Transition on Compostion and Chemical Structure: Special Implications for Flour Functionality in Cookie Baking; *Journal of Food Engineering*, 1995, 24, 431-509

Song, Y., Schowen, R. L., Borchardt, R. T., and Topp, E. M.; Effect of 'pH' on the rate of asparagine deamidation in polymeric formulations: "pH"-rate profile; *Journal of Pharmaceutical Sciences*, 2001, 90(2), 141-156

Talsma, H., Cherng, J. Y., Lehrmann, H., Kursa, M., Ogris, M., Hennink, W. E., Cotten, M., and Wagner, E.; Stabilization of gene-delivery systems by freeze-drying; *International Journal of Pharmaceutics*, 1997, 157(2), 233-238

Tang, X., and Pikal, M. J.; Design of freeze-drying processes for pharmaceuticals: practical advice; *Pharmaceutical Research*, 2004, 21(2), 191-200

Townsend, M. W., and DeLuca, P. P.; Stability of ribonuclease A in solution and the freeze-dried state; *Journal of Pharmaceutical Sciences*, 1990, 79(12), 1083-1086

Vaughan, H., Chalker, V. J., Mee, Z., Rossouw, A., and James, V.; Stability of lyophilised specimens for the molecular detection of viral DNA/RNA; *Journal of Clinical Virology*, 2006, 35(2), 135-140

Volkin, D. B., Burke, C. J., Sanyal, G., and Middaugh, C. R.; Analysis of vaccine stability; *Developments in Biological Standardization*, 1996, 87, 135-142

Wang, W.; Lyophilization and development of solid protein pharmaceuticals; *International Journal of Pharmaceutics*, 2000, 203(1-2), 1-60

Wang, W.; Protein aggregation and its inhibition in biopharmaceutics; *International Journal of Pharmaceutics*, 2005, 289(1-2), 1-30

Worrall, E. E., Litamoi, J. K., Seck, B. M., and Ayelet, G.; Xerovac: an ultra rapid method for the dehydration and preservation of live attenuated Rinderpest and Peste des Petits ruminants vaccines. *Vaccine*, 2001, 19(28-29), 4086

Yu, L.; Amorphous pharmaceutical solids: preparation, characterization and stabilization; *Advanced Drug Delivery Reviews*, 2001, 48(1), 27-42

Yu, L.; Nucleation of One Polymorph by Another; *Journal of the American Chemical Society*, 2003, 125(21), 6380-6381

Yu, L., Milton, N., Groleau, E. G., Mishra, D. S., and Vansickle, R. E.; Existence of a Mannitol Hydrate during Freeze-Drying and Practical Implications; *Journal of Pharmaceutical Sciences*, 1999, 88(2), 196-198

Zhai, S., Hansen, R. K., Taylor, R., Skepper, J. N., Sanches, R., and Slater, N. K. H.; Effect of Freezing Rates and Excipients on the Infectivity of a Live Viral Vaccine during Lyophilization; *Biotechnology Progress*, 2004, 20(4), 1113-1120

Zillies, J.; Gelatin Nanoparticles for Targeted Oligonucleotide Delivery to Kupffer Cells - Analytics, Formulation Development, Dissertation, Ludwig-Maximilians-University Munich, 2007

Zingel, C., Sachse, A., Roessling, G. L., and Mueller, R. H.; Lyophilization and rehydration of iopromide-carrying liposomes; *International Journal of Pharmaceutics*, 1996, 140(1), 13-24

4.5 Annex

Table 4.5.1 Amount of NicQb oligomers and aggregates, determined by AF4, in NicQb formulations prior to freeze-drying, freeze-dried NicQb formulations right after freeze-drying and after storage for 6, 15 and 25 weeks, respectively, at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH.

Formulation	Oligomers and aggregates [%]										
	Start		6 weeks			15 weeks			25 weeks		
	Liquid	LYO	2-8°C	25°C	40°C	2-8°C	25°C	40°C	2-8°C	25°C	40°C
A03	5.03 ± 0.19	5.13 ± 0.25	6.10 ± 0.00	6.35 ±0.07	6.55 ± 0.35	7.05 ± 0.07	6.15 ± 0.21	6.60 ± 0.00	8.00 ± 1.27	7.80 ± 0.42	7.10 ± 0.14
A06	6.13 ± 0.78	5.77 ± 0.12	6.50 ± 0.28	6.20 ±0.57	6.37 ± 0.67	7.20 ± 0.42	7.00 ± 0.14	6.60 ± 0.00	n/a	n/a	n/a
A15	5.43 ± 0.13	5.27 ± 0.21	6.95 ± 0.21	6.80 ± 0.28	6.95 ± 0.35	6.90 ± 0.14	7.10 ± 0.14	7.90 ± 0.42	8.35 ± 0.35	7.35 ± 0.07	7.75 ± 0.21
A16	5.13 ± 0.24	5.50 ± 0.00	6.45 ± 0.35	5.90 ± 0.57	6.35 ± 0.49	6.95 ± 0.21	7.15 ± 0.07	6.20 ± 0.14	n/a	n/a	n/a

Table 4.5.2 Amount of degradation products of NicQb, determined by SE-HPLC, in NicQb formulations prior to freeze-drying, freeze-dried NicQb formulations right after freeze-drying and after storage for 6, 15 and 25 weeks, respectively, at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH.

Formulation	Degradation products [%]										
	Start		6 weeks			15 weeks			25 weeks		
	Liquid	LYO	2-8°C	25°C	40°C	2-8°C	25°C	40°C	2-8°C	25°C	40°C
A03	1.54 ± 0.23	2.34 ± 0.07	2.35 ± 0.02	2.34 ±0.02	2.55 ± 0.01	2.40 ± 0.04	2.33 ± 0.01	2.59 ± 0.01	2.75 ± 0.28	2.37 ± 0.10	2.60 ± 0.03
A06	1.57 ± 0.03	1.98 ± 0.04	2.07 ± 0.03	2.02 ±0.01	2.02 ± 0.02	2.13 ± 0.02	2.07 ± 0.01	2.32 ± 0.06	n/a	n/a	n/a
A15	1.54 ± 0.23	2.47 ± 0.01	2.51 ± 0.01	2.47 ± 0.01	2.75 ± 0.00	2.55 ± 0.01	2.51 ± 0.01	2.79 ± 0.01	2.36 ± 0.01	2.37 ± 0.01	2.74 ± 0.01
A16	1.54 ± 0.23	2.69 ± 0.21	2.58 ± 0.25	2.42 ± 0.16	2.83 ± 0.13	3.01 ± 0.47	2.37 ± 0.12	3.04 ± 0.07	n/a	n/a	n/a

Table 4.5.3 Amount of free nicotine derivatives, determined by RP-HPLC, of NicQb formulations prior to freeze-drying, freeze-dried NicQb formulations right after freeze-drying and after storage for 6, 15 and 25 weeks, respectively, at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH.

Formulation	Free nicotine [% of total nicotine]										
	Start		6 weeks			15 weeks			25 weeks		
	Liquid	LYO	2-8°C	25°C	40°C	2-8°C	25°C	40°C	2-8°C	25°C	40°C
A03	<0.20*	<0.20*	0.35 ± 0.00	0.38 ±0.00	0.77 ± 0.02	0.30 ± 0.00	0.41 ± 0.00	1.05 ± 0.06	0.45 ± 0.02	0.61 ± 0.02	1.32 ± 0.02
A06	<0.20*	<0.20*	0.31 ± 0.02	0.38 ±0.00	0.75 ± 0.06	0.31 ± 0.02	0.43 ± 0.00	1.03 ± 0.00	n/a	n/a	n/a
A15	<0.20*	<0.20*	0.31 ± 0.02	0.38 ± 0.00	0.69 ± 0.06	0.29 ± 0.02	0.38 ± 0.00	0.95 ± 0.08	0.45 ± 0.02	0.57 ± 0.00	1.25 ± 0.12
A16	<0.20*	<0.20*	0.34 ± 0.02	0.43 ± 0.04	0.72 ± 0.02	0.33 ± 0.00	0.53 ± 0.06	1.35 ± 0.06	n/a	n/a	n/a

* Amount of nicotine not detectable due to detection limit

Table 4.5.4 Residual moisture content of freeze-dried NicQb formulations right after freeze-drying, and after storage for 6, 15 and 25 weeks, respectively, at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH.

Formulation	Residual moisture [%]									
	Start	6 weeks			15 weeks			25 weeks		
	LYO	2-8°C	25°C	40°C	2-8°C	25°C	40°C	2-8°C	25°C	40°C
A03	0.49 ± 0.00	0.60 ± 0.06	0.63 ±0.07	0.89 ± 0.04	0.56 ± 0.08	0.74 ± 0.08	1.20 ± 0.03	0.66 ± 0.01	0.79 ± 0.04	1.55 ± 0.00
A06	0.49 ± 0.13	0.54 ± 0.04	0.61 ±0.04	0.82 ± 0.04	0.44 ± 0.05	0.65 ± 0.18	1.12 ± 0.01	n/a	n/a	n/a
A15	0.51 ± 0.01	0.56 ± 0.11	0.60 ± 0.01	0.81 ± 0.01	0.52 ± 0.04	0.58 ± 0.01	1.20 ± 0.04	0.52 ± 0.03	0.66 ± 0.01	1.39 ± 0.07
A16	0.15 ± 0.06	0.11 ± 0.06	0.20 ± 0.01	0.52 ± 0.01	0.12 ± 0.04	0.29 ± 0.05	0.94 ± 0.06	n/a	n/a	n/a

Table 4.5.5 Tg (point of inflection) of freeze-dried trehalose based NicQb formulations right after freeze-drying, and after storage for 6, 15 and 25 weeks, respectively, at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH.

Formulation	Glass transition temperature Tg [°C]									
	Start	6 weeks			15 weeks			25 weeks		
	LYO	2-8°C	25°C	40°C	2-8°C	25°C	40°C	2-8°C	25°C	40°C
A03	98.9 ± 0.61	96.75 ± 5.44	96.27 ± 2.15	94.95 ± 4.88	98.2 ± 2.12	96.60 ± 1.84	89.27 ± 1.42	100.25 ± 0.21	97.50 ± 1.84	93.15 ± 1.34
A06	97.13 ± 1.94	97.85 ± 9.83	95.90 ± 0.14	91.05 ± 0.35	100.65 ± 0.07	97.85 ± 3.46	90.65 ± 0.21	100.55 ± 1.91	92.65 ± 2.76	89.25 ± 0.64
A15	96.35 ± 6.01	101.25 ± 3.18	94.83 ± 7.22	96.50 ± 2.40	97.10 ± 0.28	96.85 ± 0.07	90.05 ± 2.47	n/a	n/a	n/a

Table 4.5.6 Number of particles ≥ 1 and ≥ 10 µm, respectively, determined by light obscuration, in NicQb formulations prior to freeze-drying, freshly reconstituted solutions of freeze-dried NicQb formulations right after freeze-drying, and after storage for 6, 15 and 25 weeks, respectively, at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH.

Formulation	Number of particles ≥ 1 µm and ≥ 10 µm / mL							
	Start		6 weeks			15 weeks		
	Liquid	LYO	2-8°C	25°C	40°C	2-8°C	25°C	40°C
A03	1418 ± 856	1105 ± 229	818 ± 190	1190 ± 102	560 ± 354	843 ± 751	623 ± 610	788 ± 666
	30 ± 27	95 ± 53	33 ± 22	58 ± 43	35 ± 31	208 ± 223	33 ± 39	23 ± 15
A06	1425 ± 1102	1140 ± 785	678 ± 179	1225 ± 516	578 ± 158	720 ± 712	958 ± 979	1423 ± 987
	42.5 ± 34	100 ± 70	13 ± 5	18 ± 10	33 ± 15	53 ± 32	33 ± 13	18 ± 13
A15	1980 ±1337	1263 ±722	1463 ± 816	795 ± 500	1173 ± 578	1848 ± 1556	748 ± 551	1308 ± 724
	45 ± 27	50 ± 57	15 ± 6	40 ± 14	30 ± 25	245 ± 178	23 ± 33	48 ± 44
A16	1758 ± 952	788 ± 382	438 ± 107	838 ± 146	570 ± 260	1150 ± 721	1690 ± 1377	1118 ± 295
	25 ± 17	70 ± 35	10 ± 14	38 ± 31	13 ± 5	218 ± 101	13 ± 10	23 ± 10

Table 4.5.7 Proportion of main NicQb peak and polydispersity index of NicQb formulations prior to freeze-drying, freshly reconstituted solutions of freeze-dried NicQb formulations right after freeze-drying, and after storage for 6, 15 and 25 weeks, respectively, at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH.

Formulation	Proportion of main NicQb peak [%] and PI										
	Start		6 weeks			15 weeks			25 weeks		
	Liquid	LYO	2-8°C	25°C	40°C	2-8°C	25°C	40°C	2-8°C	25°C	40°C
A03	98.11 ± 1.74	99.14 ± 0.82	99.05 ± 0.84	98.85 ± 0.70	99.38 ± 0.44	98.63 ± 0.85	98.85 ± 1.02	99.30 ± 0.44	98.58 ± 0.78	98.88 ± 0.37	98.75 ± 0.81
	0.15 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.01	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
A06	99.44 ± 0.38	99.61 ± 0.30	99.39 ± 0.31	99.38 ± 0.32	99.36 ± 0.48	99.10 ± 1.07	98.78 ± 0.43	99.10 ± 1.07	n/a	n/a	n/a
	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.17 ± 0.00	0.14 ± 0.00	0.15 ± 0.00	0.15 ± 0.01	0.15 ± 0.01	n/a	n/a	n/a
A15	97.88 ± 1.05	98.23 ± 1.29	98.67 ± 0.83	99.11 ± 0.25	99.54 ± 0.27	99.45 ± 0.31	98.68 ± 1.99	99.45 ± 0.50	99.55 ± 0.17	99.40 ± 0.24	99.25 ± 0.52
	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.00	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.00	0.17 ± 0.01	0.16 ± 0.01	0.19 ± 0.03
A16	99.27 ± 0.62	98.97 ± 1.06	99.20 ± 0.70	99.78 ± 0.08	99.23 ± 0.74	99.50 ± 0.34	99.30 ± 0.43	99.55 ± 0.26	n/a	n/a	n/a
	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.00	n/a	n/a	n/a

5. DEVELOPMENT OF SUSTAINED RELEASE SYSTEMS FOR VIRUS-LIKE PARTICLE BASED VACCINES

5.1 Introduction

Vaccines aiming on the prevention or therapy of diseases typically require multiple applications to achieve the desired immune response [Bloom, 1989; Cleland, 1999; Kaufmann, 2004]. This frequent dosing is inconvenient with respect to patient's compliance and treatment costs. Hence, the development of single injection vaccine formulations is highly desirable. In 1979 Preis et al. introduced an implant formulation based on the non-degradable copolymer ethylene-vinyl acetate (EVAc), with BSA as model antigen, which was capable of releasing the antigen continuously over several months, and thus, leading to comparably high antibody titers as a multiple application of a liquid BSA formulation [Preis et al., 1979]. Based on these promising experiments great efforts have been taken during the last decades to develop single injection vaccines. A major milestone in this context was, without any doubt, the World Health Programme for Vaccine Development, initiated in the late 1980, with neonatal tetanus as its first target. This initiative clearly accelerated research in the area of controlled delivery devices for antigens, from peptides and proteins to viruses. For example Dewar et al. showed that the immune response of rabbits to HSA microspheres encapsulating Nodamura virus was significantly higher and longer lasting than for the fluid antigen [Dewar et al., 1984]. Similar observations were made by Greenway et al. in a mice model who employed PLGA microcapsules for the immunization against venezuelan equine encephalomyelitis virus [Greenway et al., 1995]. Sturesson et al. encapsulated inactivated rotavirus in PGLA microspheres and demonstrated high antibody levels in mice [Sturesson et al. 2000]. The existing literature on formulation strategies for vaccines is copious, as exemplarily displayed in Table 5.1.1., and even more research work has been done in the field of controlled release formulations for therapeutic peptides and proteins.

However, up to now only a few controlled release devices, all based on poly (lactide) (PLA) and poly (lactide-co-glycolide) (PLGA) are on the market (Lupron® Depot, Zoladex®, Eligard®, Profact® Depot, Enantone® Depot, Sandostatin® LAR

Depot, Trenantone®, Decapeptyl® Depot and Suprefact® Depot), and all of them include peptides rather than proteins and vaccines.

Table 5.1.1 Examples of single-dose vaccine systems.

Delivery system	Vaccine / immunogen	Material	Reference
Implant	Bovine serum albumin	EVAC	[Preis et al., 1979]
	Bovine serum albumin	Cholesterol and lecithin	[Khan et al., 1991]
	Recombinant dichelobacter nodosus pili	Cholesterol and lecithin	[Walduck et al., 1998]
	Avidin and clostridial toxoid	Silicone	[Lofthouse et al., 2002; Kemp et al., 2002]
	Malaria vaccine (SPf66)	PLGA	[Dorta et al., 2002]
	Plasmid DNA	Agarose	[Toussaint et al., 2007]
In situ forming implant	Plasmid DNA	PLGA	[Eliaz et al., 2002]
Minipellet	Tetanus toxoid and diphteria toxoid	Collagen	[Higaki et al., 2001]
Microparticles	Tetanus toxoid	PLA and PLGA	[Esparza et al., 1992; Alonso et al., 1994; Chang et al., 1996; Johansen et al., 1998; Sanchez et al., 1999; Katare et al., 2006]
	Tetanus toxoid	Gelatin / poloxamer core and PLGA shell	[Tobio et al., 1999a]
	Tetanus toxoid	PLGA and ABA-triblock copolymers	[Jung et al., 2002]
	Tetanus toxoid	PLGA and chitosan	[Jaganathan et al., 2005]
	Tetanus toxoid	Mineral oil core and PLGA shell	[Sanchez et al., 1996]
	Diphtheria toxoid and tetanus toxoid	PLA and PLGA	[Johansen et al., 1999; Peyre et al., 2003]
	Haemophilus influenza b conjugate, diphtheria toxoid, tetanus toxoid and pertussis toxoid	PLA and PLGA	[Boehm et al., 2002]

Delivery system	Vaccine / immunogen	Material	Reference
Microparticles	Diphteria toxoid	PLA	[Singh et al., 1991]
	Recombinant HIV envelope protein (MN gp120)	PLGA	[Cleland et al., 1994; Moore et al., 1995; Cleland et al., 1996b; Cleland et al., 1997; Cleland et al., 1998]
	Hepatitis B virus surface antigen	Hydroxypropyl cellulose core and PLGA shell	[Lee et al., 1997]
	Hepatitis B virus surface antigen	PLGA	[Singh et al., 1997; Shi et al., 2002; Feng et al., 2006]
	Hepatitis B virus surface antigen	PELA	[Li et al., 2002]
	Malaria antigen	PLA and PLGA	[Thomasin et al., 1996]
	Malaria antigen	PLA and PCL with phospholipids and lipid A	[Amselem et al., 1992]
	Staphylococcal enterotoxin B	PLGA	[Eldridge et al., 1991]
	Pneumotropic bacterial antigen	PLA and PLGA	[Kofler et al., 1996]
	PEI / DNA complexes	Chitosan	[Domb, 2006; Zhou et al., 2007]
	Nodamura virus	RSA	[Dewar et al., 1984]
	Venezuelan equine encephalitis virus	PLGA	[Greenway et al., 1995]
	Bovine serum albumin	Squalen core and PLGA or PCL shell	[Youan et al., 2001]
	Monovalent influenza vaccine	PLA and PLGA	[Coombes et al., 1998; Hilbert et al., 1999]
	Trivalent influenza vaccine	PLGA and PIBCA	[Chattaraj et al., 1999]
	Inactivated duck parvovirus	PLA and PLGA	[Palinko-Biro et al., 2001]
	Rotavirus vaccine	Poly (acryl starch) and PLGA	[Sturesson et al., 2000]
	Avidin and clostridial toxoid	Lipospheres	[Domb, 2006]

Poly (α-hydroxy esters) PLA and PLGA found most widespread use due to their excellent safety and biocompatibility [Gombotz et al., 1995; van de Weert et al., 2000; Johansen et al., 2000b; Schwendeman, 2002]. Furthermore, applying these polymers tailor-made release kinetics, like continuous or pulsed release, can be achieved by varying parameters like molecular weight of the monomers, PLA to PLGA ratio, size and shape of the final formulation (microparticles, implants), addition of further excipients like pore building substances, e.g. PEG, and different preparation methods [Johansen et al., 2000a; Kang et al., 2001; Sandor et al., 2001; Wang et al., 2002]. This is an important issue as up to now it is still unclear which release profile induces the most potent immune response [Lofthouse, 2002; O'Hagan et al., 2003]. Even though most of the scientists claim that a pulsed release, which mimics the common vaccination schedule, is preferable [Medlicott et al., 1999; Lima et al., 1999; Gander et al., 2001] other studies demonstrated that a continuous release profile also induced high antibody titers [Preis et al., 1979; Cleland et al., 1996a; Toussaint et al., 2007].

Despite these benefits several inherent shortcomings of PLA / PLGA and the resulting detrimental effects on the stability especially of complex drugs like proteins and vaccines during processing, storage and release might explain the limited success of these polymeric delivery systems [Fu et al., 2000; van de Weert et al., 2000; Schwendeman, 2002; Perez et al., 2002; Sinha et al., 2003; Bilati et al., 2005].

The broad spectrum of techniques for the preparation of implants and microparticles and linked drug stability issues are summarized in Table 5.1.2. With the exception of the manufacture of implants by simple compression all other fabrication methods implicate stress factors that may alter drug stability. The major drawback seems to be the use of organic solvents for the dissolution of the polymers leading, in dependence of the applied preparation method, to the formation of water / organic solvent or solid drug / organic solvent interfaces. Antigens, which are typically composed of proteins (substances with an amphiphilic character) tend to adsorb at such interfaces which consequently can result in protein unfolding followed by non-covalent aggregation [Schwendeman et al., 1997; Sanchez et al., 1999; Sah, 1999a; Sah, 1999b; van de Weert et al., 2000].

Table 5.1.2 Common techniques for the preparation of implants and microparticles and linked problems.

Formulation	Preparation	Challenges on drug stability	References
Implants	Direct compression of PLGA and solid drug	-	[Ramchandani et al., 1997; Takahashi et al., 2004; Onishi et al., 2005]
	Solvent casting	Exposure to organic solvent	[Garcia et al., 2002; Dorta et al., 2002; Santovena et al., 2006]
	Molding	Exposure to high temperature	[Park et al., 1995]
	Extrusion	Exposure to high temperature and pressure	[Witt et al., 2000]
In situ forming implants	Implant formation at the injection site	Exposure to organic solvent	[Eliaz et al., 2000; Ravivarapu et al., 2000; Matschke et al., 2002; Packhaeuser et al., 2004]
Microcapsules	Solvent evaporation and extraction	Exposure to organic solvent / water interfaces and shear / cavitation / forces and temperature stress during emulsification in dependence of the applied emulsification process	[Alonso et al., 1994; Greenway et al., 1995; Schwendeman et al., 1996; Schwendeman et al., 1997; Sanchez et al., 1999; Sah, 1999b; van de Weert et al., 2000; Yeo et al., 2001; Shi et al., 2002; Jiang et al., 2004; Freitas et al., 2005; Feng et al., 2006]
	Phase separation	Exposure to organic solvent / water interfaces and shear / cavitation forces	[Esparza et al., 1992; Thomasin et al., 1998; Johansen et al., 1999; Peyre et al., 2003]
	Spray drying	Exposure to organic solvent / water interfaces and shear forces	[Khan et al., 1992; Johansen et al., 1998; Johansen et al., 1999; Jain, 2000; Peyre et al., 2003]
	Supercritical fluid precipitation	Exposure to organic solvent	[Jain et al., 1998; Jain, 2000; Tamber et al., 2005]

In addition to the stress factors identified during processing several detrimental conditions for antigens evolve in PLA / PLGA based systems during release [Chang et al., 1996; Schwendeman et al., 1997; Schwendeman, 2002; Sinha et al., 2003; Jiang et al., 2005]. Poly (α-hydroxy esters) matrices undergo bulk erosion, meaning that polymer degradation is slower than the water uptake of the device. Consequently, the matrix is completely wetted before cleavage of the polymer chains begins [Gombotz et al., 1995; Goepferich, 1996; von Burkersroda et al., 2002]. Hence, upon

incubation the matrix degrades (homogeneously) throughout the device and the acidic degradation products are trapped within the matrix, accumulate, and lead to an altered microenvironment:

(1) pH drop [Zhu et al., 2000; Fu et al., 2000]

(2) Increase of osmotic pressure [Brunner et al., 1999]

(3) Accumulation of reactive species [Lu et al., 1995; Lucke et al., 2002]

These changes in the environment may cause protein unfolding, aggregation and chemical degradation, and thus, lead to a loss of efficacy. Additionally, water uptake of the polymer matrix during incubation leading to an increase in antigen mobility can result in conformational changes of epitopes and consequently to antigen aggregation [Schwendeman et al., 1995; Costantino et al., 1994]. Furthermore, interactions between PLA / PLGA or degradation products thereof with the antigen can cause inactivation of the antigen and incomplete release [Crotts et al., 1997; Johansen et al., 1999; Palinko-Biro et al., 2001].

Thus, to overcome these stability concerns several attempts have been taken. For example, basic additives such as magnesium hydroxide and calcium carbonate were introduced as buffer salts to counteract the pH drop within PLGA devices during hydrolytic polymer degradation [Johansen et al., 1998; Zhu et al., 2000; Zhu et al., 2000]. Other approaches suggest the addition of pore forming substances, like e.g. PEG, or the use of PELA copolymers to ameliorate the potential pH decrease during matrix degradation. Thereby, the porosity of the matrix is increased which in turn accounts for the accelerated diffusion of acidic degradation products out and of buffer components into the device [Jiang et al., 2001; Perez et al., 2002; Zhou et al., 2003].

Another attempt was the development of microcapsule formulations consisting of an inner oily core, in which the antigen is entrapped, surrounded by an outer PLGA shell. The oily core is deemed to protect the antigen against deleterious environmental conditions like moisture and acidic pH and to prevent permeation of PLGA degradation products until the polymer shell is degraded and releases the inner core [Sanchez et al., 1996].

Besides investigations on stabilization strategies within PLGA-based devices several alternative matrix materials for the delivery of proteins have been investigated (Table 5.1.1). Among them especially lipids, e. g. fatty acids, glycerides and waxes, gain more and more attention as matrix formers for parenteral controlled release

devices [Khan et al., 1991; Opdebeeck et al., 1993; Walduck et al., 1998; Mohl et al., 2004; Maschke et al., 2004; Koennings et al., 2005]. Lipids as natural, physiological materials, exhibit an excellent biocompatibility [Reithmeier et al., 2001; Guse et al., 2006]. A potential advantage compared to PLGA is the prevention of an acidic microenvironment, and thus, potentially preservation of drug integrity upon release.

Furthermore, with lipids additional preparation methods such as melt dispersion or spray congealing are applicable for particulate devices which do not require the use of organic solvents [Reithmeier et al., 2001; Maschke et al., 2007]. Due to the excellent compressibility of lipids the preparation of implants can be realized by compression without the employment of heat, what can be beneficial with respect to both production costs and protein stability [Wang, 1989; Mohl et al., 2004]. However, first attempts taken by Kent et al. to develop cholesterol based controlled release devices for macromolecules failed due to the poor release rates which might be related to the low diffusivity of large molecules and the lack of an interconnected pore network [Kent, 1994]. Later on, Mohl et al. demonstrated that by adding PEG as pore forming substance to tristearin implants this drawback could be overcome and release rates up to 50 % for BSA and 90 % for interferon α-2a within 30 days were attainable [Mohl, 2003]. However, a drawback of such lipidic implants is their unclear biodegradability. In vivo experiments revealed lack of erosion for pure triglyceride based devices, so that large devices would need to be removed by surgical recision after completed release [Vogelhuber et al., 2003; Guse et al., 2006]. Nevertheless, there is some information available that drawbacks related to lipid biodegradation can be solved by admixing of amphiphilic lipids, such as phospholipids, to the implant formulation [Khan et al., 1991].

Summing up, lipids are deemed to be promising matrix materials and the potential of lipid-based sustained release systems for the delivery of vaccines might be investigated as alternate to the standard PLGA. In the following chapter preliminary experiments towards the development of sustained release devices for VLP based vaccines are presented. The in vitro release behavior of two model VLP vaccines, AngQb and QbG10p33, from PLGA and lipid based implant formulations was studied and the in vivo efficacy of these provisional sustained release devices was tested in mice. Additionally, basic experiments towards the preparation of an enhanced

sustained release device, PLGA microcapsules with an oily inner core, were performed.

5.2 Results and Discussion

5.2.1 Preparation of VLP Lyophilizates as Starting Material

First, for the preparation of sustained release devices for VLP the designated VLP candidates had to be freeze-dried. Therefore, based on the findings of the formulation development studies for NicQb (compare chapter 4) it was investigated whether AngQb and QbG10p33 can be stabilized during freeze-drying by the same excipients like NicQb, i.e. polysorbate 20 and trehalose.

Preformulation studies, performed by Cytos Biotechnology AG, revealed that the optimal pH for AngQb was 7.2 and that the addition of low amounts of sodium chloride was important to preserve its integrity. Thus, in a first attempt the AngQb formulation FA01 (Table 5.2.1) was freeze-dried according to freeze-drying protocol A (see chapter 2).

Table 5.2.1: Compositions of AngQb formulation FA01 (filling volume - 0.7 mL).

Code	VLP [mg]	Trehalose [mg]	Polysorbate20 [% (w/v)]	Sodium chloride [mM]	Sodium phosphate [mM]	pH
FA01	1.40	47.25	0.005	50	20	7.2

AF4 measurements revealed that the integrity of AngQb in formulation FA01 was preserved upon freeze-drying. Neither fragmentation nor aggregation of AngQb was observed (Figure 5.2.1).

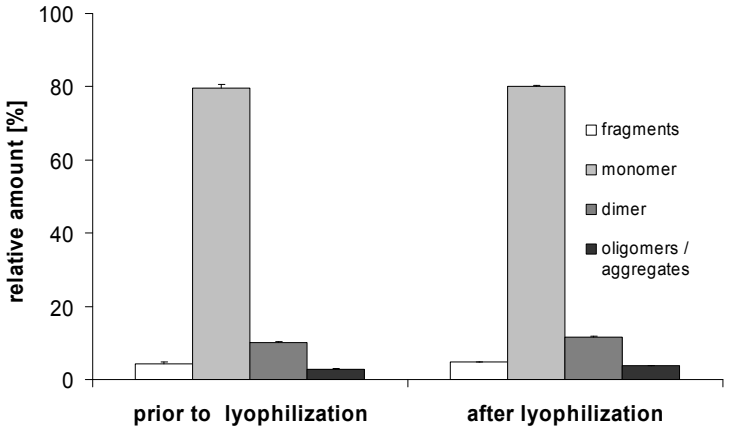


Figure 5.2.1 Stability of AngQb, determined by AF4, in formulation FA01 prior to and after FD.

Interestingly, the experiment indicated that an excipient combination of trehalose as lyoprotectant and polysorbate 20 as cryoprotectant, as it was used for NicQb, was also capable to stabilize AngQb upon freeze-drying.

However, for the preparation of sustained release devices with adequate drug payloads, there was the need to prepare highly concentrated VLP lyophilizates. Hence, in a second step the AngQb / trehalose ratio (w / w) was reduced from 1 : 33.8 (formulation FA01) to 1 : 6 (formulation FA02, Table 5.2.2) leading to an AngQb concentration of 300 µg / 2.5 mg lyophilizate. Formulation FA02 was freeze-dried according to freeze-drying protocol A (chapter 2).

Table 5.2.2 Compositions of AngQb formulation FA02 (filling volume - 0.6 mL).

Code	VLP [mg]	Trehalose [mg]	Polysorbate20 [% (w/v)]	Sodium chloride [mM]	Sodium phosphate [mM]	pH
FA02	2.24	13.50	0.005	50	20	7.2

After freeze-drying lyophilizates with excellent appearance and a residual moisture content of 1.2 % were obtained. AF4 measurements indicated that even at this low lyoprotectant / drug weight ratio the integrity of AngQb was maintained upon freeze-drying (Figure 5.2.2).

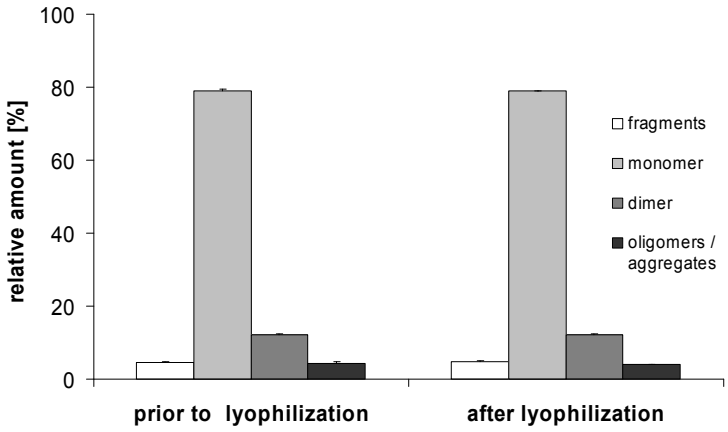


Figure 5.2.2 Stability of AngQb, determined by AF4, in formulation FA02 prior to and after FD.

Consequently, the freeze-dried AngQb formulation FA02 was used as starting material for the preparation of sustained release systems.

For QbG10p33 no data were available concerning its stability with respect to varying pHs or the presence of sodium chloride. Thus, according to the experiences made with NicQb (see chapter 4) and AngQb (see above) formulation FQ01 (Table 5.2.3), with and QbG10p33 concentration of 300 µg / 2.5 mg (QbG10p33 / trehalose weight ratio 1 : 7.5), was freeze-dried according to protocol A (chapter 2).

Table 5.2.3 Compositions of QbG10p33 formulation FQ01 (filling volume - 0.6 mL).

Code	VLP [mg]	Trehalose [mg]	Polysorbate20 [% (w/v)]	Sodium phosphate [mM]	pH
FQ01	1.80	11.56	0.005	20	7.2

After freeze-drying lyophilizates with excellent appearance and a residual moisture content of 1.7 % were obtained. AF4 measurements revealed that the integrity of QbG10p33 was maintained upon freeze-drying (Figure 5.2.3). The API bulk already contained relatively large amounts of QbG10p33 oligomers and aggregates but the proportion of this VLP fraction was not altered during freeze-drying.

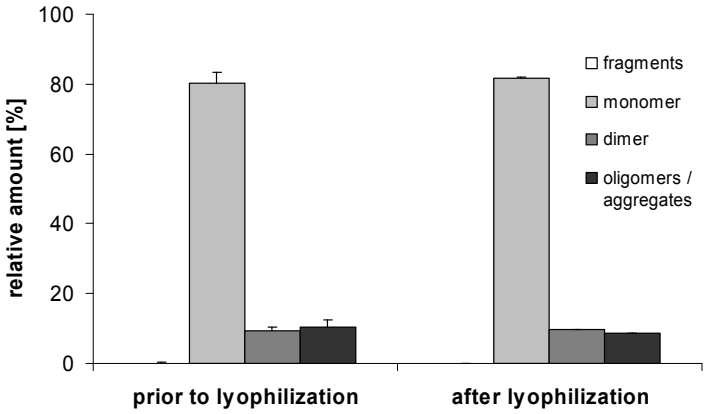


Figure 5.2.3 Stability of QbG10p33, determined by AF4, in formulation FQ01 prior to and after FD.

It can be stated that the excipient composition of trehalose as lyoprotectant and polysorbate 20 as cryoprotectant was, according to the results obtained for NicQb and AngQb, also capable of stabilizing QbG10p33. Hence, formulation FQ01 was used as starting material for the manufacture of sustained release devices.

5.2.2 Definition of Release Study Parameters

Selection of container and release medium

The release medium used for in vitro release studies with AngQb and QbG10p33 sustained release formulations was phosphate buffered saline composed of 50 mM sodium chloride and 20 mM sodium phosphate buffer at a pH of 7.2. Furthermore, sodium azide was added at a concentration of 0.05 % as bacteriostatic agent. This composition was chosen based on the results obtained during preformulation studies, performed by Cytos Biotechnology AG, which indicated the highest stability of AngQb at this salt concentration and pH. Preliminary experiments performed with AngQb solutions (1 mg / mL) at 37 °C indicated that it tended to fragmentate even at this "optimum" pH (Figure 5.2.4). Based on these results the release media was exchanged at least every third day.

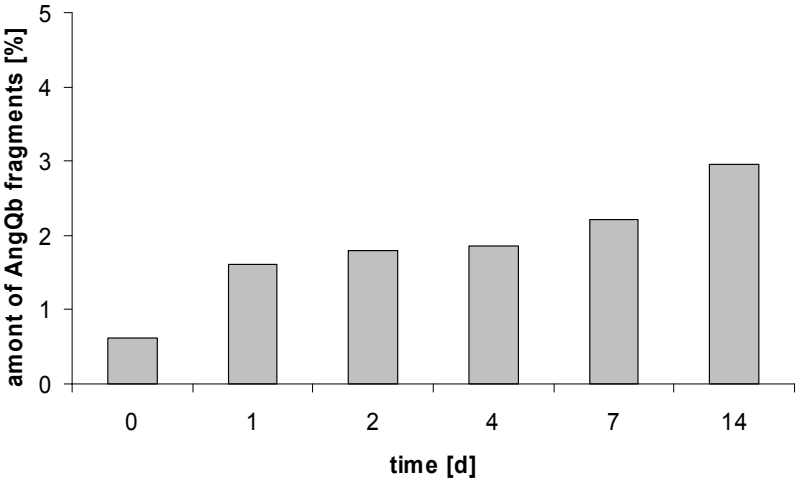


Figure 5.2.4 Stability of AngQb (1 mg / mL) at 37 °C at pH 7.2, determined via SE-HPLC.

The determination of the most appropriate container for release studies is another crucial parameter, because upon long-term release studies the amount of VLP in the release medium was expected to be extremely low (< 20 µg / mL). Adsorption of VLP to the container surface might result in lower drug concentrations detected in the release medium. Therefore, the adsorption behavior of AngQb to four different container types was investigated: (1) Standard Eppendorf reaction tubes, (2) LoBind Eppendorf reaction tubes (Eppendorf AG, Hamburg, Germany), (3) 2R class 1 glass vials (Schott AG, Mainz, Germany), and (4) TopPac® vials (Schott AG, Mainz,

Germany). Adsorption of AngQb to the different containers was analyzed at two drug concentrations, 20 µg / mL and 100 µg / mL, respectively, upon incubation at 37 °C for 7 days. As surfactants are often used to prevent adsorption to surfaces [Cleland et al., 1997; Tobio et al., 1999b; Palinko-Biro et al., 2001; Jaganathan et al., 2005], the effect of polysorbate 20 (0.01 % w / w), on the adsorption behavior of AngQb to the above mentioned containers was additionally examined. The AngQb amount in the respective samples was determined via RP-HPLC (see chapter 2). The results obtained revealed a strong adsorption of AngQb to all container types in the absence of polysorbate 20, where glass vials and TopPac® vials exhibited the worst results with more than 50 % adsorption after 7 days at an AngQb concentration of 20 µg / mL. By adding polysorbate 20 to the AngQb solutions adsorption to the container surfaces could be clearly reduced, exemplarily displayed in Figure 5.2.5 for the AngQb solution of 20 µg / mL. The results indicated that the most favorable container was the LoBind Eppendorf reaction tube. Here, less than 10 % of AngQb were adsorbed to the container after 3 days. Thus, LoBind Eppendorf reaction tubes were used as container for the release studies with AngQb and QbG10p33 sustained release devices and polysorbate 20 was added in a concentration of 0.01 % (w / w) to the release medium.

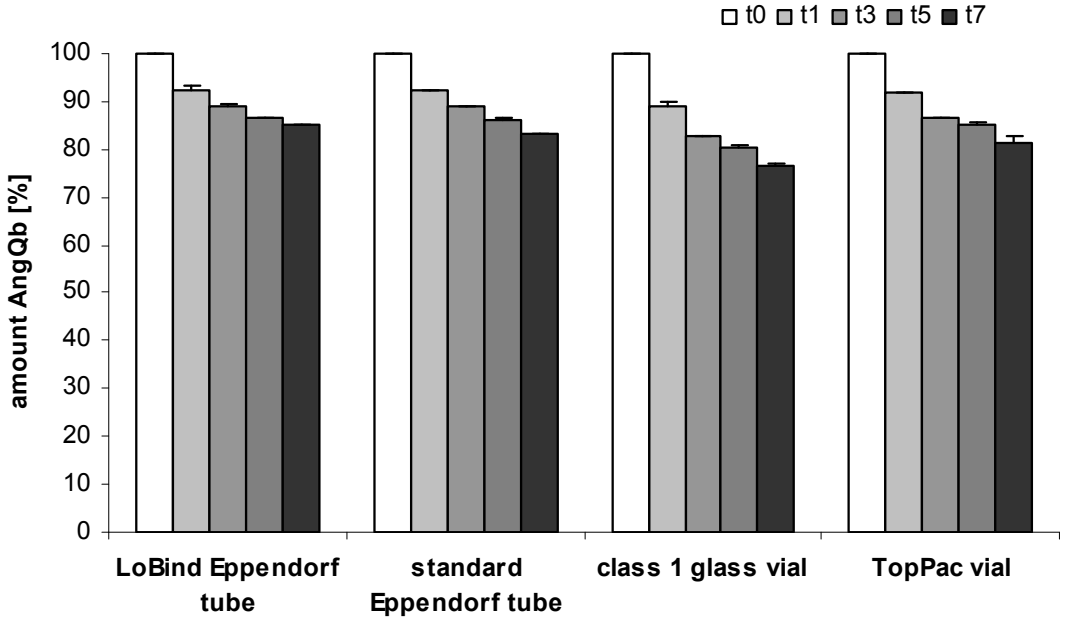


Figure 5.2.5 Adsorption behavior of AngQb (20 µg / mL) in the presence of 0.01 % polysorbate 20 to different container types upon 7 days storage. AngQb concentration determined via RP-HPLC.

Evaluation of quantification method

For in vitro release studies an adequate analytical method for drug quantification is inevitable. Common methods for protein based drugs are the bicinchoninic acid (BCA) [Chattaraj et al., 1999; Higaki et al., 2001; Feng et al., 2006] and Bradford [Thomasin et al., 1996] assays, which are both applicable for protein concentrations down to 1 µg / mL. Besides these colorimeteric assays ELISA es a further commonly used quantification method which is applicable even for antigen concentrations in the ng range [Singh et al., 1991; Jung et al., 2002; Katare et al., 2006].

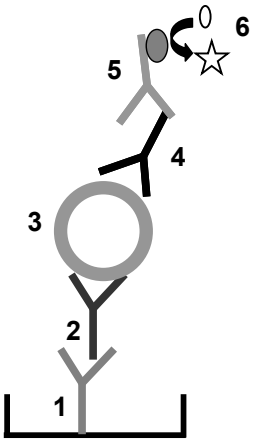
However, as all of these methods have some specific drawbacks their applicability for the quantification of VLP was investigated.

The Bradford assay is a dye binding assay in which a differential color change of Coomassie Brilliant Blue dye from 465 nm to 595 nm occurs due to stoichiometric protein binding. From the optical absorbance the protein concentration can be calculated by using a protein standard curve [Bradford, 1976]. This assay is very sensitive to surfactants especially at very low protein concentrations [Compton et al., 1985]. Preliminary experiments with AngQb solutions indicated that even slight variations in the surfactant concentration had clear effects on the determined VLP amount. However, the addition of polysorbate 20 to the release medium was essential to prevent the adsorption of the VLP to the container. As potentially different amounts of polysorbate might adsorb to the container surfaces leading to varying amounts of polysorbate in the release medium it was assumed that this method was inappropriate for the quantification of VLP in the samples obtained during release studies.

The BCA method involves the reduction of Cu^{2+} to Cu^{+} by peptidic bonds of proteins. The BCA chelates Cu^{+} ions with very high specificity to form a water soluble purple colored complex. This reaction can be measured by optical absorbance of the final Cu^{+} complex at 562 nm and is used for the determination of the protein concentration by applying a standard curve [Smith et al., 1985]. The method is non-sensitive to surfactants but susceptible to chelating substances, lipids and reducing substances [Smith et al., 1985; Kessler et al., 1986; Wiechelman et al., 1988]. Preliminary experiments performed with AngQb solutions spiked with PEG (as potent release modifier) or lactic acid (as degradation product of PLGA) revealed that

both substances lead to deviant results. Hence, this protein quantification assay was useless as well.

As the two commonly used colorimetric protein quantification methods were not well applicable a multi-step sandwich ELISA, as displayed in Figure 5.2.6, was developed for the quantification of VLP.



1) Coating with goat anti-rabbit IgG
2) Incubation with rabbit anti-Qb serum
3) Incubation with VLP standard or VLP sample
4) Incubation with mouse anti-Qb serum
5) Incubation with goat anti-mouse IgG coupled to horse-radish peroxidase
6) Addition of substrate (O-phenylendiamine-dihydrochloride)
7) Measurement of absorbance at 450 nm

Figure 5.2.6 Organization of sandwich ELISA for the quantification of VLP.

After optimization of the dilutions of the specific sera and antibody solutions linear standard curves (absorbance vs. concentration plot) for both VLP, AngQb and QbG10p33, exemplarily shown for AngQb in Figure 5.2.7, were established.

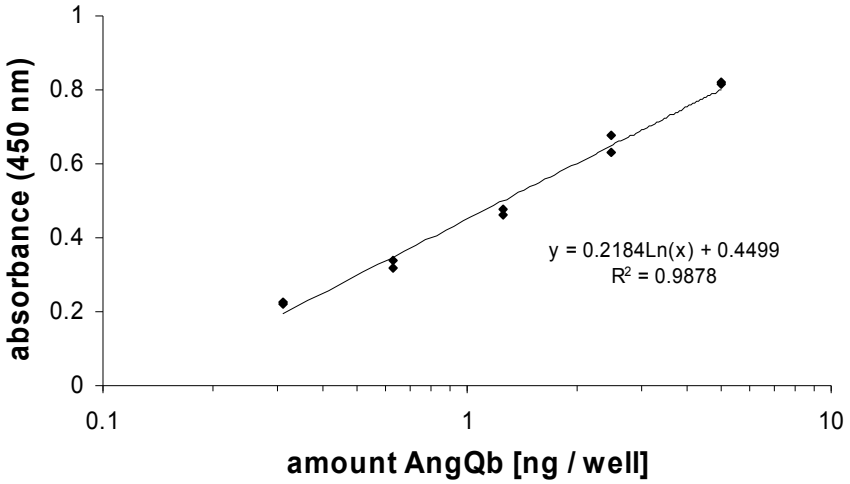


Figure 5.2.7 AngQb standard curve for the developed ELISA.

Even though ELISA is a very specific quantification method for small antigens with single epitopes [Reid et al., 2000; Breuer, 2007], a problem can arise when working with large antigens, or especially large antigens that consist of multiple similar

subunits with potentially similar epitopes. For complex structures there is the risk of detecting not only the complete, intact antigens, but additionally fragmented species or single subunits. If e.g. a VLP is dissembled into 20 subunits that are all recognized by the ELISA, 20 fold too high results might be achieved. Therefore, it was investigated whether the developed ELISA detects purified, single Qb coat protein dimers (obtained by Cytos Biotechnology AG) as well. Unfortunately, this was the case so that even this highly sensitive quantification method could not be used.

Finally, it could be stated that none of the commonly used methods was applicable for the quantification of VLP during release studies.

Fortunately, a different approach could be applied. Cytos Biotechnology AG developed a RP-HPLC method for the quantification of Qb coat proteins (see chapter 2). The Qb VLP are assembled from 180 units of Qb coat protein monomer and are stabilized by tight non-covalent Qb coat protein dimer interactions and disulfide bonds linking each Qb coat protein dimer covalently to the rest of the coat [Golmohammadi et al., 1996]. For sample preparation the Qb VLP are first disassembled with dithiothreitol (DTT) and guanidine hydrochloride. Thereby, dithiothreitol, a reducing agent [Cleland, 1964], cleaves the disulfide bonds linking the coat proteins together, and guanidine hydrochloride, a chaotropic salt [Hatefi et al., 1969], disrupts the non-covalent interactions and increases protein solubility. Subsequently, the Qb coat proteins are separated by RP-HPLC from potential impurities using an acetonitrile gradient under acidic conditions and are detected by UV absorption at 215 nm. Finally, the Qb coat protein content is determined by external calibration with a Qb standard. The concentration of the standards was determined by amino acid analysis. In Figure 5.2.8 a typical standard curve is shown, which reveals that this quantification method is applicable for Qb concentrations down to 0.1 µg / mL. Consequently, this RP-HPLC method was used for the quantification of VLP in samples obtained during release studies.

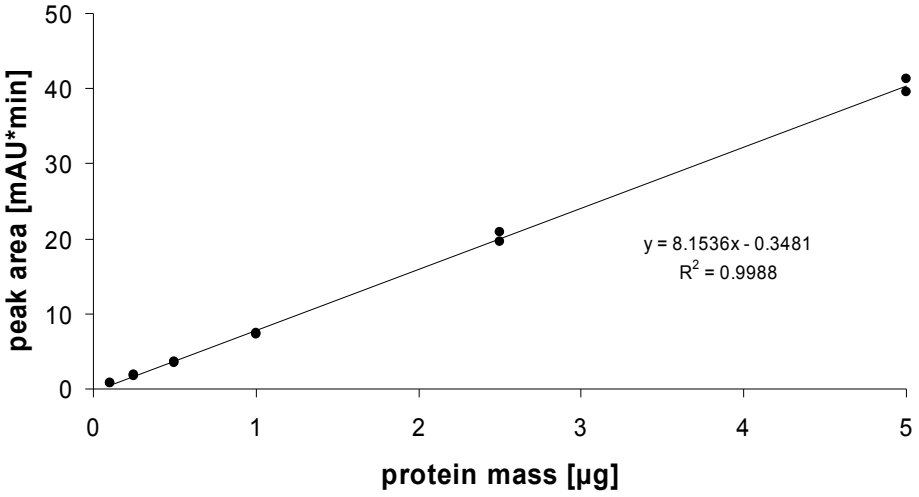


Figure 5.2.8 Qb coat protein standard curve obtained by RP-HPLC analysis.

5.2.3 Implants

The aim of these initial experiments was to study VLP release from implants, composed of PLGA or lipids as matrix materials, as simple models for sustained release devices. Implants were manufactured by simple compression of the matrix material and other excipients like pore forming substances and lyophilized VLP. This preparation method was chosen in order to prevent the exposure of the drug to stress factors like organic solvents and water / organic interfaces, shear stress or temperature, which are connected to preparation methods for microparticulate systems [Jain, 2000; Yeo et al., 2001; Sinha et al., 2003]. In addition such systems can be manufactured fast and easy without great operating expenses [Mohl, 2003; Onishi et al., 2005].

AngQb, a therapeutic VLP for the treatment of hypertension, acting upon the induction of a specific anti-angiotensin antibody response [Ambuehl et al., 2007], and QbG10p33, a model VLP which was designed to induce T-cells additionally to an antibody response [Storni et al., 2004], were used for this purpose. The release behavior in dependence of the matrix material and the addition of further excipients like, e.g. pore forming agents, was investigated. Furthermore, the stability of the VLP upon processing and release was analyzed. Finally, the in vivo efficacy of VLP implants in comparison to multiple applications of a liquid formulation was studied.

AngQb implants

Implants of 25 mg total weight each were produced according to the protocol introduced in chapter 2. In brief, five different formulations (Table 5.2.4, n = 3) were compressed (15 seconds, 2.0 kN) to small cylindric implants with a diameter of 3 mm, exemplarily shown for formulation IA01 and IA05 in Figure 5.2.9. The height of the PLGA based implants was about 2.6 mm whereas the tristearin based implants were about 3.3 mm high. The payload of AngQb was throughout all formulations 300 µg / implant.

Table 5.2.4: Compositions of AngQb implants (25 mg) used for in vitro release studies.

Code	AngQb lyophilizate	Resomer® RG 502H	Resomer® RG 502	Dynasan 118	PEG 6,000	$Mg(OH)_2$
IA01	10 %	80 %	-	-	10 %	-
IA02	10 %	-	80 %	-	10 %	-
IA03	10 %	80 %	-	-	7 %	3 %
IA04	10 %	80 %	-	-	6 %	4 %
IA05	10 %	-	-	80 %	10 %	-

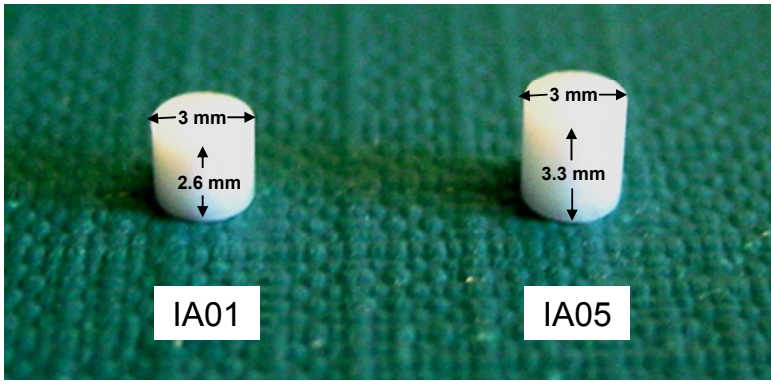


Figure 5.2.9 Compressed implants – formulation IA01 and IA05.

Formulations IA01 and IA02 were chosen to study the effect of different PLGA materials on the release kinetics of VLP. Both polymers are composed of equivalent molar ratios of lactic acid to glycolic acid and have an average molecular weight of about 12 kDa and an inherent viscosity of 0.2 dL / g in chloroform. The difference between these two PLGAs is that Resomer® RG 502H exhibits free carboxylic acid end groups whereas in the case of Resomer® RG 502 these groups are end-capped.

As free carboxylic acid end groups autocatalytically accelerate the hydrolytic degradation of PLGA [Park, 1995] a faster release of the VLP was expected for Resomer® RG 502H. PEG 6,000 was added to these formulations as pore forming agent in order to enable an adequate release of the VLP by diffusion through a continuous porous network, formed within the matrix by fast dissolution of the hydrophilic PEG. Furthermore, by increasing the porosity of the matrix diffusion of acidic degradation products out of the device, and thus, prevention of a pH drop within the device, is described to potentially increase drug stability upon release [Bilati et al., 2005; Jiang et al., 2005]. The PLGA based formulations IA03 and IA04 were selected based on the findings of Zhu et al. who showed that alteration of BSA upon release from PLGA implants and microspheres could be significantly reduced by the addition of poorly water-soluble basic salts like $Mg(OH)_2$ which were coincorporated to neutralize the acidic microclimate pH [Zhu et al., 2000]. Formulation IA05, a lipid based formulation, was included as alternative to the standard PLGA. Such a tristearin / PEG based implant formulation was introduced by Mohl et al. who showed that it was capable of releasing BSA and interferon α-2a continuously up to 30 days [Mohl, 2003]. The potential advantage of lipids as matrix material in comparison to PLGA might be the prevention of an acidic environment, and thus, potentially preservation of drug integrity upon release. A drawback of lipids as matrix materials for sustained controlled release devices is their slow biodegradability.

The release kinetics of the five AngQb implant formulations were investigated in an in vitro study over a time frame of 3 months. The cumulative release profiles of AngQb from the presented formulations are depicted in Figure 5.2.10. Importantly, it has to be mentioned that the RP-HPLC method applied can be used for the determination of the amount of VLP coat proteins in the release medium but not for stability testing as AngQb is disassembled before analysis (see page 136). PLGA implants swelled upon incubation and were almost completely degraded after 3 months. By contrast, tristearin implants remained macroscopically unaltered throughout the release studies.

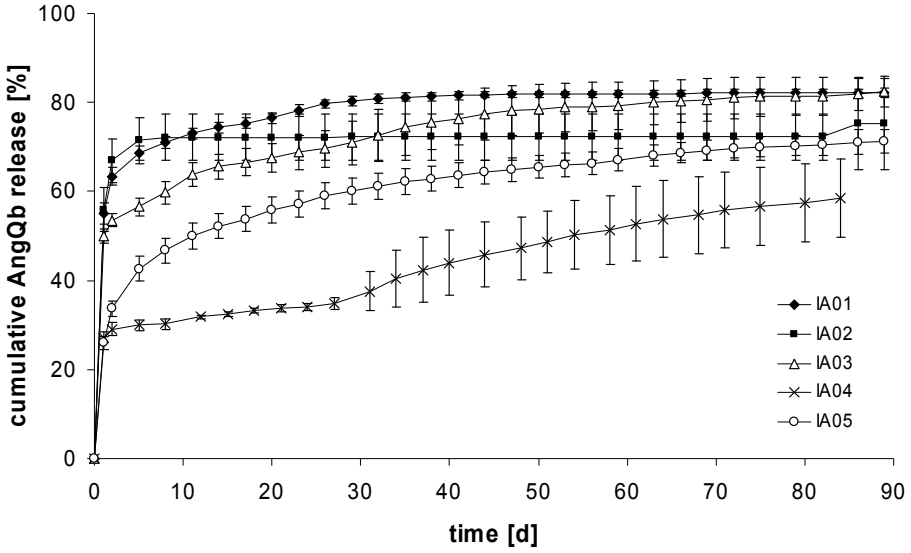


Figure 5.2.10 Cumulative in vitro release of AngQb from tristearin / PEG and PLGA / PEG implants over a time frame of 3 months.

Following conclusions can be drawn from the presented data:

(1) The PLGA / PEG based formulations IA01 and IA02 (10 % PEG) showed a high initial burst of about 55 % within the first day; A lower initial burst of ~ 50 % and ~ 30 %, respectively, was observed for formulations IA03 (7 % PEG) and IA04 (6 % PEG); The tristearin / PEG based formulation IA05 revealed an initial burst of ~ 25 %.

(2) All formulations with exception of formulation IA04 showed a continuous release after the initial burst phase:
- IA01 another 25 % within day 2 – 26
- IA02 another 15 % within day 2 – 8
- IA03 another 30 % within day 2 – 50
- IA05 another 45 % within day 2 – 70

which leveled off after the respective release periods.

(3) For formulation IA04 a triphasic release behavior was observed. The initial burst was followed by a lag period up to day 27. Then, from day 27 to 84 another 30 % were released.

(4) All formulations revealed an incomplete release of AngQb.

Formulations IA01 and IA02 showed, after a primary burst, no long-lasting or pulsed release of AngQb and might thus be no appropriate sustained release devices. Formulations IA03 and IA05 revealed a primary burst followed by a continuous AngQb

release up to 7 and 10 weeks, respectively. An adequate primary burst, which mimics the first vaccine dose, is desirable for sustained release devices for vaccines. Furthermore, a continuous antigen release was shown to be capable of inducing long-lasting high antibody titers [Preis et al., 1979; Cleland et al., 1996a; Toussaint et al., 2007], and thus, the respective formulations seemed to be promising sustained release devices for AngQb. However, the composition of formulation IA03 is still optimizable by reducing the high initial burst rate. Formulation IA04 might be a promising controlled release device due to its pulsed release profile. For example Thomasin et al. showed that a pulsed release of tetanus toxoid and malaria antigens from PLGA microspheres initiated high, long-lasting antibody titers in mice [Thomasin et al., 1996].

Stability of AngQb during release from PLGA and lipid based implants

From a quality and safety perspective the investigation of drug integrity upon release is an important issue. Appropriate analytical methods for the investigation of VLP degradation and aggregation are SE-HPLC and AF4 (compare chapter 3). However, both analytical methods have a limit of quantification of about 20 µg / mL. As the amount of AngQb released from the implant formulations investigated was after one ore two days below this limit, the integrity of AngQb could only be assessed by SE-HPLC and AF4 during the first days. Unfortunately, the analysis of the AngQb samples by AF4 was not feasible, probably due to interference of the excipients and / or degradation products of the matrix with the ultrafiltration membrane material. The SE-HPLC data for day 1 and 2 were compared to the starting material (Table 5.2.5). For all samples a slight increase of aggregated species and fragments was observed, but the proportion of the main AngQb peak was still > 90 %. As AngQb is rather unstable in solution (compare Figure 5.2.4 and Figure 5.2.14) it was not clear whether the observed degradation of AngQb was related to specific detrimental conditions upon release from the devices or was just a result of liquid storage.

Table 5.2.5 Stability of AngQb, determined via SE-HPLC, upon release from PLGA and lipid implants.

Formulation	Time [d]	Aggregates	Main peak	Fragments
			Amount [%]	
AngQb lyophilizate FA02	-	0.1	98.7	1.1
IA01	1	0.6	95.1	4.3
	2	0.7	92.8	6.4
IA02	1	0.6	94.4	5.1
	2	0.9	92.0	7.1
IA03	1	0.3	95.9	3.8
IA04	1	0.1	96.7	3.2
IA05	1	0.5	92.9	6.6
	2	0.2	90.7	9.1

Further discussion of release from PLGA implants

The release of antigens from the bulk eroding, degradable polymer PLGA is generally controlled by a primary release from the surface and surface-near areas (initial burst) followed by a further release governed by three mechanisms: (1) liberation through a water-filled porous network, (2) degradation of the polymer, and / or (3) swelling of the system [Pitt, 1990; Cleland et al., 1994; Gombotz et al., 1995; Park, 1995; Johansen et al., 1999].

The PLGA based AngQb implant formulations investigated in the present study exhibited different initial release rates. The primary burst relies on the initial penetration of water into the polymer matrix which initiates release of the drug entrapped very close to the surface of the matrix. Water dissolves the drug and enables its liberation through the developing interconnecting porous network. In general, the water uptake and consequently the initial release increases with increasing drug loadings, increasing amounts of hydrophilic porogens and increasing hydrophilicity of the matrix material [Perez et al., 2002; Yeo et al., 2004]. In our case the hydrophilicity of the matrix seemed to have no effect on the amount of initially liberated VLP. A similar burst of approximately 55 % was observed for formulation IA02, prepared with the end-capped PLGA Resomer® RG 502, and IA01, composed of the PLGA Resomer® RG 502H, with similar molar ratios of lactic to glycolic acids and the same molecular weight as Resomer® RG 502, but free carboxylic end-groups,

which increase the hydrophilicity of the polymer [Johansen et al., 1999]. As all formulations contain the same amount of VLP lyophilizate the different initial burst rates seemed to be solely depending on the PEG amount. With decreasing PEG amounts the initial burst decreased from 55 % for formulation IA01 and IA02 (10 % PEG) over 50 % for formulation IA03 (7 % PEG) to 30 % for formulation IA04 (6 % PEG).

For formulations IA01, IA02 and IA03 a biphasic release profile was observed whereas formulation IA04 exhibited a triphasic release behavior. Formulations IA01 and IA02 showed a short continuous release period of 26 and 8 days, respectively. The more prolonged release of AngQb from formulation IA01 (PLGA with free carboxylic end groups) in comparison to formulation IA02 (end capped PLGA) can be explained by the more pronounced water uptake and swelling of the more hydrophilic matrix, as illustrated in Figure 5.2.11, which facilitated the formation of larger pores, and thus, enabled increased liberation of the drug. After the short continuous release periods the release of AngQb leveled off due to the formation of acid and moisture induced AngQb aggregation and adsorption to the matrix (compare pages 145 – 148).

For formulation IA03 a continuous release of AngQb was observed up to day 50. This continuous release of AngQb in comparison to formulation IA01 seemed to be related to the incorporation of $Mg(OH)_2$ which was deemed to prevent AngQb aggregation upon incubation enabling prolonged release. These data are in good correlation to the findings of Zhou et al. who showed that BSA release from PLGA implants could be clearly increased by incorporation of poorly soluble basic additives. The postulated mechanism was that basic additives neutralize the acidic microclimate pH within the PLGA implant, consequently inhibit acid-induced BSA aggregation, and thus, facilitate a prolonged release of BSA [Zhu et al., 2000].

The lag-phase obtained for formulation IA04 can be explained by its lower PEG (6 %) content in comparison to formulations IA01 (10 %) and IA03 (7 %). For formulation IA04 the water uptake was slowed down as illustrated by the swelling behavior of the implants (Figure 5.2.11), and thus, the formation of an interconnected pore network with sufficiently large pores was reduced until the matrix was degraded to such an extend so that the VLP could be released. However, after this lag phase a continuous, long-lasting AngQb release was observed which, following the theory of the hindrance of the release of aggregated species due to their size, led to the

assumption that in contrast to formulations IA01 and IA02 AngQb was protected to a certain level against acid-induced aggregation by coincorporated $Mg(OH)_2$.

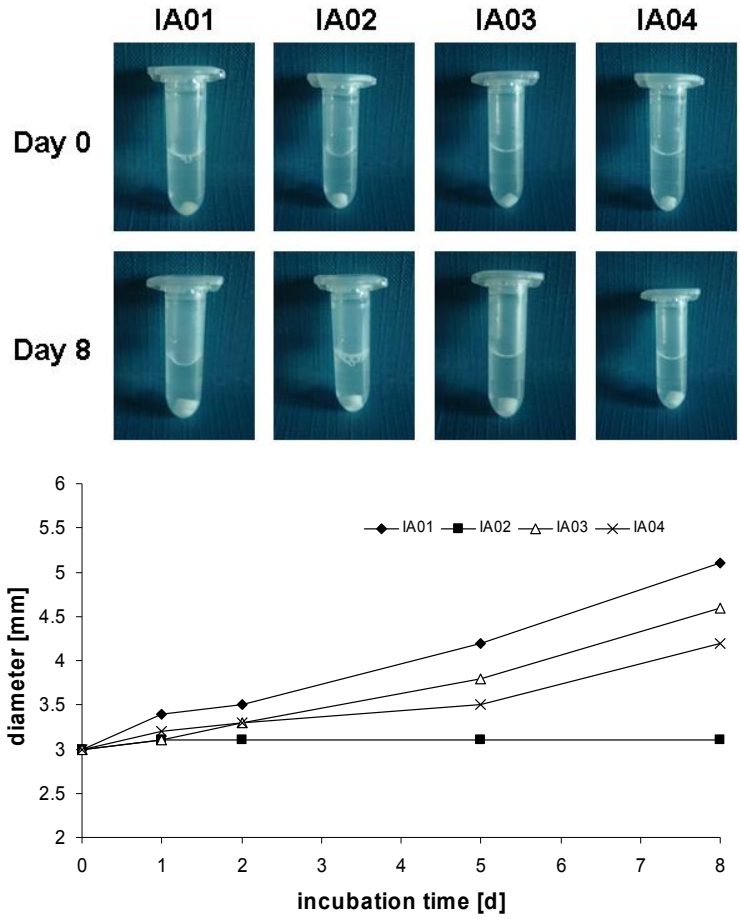


Figure 5.2.11 Swelling behavior of AngQb implants IA01, IA02, IA03 and IA04 upon 8 days incubation time.

However, similar to formulations IA01 and IA02 the formulations IA03 and IA04 revealed an incomplete release which might probably be affected by moisture induced aggregation, in later release periods acid induced aggregation (depletion of the $Mg(OH)_2$ reservoir) and / or adsorption to the matrix.

Discussion of release from tristearin implants

The release profile from very slow degrading lipid matrices is generally controlled by release of the drug from the surface and surface-near areas, followed by leaching out of the drug through pores. As the matrix is impermeable for macromolecules the formation of an interconnected network is essential to facilitate the release of adequate amounts of the incorporated drug. This can be achieved by high drug loadings or the incorporation of hydrophilic substances, such as PEG. Thereby, first

the drug and the water soluble excipient located at the surface of the device and in surface-near areas are dissolved and released very fast. Then, water penetrates into the system and further dissolves the incorporated drug and the porogen leading to the creation a porous microstructure which in turn enables further leaching of the drug. At low drug loadings and / or too low amounts of porogen an interconnected network might not be achieved, so that not the entire drug has access to the water-filled pores, and thus, cannot be released [Kaewvichit et al., 1994; Vogelhuber et al., 2003; Mohl et al., 2004; Koennings et al., 2006; Herrmann et al., 2007b].

The tristearin / PEG based AngQb implant formulation IA05 was produced following the findings of Mohl et al. who showed that interferon α-2a could be released almost completely (~ 90 %) upon a time span of 30 days from an almost similar implant formulation (10 % lyophilized interferon α-2a, 80 % tristearin, 10 % PEG 6,000) [Mohl et al., 2004]. Comparing the release profiles from AngQb formulation IA05 to the interferon α-2a release data it is apparent that despite a comparable initial burst (~ 25 %) a lower amount of AngQb was released upon a clearly longer time span. A reason for this finding might be the remarkably higher molecular weight of AngQb (~ 4.1 MDa) in comparison to interferon α-2a (19 kDa). Even though smaller implants (3 mm vs. 5 mm diameter) were produced by applying lower compressions force (2.0 kN vs. 19.6 kN) and shorter compression time (15 s vs. 30 s), in order to facilitate the formation of larger pore diameters, liberation of the larger VLP was hindered. Other possible reasons for an incomplete release are adsorption of AngQb to the matrix and / or moisture induced aggregation of the drug induced by the slow hydration upon release (compare pages 146-147).

Reasons for incomplete release

In order to examine the adsorption behavior of AngQb to the specific matrix materials, placebo implants composed of 10 % placebo lyophilizate, 80 % Resomer® RG 502, Resomer® RG 502H or tristearin, respectively, and 10 % PEG 6,000 were produced according to the same protocol used for the preparation of the verum implants (see chapter 2). The placebo implants were placed in LoBind Eppendorf reaction tubes together with 1 mL AngQb solution (0.3 mg / mL – amount equal to payload of the verum implants, 20 mM sodium phosphate buffer, 0.01 % polysorbate 20 and 0.05 % sodium azide, pH 7.2) and incubated for 7 days at 37 °C and an

agitation speed of 40 rpm. The AngQb concentration was monitored via RP-HPLC at least in duplicate. The results, depicted in Figure 5.2.12., revealed that almost 15 % of the material was adsorbed to all placebo implant formulations after 7 days incubation time. Thus, it was assumed that adsorption of AngQb to PLGA and tristearin is a potential cause for the incomplete release during in vitro studies.

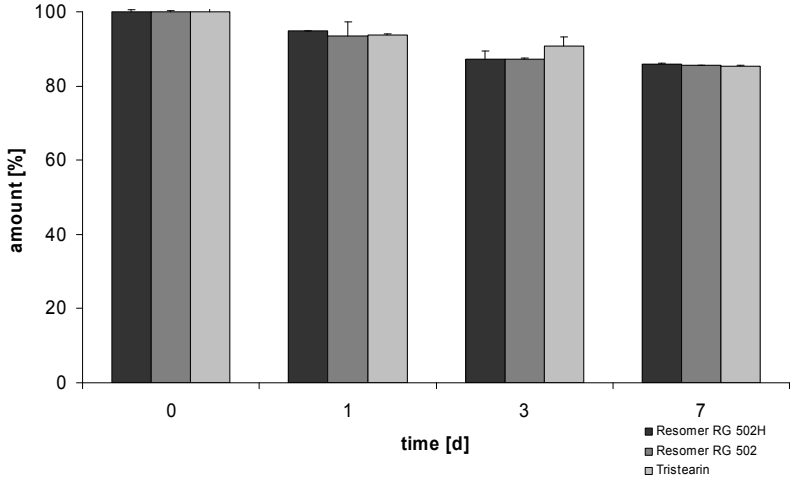


Figure 5.2.12 Adsorption of AngQb (0.3 mg / mL) to placebo implants upon incubation for 7 days at 37 °C.

Now the effect of moisture induced aggregation of AngQb within the implants upon incubation was investigated. To simulate the slow hydration of dried AngQb within the implants upon release AngQb lyophilizates (FA02, Table 5.2.2) were stored unsealed for four weeks at 40 °C in a controlled humidity environment (75 % RH). Additionally, AngQb lyophilizates were stored in closed containers under the same conditions. After storage, the samples were reconstituted in 0.6 mL purified water. In contrast to the lyophilizates stored sealed the lyophilizates stored open could not be reconstituted completely, and large particles were macroscopically visible. Samples were filtered through a PVDF filter (0.22 µm pore size). All samples were analyzed by SE-HPLC at least in duplicate. In Figure 5.2.13 the proportions of the specific VLP fractions, in relation to the total amount of VLP in the untreated samples, are displayed. The results revealed that the most of the material aggregated upon open storage; the loss of about 90 % AngQb was ascribed to the formation of insoluble VLP aggregates which were removed by the filtration step.

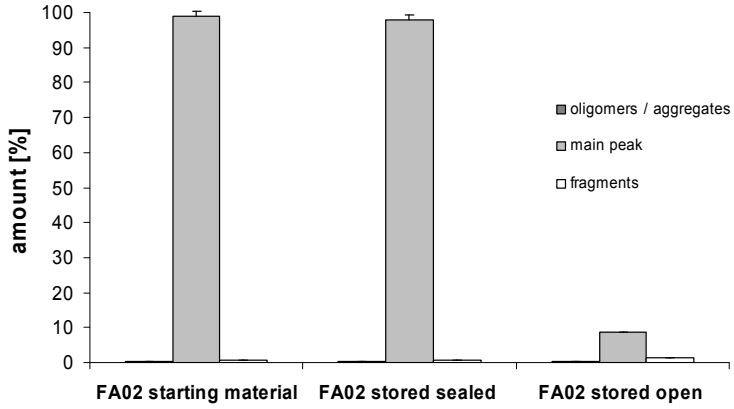


Figure 5.2.13 Stability of AngQb lyophilizates, determined by SE-HPLC, after storage for 4 weeks open or sealed at 40 °C and 75 % RH. The proportions of the different VLP fractions are expressed as relative amount to the total amount of the untreated sample.

Moisture induced aggregation can be related to non-covalent aggregation induced by conformational changes of the drug due to an increased mobility [Schwendeman et al., 1995; Costantino et al., 1995; Perez et al., 2002] or covalent aggregation such as disulfide interchange [Liu et al., 1991]. As the leaching of huge aggregated VLP species from the specific implants might be more improbable than for the intact VLP, it was assumed that moisture induced aggregation was a further cause for the incomplete release of AngQb from the implant formulations investigated.

With the aim to investigate whether acid induced aggregation was a further cause of incomplete AngQb release from PLGA implants, as for example proposed for BSA and tetanus toxoid [Johansen et al., 1998; Zhu et al., 2000], the stability of AngQb in dependence of the pH was investigated. Therefore, four AngQb solutions (0.3 mg / mL, 20 mM sodium phosphate buffer) with pHs of 4.0. 5.0. 6.0 and 7.2, respectively, were prepared by dilution of the API bulk with pH adjusted buffers. Right after manufacture, the AngQb solutions with a pH $\leq$ 6.0 were turbid indicating aggregation of AngQb. Consequently, the formulations were filtered through a PVDF filter (0.22 µg pore size) and subsequently incubated for 7 days at 37 °C and an agitation speed of 40 rpm. The stability of AngQb upon storage was investigated via AF4 and SE-HPLC at least in duplicate.

Initial measurements showed that the whole AngQb material was aggregated in the solutions with a pH $\leq$ 5.0. The results obtained for the AngQb solutions with a pH of 6.0 and 7.2 are illustrated in Figure 5.2.14 and Figure 5.2.15. The results showed

that AngQb degraded upon incubation at both pHs and aggregated at a pH of 6.0. The loss of about 45 % AngQb observed for the liquid formulation with a pH of 6.0 was ascribed to the formation of insoluble VLP aggregates which were removed by filtration. Thus, it was assumed that acid induced aggregation played a major role for the incomplete release of the drug from PLGA based implants.

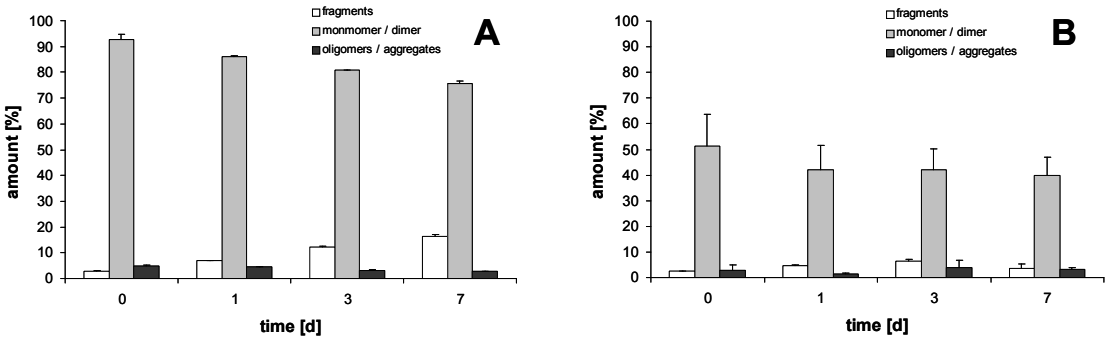


Figure 5.2.14 Stability of AngQb at a pH of 7.2 (A) and 6.0 (B), determined by AF4, upon storage for 7 days at 37 °C.

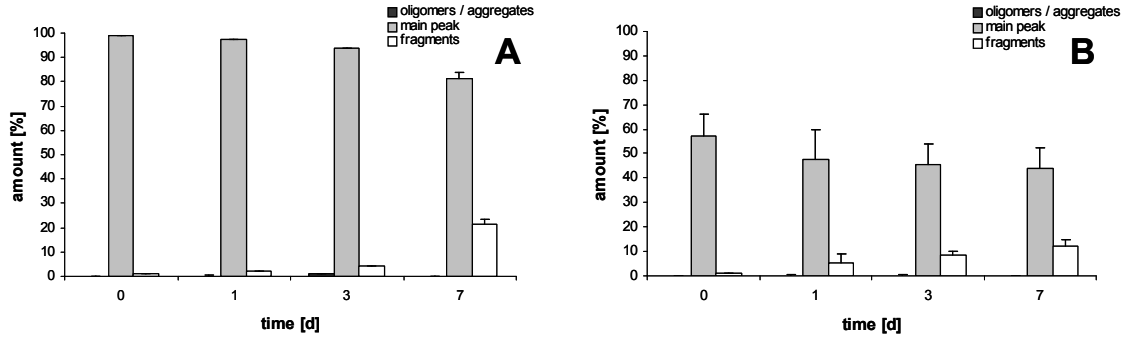


Figure 5.2.15 Stability of AngQb at a pH of 7.2 (A) and 6.0 (B), determined by SE-HPLC, upon storage for 7 days at 37 °C.

Summary

The in vitro release study revealed that the liberation of AngQb from PLGA or lipid based implants, as provisional sustained release devices, was feasible for several weeks. For PLGA based implants both, triphasic and biphasic release profiles, were achieved by variations of the matrix composition. The lipid based implant formulation exhibited a biphasic release profile. As literature describes the induction of high antibody titers for both release patterns for various antigens the development of single-dose formulations for VLP seemed to be feasible.

The integrity of AngQb released upon time could not be investigated due to low concentrations of the drug in the release media and the limitations of the accessible analytical methods. Additional experiments indicated that AngQb is rather unstable at pH < 7 that might develop upon degradation of the PLGA devices, and that the drug tends to moisture induced aggregation, a challenge that is connected to both matrix materials, and to adsorption to PLGA and tristearin. Thus, it was assumed that AngQb was an inappropriate VLP drug candidate for the investigated devices. As the stability issues of AngQb were all associated with an aqueous phase, “water-free” sustained release devices might overcome the challenges.

QbG10p33 implants

The experiments performed with AngQb implants (compare previous section) indicated that its stability was affected by environmental conditions like water uptake and / or acidic pHs evolving during incubation of PLGA and lipid implants. Therefore, it was investigated whether the second VLP drug candidate QbG10p33 is more stable than AngQb and might thus enable further in vitro and in vivo studies with implant formulations.

Stability studies QbG10p33

In order to study the effect of different pHs on the stability of QbG10p33 four solutions (0.3 mg QbG10p33 / mL, 20 mM sodium phosphate buffer) with pHs of 4.0. 5.0. 6.0 and 7.2, respectively, were prepared by dilution of the API bulk with buffer solutions. In contrast to the observations made for AngQb (clouding of the solutions at pH ≤ 6.0, see page 147) all QbG10p33 solutions were clear after preparation, indicating a superior physical stability. The solutions were incubated for 7 days at 37 °C and an agitation speed of 40 rpm. The stability of QbG10p33 upon storage was investigated via AF4 and SE-HPLC. The results obtained for the different QbG10p33 solutions before and after storage are depicted in Figure 5.2.16 and Figure 5.2.17. The SE-HPLC measurements revealed no alteration of QbG10p33 integrity in any of the solutions tested (Figure 5.2.17). The results obtained by AF4 (Figure 5.2.16) showed slightly increased aggregation levels of QbG10p33 after storage at formulation pHs of 6.0, 5.0 and 4.0, respectively. At a pH of 7.2 no changes in VLP purity were observed. Thus, it was assumed that QbG10p33 was, in comparison to

AngQb (compare Figure 5.2.14 and Figure 5.2.15) more stable in the whole pH range examined.

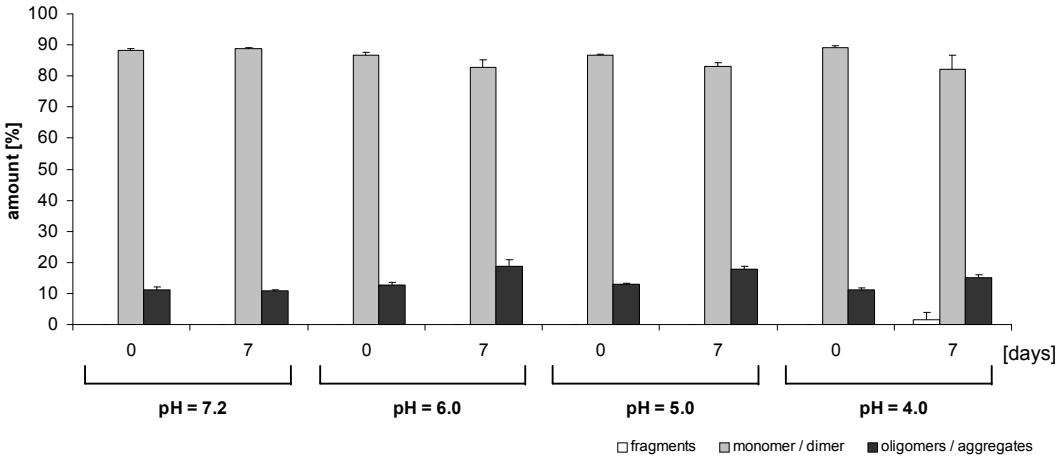


Figure 5.2.16 Stability of QbG10p33 at a pH of 7.2, 6.0, 5.0 and 4.0, respectively, determined by AF4, before and after storage for 7 days at 37 °C.

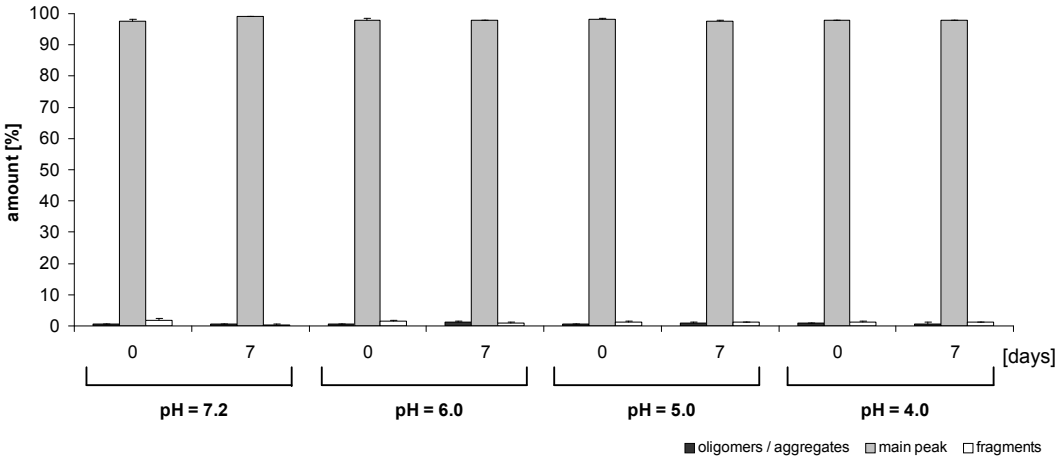


Figure 5.2.17 Stability of QbG10p33 at a pH of 7.2, 6.0, 5.0 and 4.0, respectively, determined by SE-HPLC, before and after storage for 7 days at 37 °C.

To investigate a possible moisture induced aggregation of QbG10p33, QbG10p33 lyophilizates (FQ01,Table 5.2.3) were stored unsealed and sealed for four weeks at 40 °C in a controlled humidity environment (75 % RH). After storage, the samples were reconstituted in 0.6 mL purified water. In contrast to the lyophilizates stored sealed the lyophilizates stored open could not be reconstituted completely, some particles were macroscopically visible. Therefore, these samples were filtered through a PVDF filter (0.22 µm pore size). All samples were analyzed by SE-HPLC and AF4, at least in duplicate, and the results obtained were compared to the starting material. In Figure 5.2.18 the proportions of the specific QbG10p33 fractions, in

relation to the total amount of QbG10p33 in the untreated samples, are displayed. For the QbG10p33 lyophilizates stored open a loss of about 20 % was observed. This loss was ascribed to the formation of insoluble aggregates which were removed by filtration. The amount of VLP oligomers and higher aggregates in the remaining material was determined to be 20 % (SE-HPLC analysis) and 50 % (AF4 analysis), respectively. The lower amount of detectable aggregated species in the SE-HPLC analysis might be related to either inaccurate integration of the oligomer / aggregate peak due to the poor resolution, abrasion of VLP aggregates due to the harsher conditions, i.e. high shear forces connected with SE-HPLC, and / or generation of VLP aggregates during the focusing step of the AF4 analysis. However, both analytics showed that QbG10p33 tends to moisture induced aggregation, but in comparison to the results obtained for AngQb (see Figure 5.2.13) the proportion of “intact” VLP was remarkably higher (50 % vs. 9 % main peak, determined by SE-HPLC).

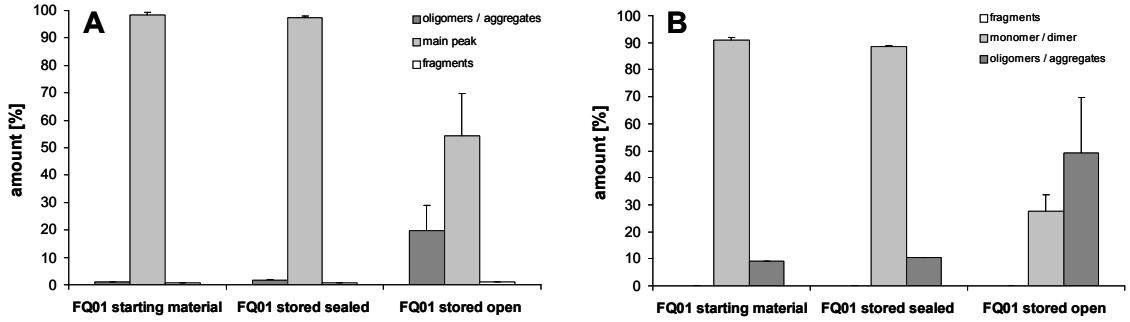


Figure 5.2.18 Stability of QbG10p33 lyophilizates, determined by SE-HPLC (A) and AF4 (B), after storage for 4 weeks open or sealed at 40 °C and 75 % RH. The proportions of the different VLP fractions are expressed as relative amount to the total amount of the untreated sample.

In summary it can be stated that QbG10p33 is far more stable than AngQb. Consequently, further in vitro release experiments with QbG10p33 implant formulations were conducted.

In vitro release study

QbG10p33 implants of 25 mg total weight each were produced according to the protocol introduced in chapter 2. In brief, four different formulations (Table 5.2.6, n = 3) were compressed (15 seconds, 2.0 kN) to small cylindric implants with a diameter of 3 mm. The payload of QbG10p33 was throughout all formulations 300 µg / implant.

Table 5.2.6: Compositions of QbG10p33 implants (25 mg) used for in vitro release studies.

Code	QbG10p33 lyophilizate	Resomer® RG502H	Dynasan 118	PEG 6,000	$Mg(OH)_2$
IQ01	10 %	84 %	-	6 %	-
IQ02	10 %	86 %	-	-	4 %
IQ03	10 %	80 %	-	6 %	4 %
IQ04	10 %	-	80 %	10 %	-

The formulations were selected on the basis of the in vitro release studies performed with AngQb implants (see previous section). Formulation IQ01 was adopted from formulation IA01 which exhibited a biphasic release profile. The content of the release modifier PEG was reduced from 10 % to 6 % to reduce the high primary burst observed for formulation IA01 (55 % within the first day). Formulation IQ02, similar to IQ01 but without PEG, was included to further investigate the effect of pore forming substances on the release behavior of VLP. IQ03 and IQ04 were similar to the AngQb implant formulations IA04 and IA05, respectively.

The release kinetics of the four QbG10p33 implant formulations were investigated in an in vitro study over a time frame of 3 months. The cumulative release profiles of QbG10p33 from the presented formulations are depicted in Figure 5.2.19. The PLGA implants swelled upon incubation and were almost completely degraded after 3 months. By contrast, the tristearin implants remained macroscopically unaltered throughout the release studies.

Following conclusions can be drawn from the presented data:

(1) Formulations IQ01 and IQ02 revealed a triphasic release profile, with an initial burst of 30 % (IQ01) and 10 % (IQ02), followed by a lag phase lasting for 44 days (IQ 01) and 31 days (IQ02); After the lag phase another 30 % (IQ01) and 60 % (IQ02) QbG10p33, respectively, were released continuously up to day 90;

(2) Formulations IQ03 and IQ04 showed a biphasic release behavior with an initial burst of about 10 % followed by a further continuous release of another 50 % within day 2 – 80 (IQ03) and 2 – 63 (IQ04), respectively;

(3) All formulations revealed an incomplete release of QbG10p33.

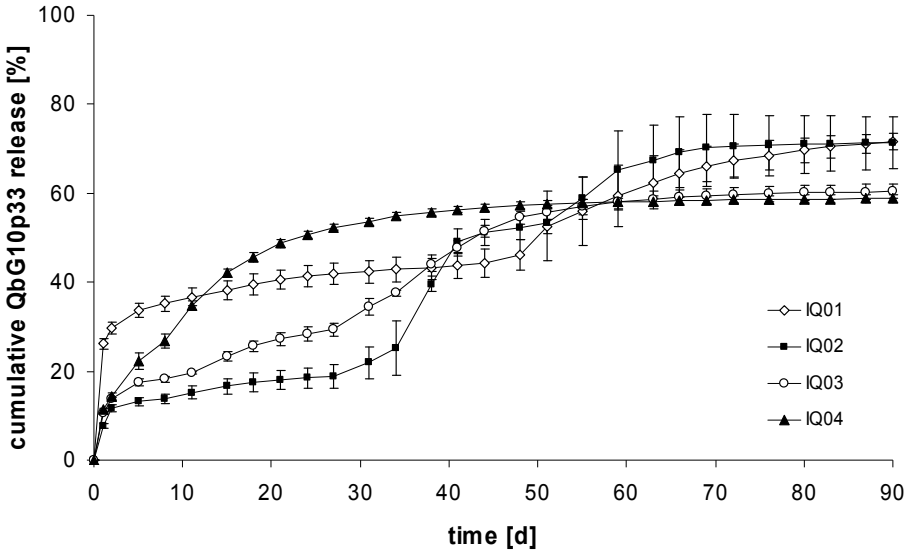


Figure 5.2.19 In vitro release profiles (cumulative release) – QbG10p33 implants.

The reasons for the different release behaviors of VLP from PLGA or lipid implants were already discussed in detail in previous sections. For QbG10p33 formulations IQ03 and IQ04 almost similar release profiles where obtained as for the comparable AngQb formulations IA04 and IA05, respectively. As expected the initial burst of formulation IQ01 was reduced in comparison to formulation IA01 (30 % vs. 55 %) due to the lower amount of release modifier PEG (6 % vs. 10 %). Furthermore, formulation IQ01 showed in contrast to formulation IA01, where the release leveled off after 26 days, a second continuous release period from day 44 to day 90 which might be promoted by the increased stability of QbG10p33. The more pronounced triphasic release profile observed for formulation IQ02 might be explained by the absence of an additional pore forming substance. Porogens typically favor swelling of the matrix and formation of an interconnected porous network with large pore diameters [Wang et al. 2002]. Hence, the release of the VLP seemed to be delayed until advanced stages of matrix wetting, swelling and polymer degradation.

The major cause for incomplete release of QbG10p33 seemed to be moisture induced aggregation (compare pages 150 - 151). To investigate whether adsorption was a further reason for incomplete release adsorption of QbG10p33 to placebo implants composed of 10 % placebo lyophilizate, 80 % Resomer RG 502H or tristearin, respectively, and 10 % PEG 6,000 was tested. Therefore, placebo implants were placed in LoBind Eppendorf reaction tubes together with 1 mL QbG10p33

solution (0.3 mg / mL – amount equal to payload of the verum implants, 20 mM sodium phosphate buffer, 0.01 % polysorbate 20 and 0.05 % sodium azide, pH 7.2) and incubated for 7 days at 37 °C and an agitation speed of 40 rpm. The QbG10p33 concentration was monitored via RP-HPLC at least in duplicate. The results, illustrated in Figure 5.2.20, revealed that about 13 % and 7 % of the material was adsorbed to PLGA and lipid implants, respectively, after 7 days incubation time. Consequently, it was assumed that adsorption of QbG10p33 to the different matrix materials was a further cause for the incomplete release during in vitro studies.

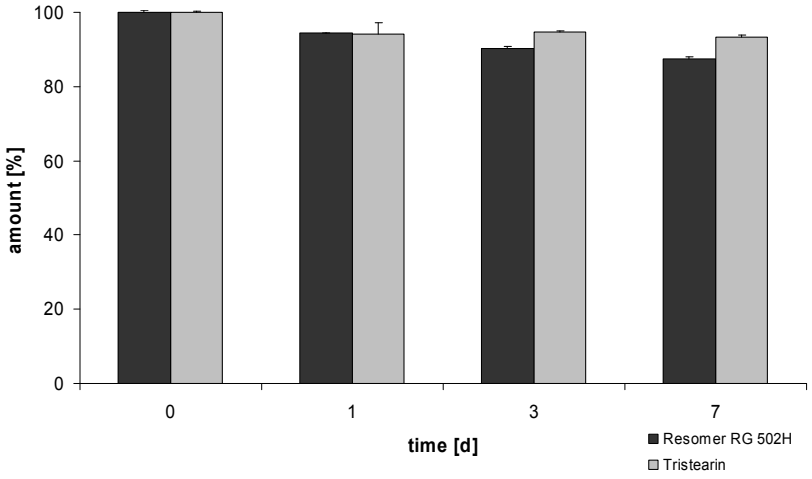


Figure 5.2.20 Adsorption of QbG10p33 (0.3 mg / mL) to placebo implants upon incubation for 7 days at 37 °C.

Stability of QbG10p33 during release from PLGA and lipid based implants

The stability of QbG10p33 during release from the different implant formulations was investigated via SE-HPLC. In contrast to the studies performed with AngQb implant formulations the concentration of QbG10p33 was high enough (> 20 µg / mL) to make the analysis of VLP integrity possible, even after several weeks of incubation. The results revealed that QbG10p33 released from the PLGA based formulations IQ01, IQ02 and IQ03 material was even after 1-2 months of incubation almost intact with 91 – 98 % of the main peak (Table 5.2.7). The analysis of QbG10p33 released from the lipid based implant formulation IQ04 was not possible due to the low amount liberated per time interval. However, it was assumed that QbG10p33 remained in the lipid matrix at least as stable as in the PLGA matrices [Schwendeman et al., 1997; Sanchez et al., 1999; Mohl, 2003].

Table 5.2.7 Stability of QbG10p33upon release from PLGA and lipid implants, determined via SE-HPLC.

Formulation	Time [d]	Aggregates	Main peak	Fragments
			Amount [%]	
QbG10p33 lyophilizate FQ01	-	1.3	98.0	0.7
IQ01	1	0.5	97.6	1.9
	5	1.2	96.6	2.2
	51	3.1	96.5	0.4
IQ02	1	0.4	97.8	1.8
	2	0.5	99.4	0.3
	38	0.4	99.3	0.3
	55	8.1	91.2	0.7
IQ03	1	0.5	98.2	1.3
	2	0.2	99.5	0.3
	38	1.1	98.2	0.7
IQ04	1	0.6	98.6	0.8

Summary

The controlled release of QbG10p33 from implants with either PLGA or lipid as matrix material was feasible up to several weeks. For the lipid based implant formulation a biphasic release of QbG10p33 was achieved whereas in dependence of additives both biphasic and triphasic release profiles were obtained for PLGA based formulations. Furthermore, it was shown that QbG10p33 released from the different PLGA devices was almost intact during the first two months of incubation. An at least comparable stability of the drug was assumed for the lipid devices, as here less detrimental conditions evolve upon incubation than with PLGA devices.

Hence, having different sustained release systems for QbG10p33 with different release kinetics on hand the efficacy of these provisional single dose formulations was investigated in vivo.

In vivo studies - QbG10p33 implants

For bioactivity testing four QbG10p33 implant formulations (Table 5.2.8) were prepared according to the protocol described in chapter 2. The payload of all formulations was 300 µg QbG10p33 / implant. Formulations IQ02, IQ03 and IQ04 were chosen because they revealed the most promising release profiles in vitro with a primary burst of about 10 % (~ 30 µg QbG10p33) followed by either continuous (IQ03 and IQ04) or pulsed release (IQ02) of further 50 – 60 % of incorporated VLP (~150-180 µg QbG10p33) within 3 months (compare Figure 5.2.19). Formulation IQ05 was similar to IQ03 with exception of the used PEG, here PEG 6,000 (IQ03) was exchanged by the FDA approved PEG 3,350 (like in Depo-Provera™).

Table 5.2.8: Compositions of QbG10p33 implants (25 mg) used for in vivo experiments.

Code	QbG10p33 lyophilizate	Resomer® RG 502H	Dynasan 118	PEG 6,000	PEG 3,350	$Mg(OH)_2$
IQ02	10 %	86 %	-	-	-	4 %
IQ03	10 %	80 %	-	6 %	-	4 %
IQ04	10 %	-	80 %	10 %	-	-
IQ05	10 %	80 %	-	-	6 %	4 %

The in vivo experiments were carried out as described in chapter 2. In brief, the different implant formulations were administered to female C57BL / 6 mice (n = 4). Additionally, as a positive control, liquid QbG10p33 formulations (reconstituted lyophilizate FQ01) containing 50 µg VLP were applied either once (day 0) or three times (day 0, 7 and 14) to groups of 3 (single application) or 5 mice (threefold application), respectively. Furthermore, as negative control one placebo implant formulation, composed of 10 % placebo lyophilizate, 80 % Resomer® RG 502H, 6 % PEG 6,000 and 4 % $Mg(OH)_2$ was applied to a group of 4 mice. Sera from the different test groups were collected up to 12 weeks and analyzed by ELISA, to measure the specific anti-p33 IgG titers, and by FACS, to study the activation of p33-specific $CD8^+$ T cells. In parallel in vitro release studies were performed with 3 implants / formulation, respectively.

After three months the in vivo experiments were terminated and the mice were sacrificed. The implantation site was macroscopically examined and it was found that the PLGA implants were almost completely degraded whereas the appearance of the lipid implants was unaltered. No signs of inflammation or encapsulation were observed for none of the implant formulations tested.

In vitro release profile

The cumulative release profiles of QbG10p33 from the four implant formulations are illustrated in Figure 5.2.21. The PLGA implants swelled upon incubation and were almost completely degraded after 3 months. The tristearin implants remained macroscopically unaltered throughout the release studies.

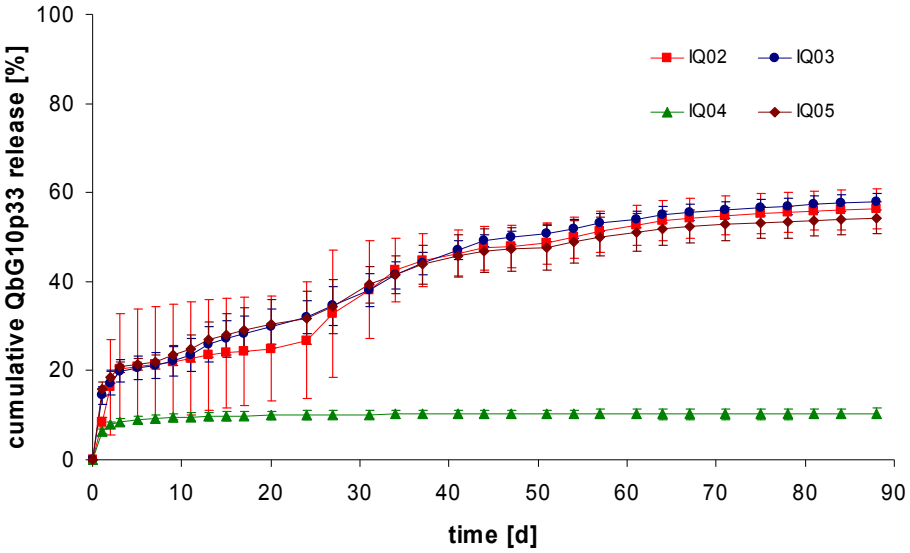


Figure 5.2.21 In vitro release profiles (cumulative release) – QbG10p33 implants.

The release profiles obtained for formulations IQ02, biphasic release, and IQ03, triphasic release, were despite slightly decreased total amounts, comparable to the previously performed in vitro study (compare Figure 5.2.19). The substitution of PEG 6,000 in formulation IQ03 by PEG 3,500 in formulation IQ05 had no effect on the release behavior of QbG10p33. Similar release profiles were obtained for both formulations. Formulation IQ04 showed in contrast to the previously performed in vitro studies a changed release profile. After a similar initial burst of about 10 % VLP, no further QbG10p33 release was observed in this study whereas in the previous experiments a continuous release of further 50 % within 9 weeks was obtained. As

similar prepared QbG10p33 lyophilizates and the same excipients were used, and the same preparation protocol was applied the reason for this changed release behavior is unclear. Importantly, in former in vitro studies similar release profiles were obtained for comparable AngQb and QbG10p33 lipid implant formulations (see Figure 5.2.10 and Figure 5.2.19). Furthermore, Mohl et al and Herrmann et al showed that reproducible release profiles of interferon α-2a from comparable lipid implant formulations (10 % lyophilized protein, 80 % tristearin, 10 % PEG 6,000) could be achieved when applying similar preparation protocols (e.g. compression force and duration) [Mohl et al., 2004; Herrmann et al., 2007a]. Thus, further investigations are necessary to determine the cause of the changed release profile.

Antibody response

The antibody titers obtained for the four implant formulations as well as for the positive and negative controls are illustrated in Figure 5.2.22.

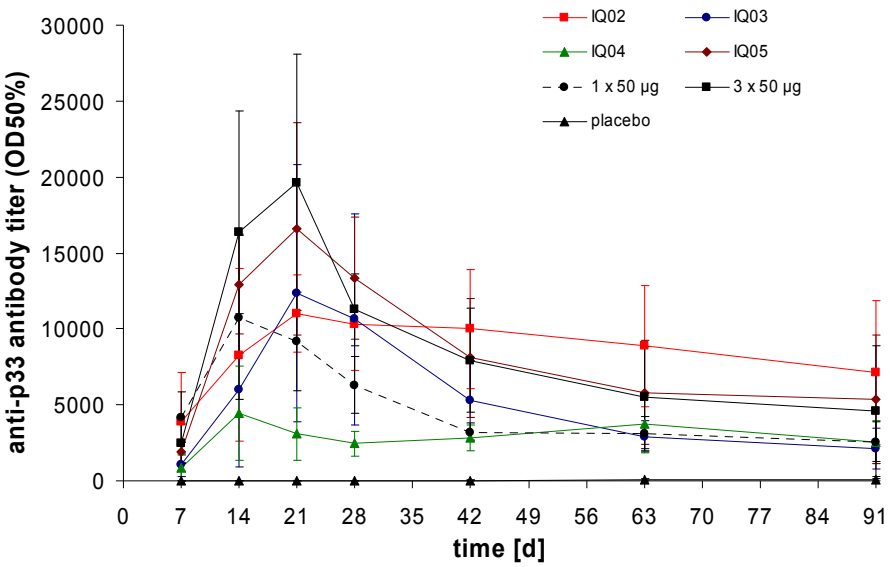


Figure 5.2.22 Anti-p33 antibody response following a single or threefold s.c. administration of a liquid QbG10p33 formulation, or s.c. application of QbG10p33 implant formulations IQ02, IQ03, IQ04 and IQ05.

Following conclusions can be drawn from the presented data:

(1) All verum implant formulations as well as the single and threefold shots of liquid vaccine induced an anti-p33 specific antibody response within the first two weeks whereas no antibody response was observed for the placebo formulation;

(2) A further increase of the antibody titers within day 14 to 21 was observed for the threefold shot of liquid vaccine and the implant formulations IQ02, IQ03 and IQ05;

(3) The antibody titers declined after 14 days for the single shot liquid vaccine and after 21 days for the implant formulations IQ03 and IQ05 and the threefold shot of liquid vaccine;
(4) The antibody titers remained almost stable for formulation IQ02 from day 21 to 91 and for formulation IQ04 from day 14 to 91.

The results obtained for the single shot of the liquid formulation showed after a primary induction of an antibody response, within the first two weeks after application, declining antibody titers. A further increase of the antibody titers was achieved by the threefold weekly application of the liquid vaccine, but even for this group a decrement of the antibody titers was observed after three weeks. Hence, it was assumed that a multiple application of the liquid vaccine, at least for the chosen interval, leads to an increase of the antibody titers but does not lead to long-lasting high antibody titers. The PLGA implant formulations IQ03 and IQ05 induced similar to the threefold application of the liquid vaccine increasing antibody titers, within the first three weeks. The lower initial anti-p33 antibody titers in comparison to the threefold applied liquid formulation can be explained by the lower vaccine release in vitro (Figure 5.2.22). Both implant formulations released about 30 % of the incorporated vaccine (~ 90 µg VLP) within the first three weeks whereas 150 µg were applied with three shots of the liquid vaccine. However, after three weeks the antibody titers declined, no long-lasting high antibody titers were observed. By contrast, long-lasting high antibody titers were obtained for the implant formulation IQ02, here, initially achieved antibody titers remained on the same level for 3 months. As formulation IQ02 was the only formulation which exhibited a pulsed release of the drug in vitro, it seemed that for a long-lasting anti-p33 antibody response this pulsed release with a second burst after four weeks was beneficial to the more continuous release observed for formulations IQ03 and IQ05. The potential of inducing long-lasting antibody responses by sustained release systems which exhibit a pulsed release of the antigen was described in literature e.g. for influenza A vaccine, tetanus toxoid and malaria antigen [Thomasin et al., 1996; Hilbert et al., 1999]. Formulation IQ04 showed after the primary induction of antibody response also long-lasting antibody titers, but on a notably low level. The reason for this long-lasting antibody response is not clear as in vitro no further release of QbG10p33 was observed. Thus, after clarification of the

reasons for the changed in vitro release behavior further in vivo investigations with this implant formulation are necessary.

It has to be mentioned that the vaccination schedule of the test group (threefold application of 50 µg QbG10p33 at day 0, 7 and 14) was different from the observed release profiles obtained for the different implant formulations. For example formulation IQ02 released about 60 µg QbG10p33 within the first three days, a dose similar to a single application of the liquid drug, but then the "second" dose (~ 60 µg) was liberated not as a sharp bolus but over a prolonged period of about two weeks after a three week latency period (see in vitro release profile - Figure 5.2.21). Consequently, the comparison of the implant formulations to the three shot liquid vaccine group is problematic. Therefore, for further studies more comparable vaccination schedules should be used for the positive controls (multiple application of liquid vaccine). Additionally, a more proper investigation of the efficacy of a pulsed vs. continuous release of antigen is necessary. This might for example be feasible by using implantable mini-pumps as introduced by Walduck et al. [Walduck et al., 1997].

Induction of p33-specific CD8$^+$ T cells

The induction of p33-specific CD8+ cells by the four implant formulations as well as for the positive and negative controls are illustrated in Figure 5.2.23.

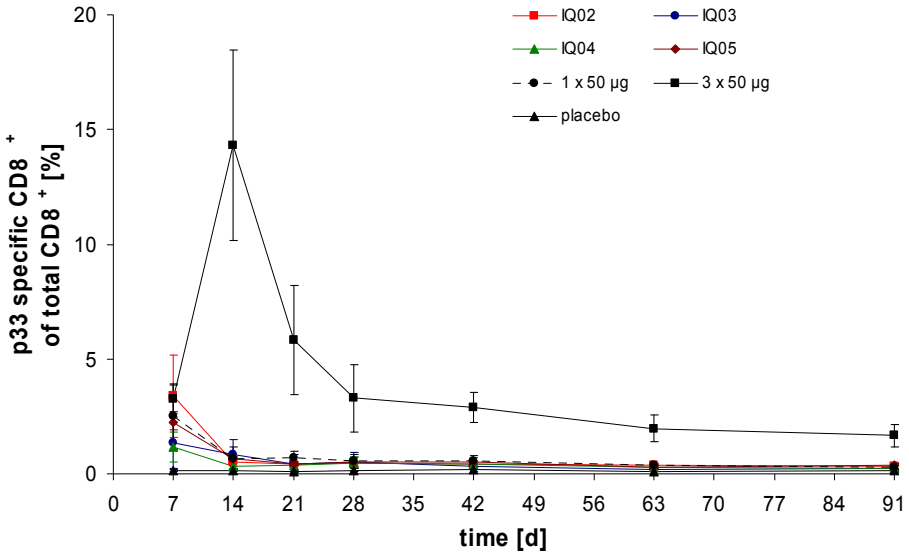


Figure 5.2.23 Induction of p33 specific CD8+ cells after single or threefold s.c. administration of a liquid QbG10p33 formulation, or s.c. application of QbG10p33 implant formulations IQ02, IQ03, IQ04 and IQ05.

The ability of QbG10p33 to induce p33 specific $CD8^+$ T cells was previously shown by Schwarz et al. [Schwarz et al., 2003]. In comparison to recombinantly produced Qbp33 VLP (mainly containing RNA) the application of QbG10p33 VLP (RNA substituted by CpG oligonucleotides) substantially increased frequencies of p33 specific $CD8^+$ T cells. This increased efficacy was explained by the activation of dendritic cells by CpGs via stimulation of Toll like receptor 9 allowing a stronger induction of T cell responses. In the current study it was investigated whether a multiple application of QbG10p33 and a continuous or pulsed release of QbG10p33 from implants can further increase the T cell response. The results obtained from the in vivo study showed that all implant formulations as well as the application of the liquid vaccine was capable to induce p33 specific $CD8^+$ T cells within the first week which is in good correlation to previous studies [Schwarz et al., 2003]. However, after this primary T cell response the amount of p33 specific $CD8^+$ T cells declined for the implant test group and the group that received a single shot of the liquid vaccine. A further increase of p33 specific $CD8^+$ T cells within the second week was only observed for the group of mice which received three times the liquid vaccine. After two weeks a decrease of p33 specific $CD8^+$ T cells was observed as well. The reason for the inability of the implant formulations to further increase the T cell response upon incubation is not clear. Possible reasons for the inhibition of a recall response might be: (1) The amount of QbG10p33 delivered per time interval was too low to restimulate effector T cells; (2) Clearance of the VLPs by neutralizing antibodies [Da Silva et al., 2001] before they could reach lymphoid tissue , which is believed to be unique for T cell priming and expansion [Kaufmann, 2004]; and / or (3) Death or anergy of effector T cells induced by prolonged stimulation [Iezzi et al., 1998; Kaech et al., 2002]. Finally, it can be stated that the achievement of a long-lasting T cell response by sustained release devices is extremely challenging due to the high complexity of the immune system, and thus, further, extensive investigations are necessary.

Summary

The aim of the current in vivo study was to investigate whether long-lasting antibody and / or T cell responses against QbG10p33, as model VLP, can be achieved by PLGA and lipid based implants as provisional single dose vaccine

formulations. The experiments showed that high antibody titers could be achieved by all PLGA based formulations. Long-lasting high antibody levels were obtained by a PLGA formulation which exhibited a triphasic release profile whereas for PLGA formulations with a continuous release profile the antibody titers leveled off after the primary activation. The results obtained for the lipid based formulation were not meaningful due to the in comparison to previous in vitro studies altered release profile. A primary T cell induction was observed for all implant formulations, but no long-lasting effect was observed for any implant formulation. In conclusion, it was shown that the development of single dose formulations for VLP based vaccines intended to induce antibody-mediated immunity is feasible, and thus, the performance of further studies with therapeutic VLP are recommendable. The generation of long-lasting T cell responses by sustained release systems with VLP based vaccines seemed to be more challenging and requires further fundamental research.

5.2.4 Evaluation of Biodegradable PLGA Microcapsules Containing an Oily Core as an Enhanced Controlled Release System for AngQb

Previous experiments revealed that the stability of AngQb, a therapeutic VLP vaccine intended for the treatment of high blood pressure [Ambuehl et al., 2007], was affected by detrimental environmental conditions arising during release from PLGA and lipid based implant formulations. Further in vitro and in vivo studies were performed with QbG10p33, a model VLP that is far more stable than AngQb. It was shown that long-lasting antibody responses against QbG10p33 could be achieved by PLGA implants. Encouraged by this “proof of concept” it was now the objective to develop a formulation which is on the one hand capable to protect the drug from the above mentioned deleterious conditions and on the other hand enables a pulsatile release. In 1996 Sanchez et al. introduced biodegradable PLGA microcapsules with an oily inner core in which a lyophilized antigen, tetanus toxoid, was entrapped. It was considered that the antigen is protected in the oily core from deleterious conditions, e.g. moisture, acidic pH and / or degradation products of PLGA, until the polymer shell is degraded to such a degree that the drug can be liberated. Sanchez et al. showed that depending on the copolymer composition, a pulsed release of immunochemically detectable antigen could be achieved after different times, 3 and 7 weeks, respectively. It was proposed that a single-step immunization might be feasible by

single administration of a composition containing a priming dose and two types of antigen loaded microcapsules with different coating composition [Sanchez et al., 1996]. This seemed to be a very promising approach for VLP, and thus feasibility studies were carried out.

Stability of AngQb in mineral oil

In a first step the stability of AngQb in lyophilized form suspended in mineral oil was assessed as this is the prerequisite for the development of the above described microcapsule formulation. Therefore, 1 mL of mineral oil were added to AngQb lyophilizates (formulation FA02, Table 5.2.2), sealed under nitrogen atmosphere, and stored for 10 weeks at 40 °C to imitate in vivo conditions. For comparison stability of pure AngQb lyophilizates was assessed. Right after preparation and after storage for 4 and 10 weeks, respectively, samples were reconstituted with purified water. To allow extraction of AngQb from the lyophilizate / mineral oil suspensions the samples were agitated gently for 10 minutes and subsequently centrifuged. The reconstituted lyophilizates and the aqueous phase of the extracted samples were analyzed by AF4 at least in duplicate.

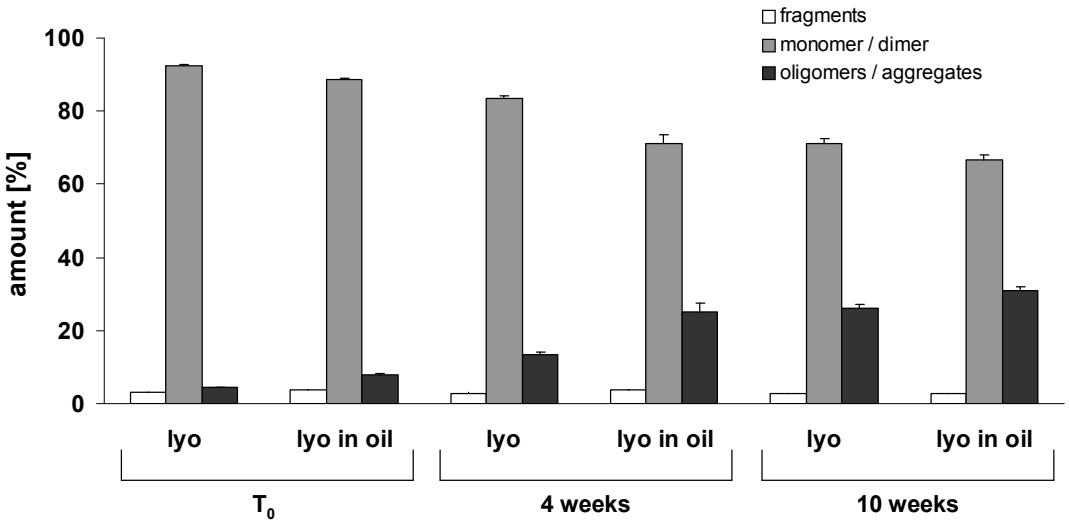


Figure 5.2.24 Stability of AngQb in pure lyophilized form and in lyophilizate / mineral oil suspension during 10 weeks storage at 40 °C, determined by AF4.

In Figure 5.2.24 the respective storage stabilities at 40 °C of AngQb in the different environments are illustrated. AF4 measurements revealed an increase of the aggregation level for both, AngQb lyophilizates and AngQb lyophilizate / mineral oil suspensions. Importantly, after 10 weeks storage time almost similar amounts of

aggregated species were detected for both sample sets. Hence, it was assumed that the stability of AngQb was not affected by mineral oil but depends on the lyophilizate composition. Based on these results it is expected that with an enhanced lyophilized formulation the stability of AngQb in the lyophilizate / oil suspension might be improved as well. However, as the optimization of the lyophilized AngQb formulation was beyond the scope of the present work the above presented formulation (FA02) was used for further basic experiments.

Preparation of microcapsules

Basic principle of microcapsule preparation

The preparation method proposed by Sanchez et al. is a solid / oil_1 / oil_2 / water emulsion technique. In brief, the lyophilized antigen is first dispersed in mineral oil. The resulting suspension is next dispersed in an organic solvent containing PLGA. This organic phase is then emulsified in an aqueous PVA solution. Thereby, microdroplets are formed which gradually harden by precipitation of the PLGA around the oily droplets induced by solvent extraction and evaporation. Finally, the microcapsules are collected, washed and lyophilized [Sanchez et al., 1996] (compare flow chart depicted in chapter 2).

The suggested process is highly complex and includes several factors that influence the morphology, structure, size distribution and encapsulation efficiency of the final microcapsules. Thus, in the following investigations on the diverse processing steps were carried out.

Homogenization of AngQb lyophilizate in mineral oil

First, homogeneous dispersion of the AngQb lyophilizate in mineral oil had to be achieved. For this purpose the centrifugal mixer SpeedMixer™ DAC 150 FVZ (Hauschild Engineering, Hamm, Germany) was evaluated. This device is described to enable extremely fast homogenization (seconds to minutes) of low sample volumes in disposable cups e.g. reaction tubes[#]. For suitability testing a suspension with an lyophilizate / oil weight ratio of 5 : 95 was produced. First AngQb lyophilizate FA02

[#]http://www.speedmixer.co.uk/dac3000.php

was levigated in an agate mortar in a dry-nitrogen-purged glove box (to prevent moisture uptake from the ambient atmosphere). Then, 25 mg of the powder were placed together with 475 mg mineral oil in a 0.5 mL LoBind Eppendorf Cap and homogenized with the Speedmixer^{TM} DAC 150 FVZ at 3500 rpm for 30 s. Then, 3 samples of the resulting suspension, 50 mg each, were extracted within 10 minutes with 1 mL PBS buffer. The aqueous phase was analyzed by SE-HPLC for the determination of AngQb content (by calculation from the regression of an AngQb standard curve) and for the investigation of VLP integrity. The results showed that homogeneous dispersion was feasible, 96.5 ± 4.7 % of the estimated amount of AngQb were determined. Furthermore, it was found that the liberated VLP remained intact during homogenization and liberation from the oil, in comparison to the starting material no increase of VLP fragments or aggregated species was observed.

Selection of organic solvent for PLGA

For manufacture of the above described microcapsules the organic solvent used for PLGA had to possess several features. It had to be volatile, so that it can finally be removed from the microcapsule formulation. Furthermore, it needed to be immiscible with mineral oil and should not harm VLP stability. Commonly used volatile organic solvents for the preparation of PLGA microparticles are methylene chloride [Chattaraj et al., 1999; Kim et al., 1999; Feng et al., 2006], ethyl acetate [Lee et al., 1997; Shi et al., 2002; Li et al., 2002], acetonitrile [Tobio et al., 1999] and acetone [Coombes et al., 1998; Zhuang et al., 2002]. Dissolution experiments revealed that methylene chloride and ethyl acetate as single solvents for PLGA were not possible because both dissolved mineral oil. By contrast mineral oil was not soluble in pure acetone or acetonitrile, and in an acetonitrile / ethyl acetate mixture, which was used e.g. by Sanchez et al. [Sanchez et al., 1996] and Youan et al. [Youan et al., 2001] for microcapsule preparation.

In order to investigate the stability of AngQb suspended in selected organic solvents AngQb lyophilizates were incubated for 10 minutes with 1.0 mL of acetone, ethyl acetate or acetonitrile, respectively. Subsequently, the samples were centrifuged, the organic solvent was removed and the remaining VLP lyophilizate was dissolved in purified water and analyzed by SE-HPLC. The SE-HPLC measurements revealed that AngQb remained intact in all samples, no alteration of AngQb

composition was observed. Thus, it was concluded that acetone, acetonitrile and a acetonitrile / ethyl acetate mixture (1 : 1) were potent solvents for the preparation of microcapsules.

Consequently, three plain microcapsule formulations were prepared with the selected solvents to investigate their effect on the microcapsule morphology. In brief, 50 mg mineral oil were dispersed by vortexing for 3 s in 2 mL of either acetonitrile, acetone or an acetonitrile / ethyl acetate mixture (1 : 1), respectively, containing 10 % Resomer® RG 503, a PLGA with lactide : glycolide molar ratios of 50 : 50 (inherent viscosity in chloroform of 0.42 dL / g). The resulting mixtures were then added through a capillary (0.8 mm inner diameter) to 10 mL of an aqueous PVA solution (0.75 %) under vigorous magnetic stirring. After 3 minutes light microscopic pictures of the initially formed microcapsules were taken.

The pictures showed that by using acetone as solvent for PLGA large, undefined structures were formed (Figure 5.2.25 A and B). A similar observation was made for acetonitrile but besides undefined structures intermediate microcapsules were obtained (Figure 5.2.25 C and D). It was assumed that the reason for the formation of such irregular PLGA structures was the excellent miscibility of acetone and acetonitrile with water enabling a very fast diffusion of these solvents from the organic droplets into the aqueous phase. Hence, the polymer precipitated before microdroplets were formed. In contrast, by using a mixture of acetonitrile and ethyl acetate microdroplets were formed (Figure 5.2.25 E and F). As ethyl acetate is not miscible with water, precipitation of PLGA might be slowed down, so that the organic phase could be emulsified in the surfactant solution before uncontrolled polymer precipitation. Then, by diffusion of acetonitrile out of the organic microdroplets a polymer film at the water / oil interphase is formed entrapping the mineral oil inside the microdroplets and leading to the formation of preliminary microcapsules (Figure 5.2.25 E and F). Hence, a mixture of ethyl acetate / acetonitrile (1 : 1) was used for further studies as solvent for PLGA.

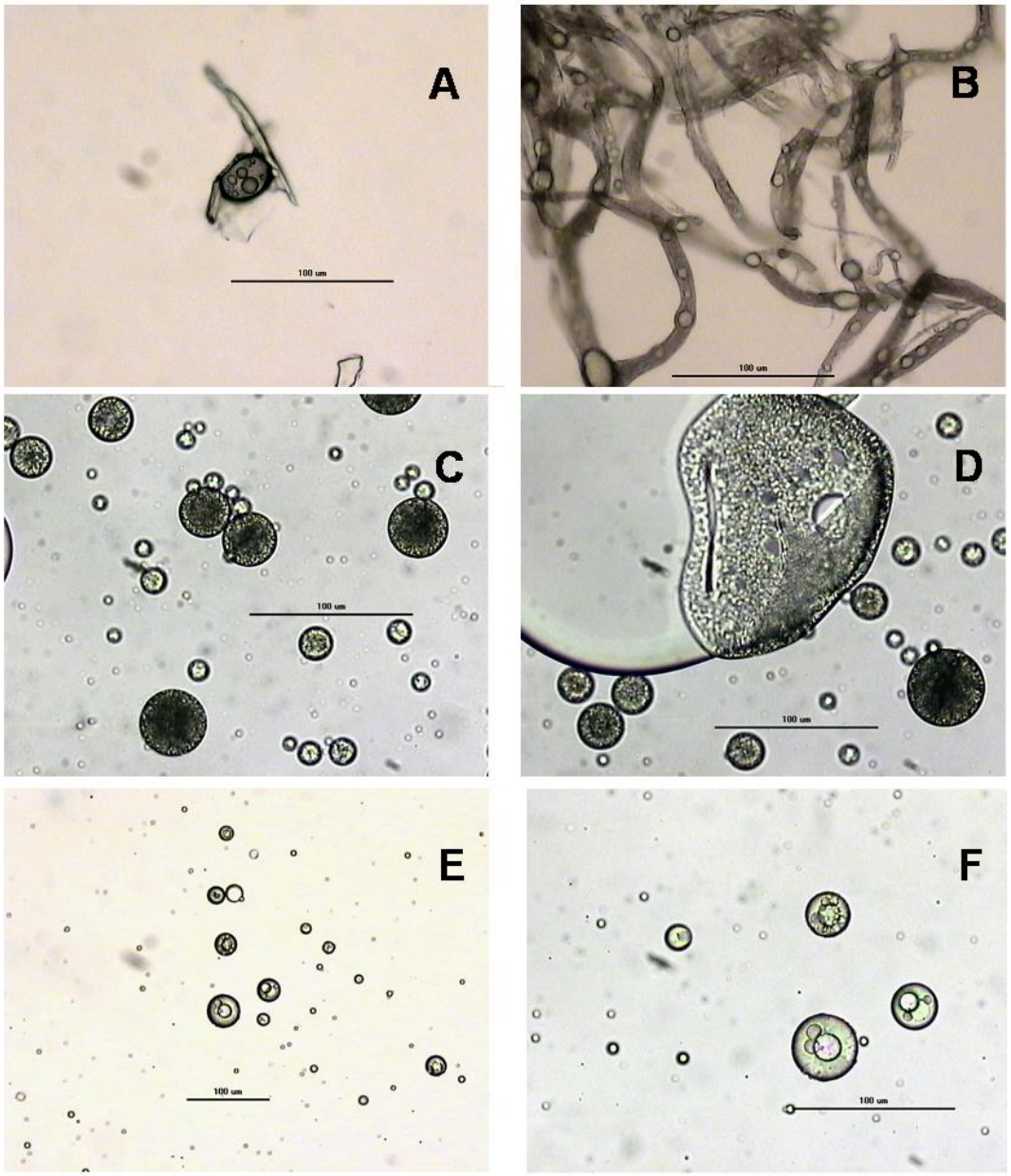


Figure 5.2.25 Photomicrographs of PLGA microcapsules with an oily inner core obtained by light microscopy during the initial phase of the microencapsulation process by using either acetone (A / B), acetonitrile (C / D) or a mixture of acetonitrile / ethyl acetate (1 : 1) as solvent for PLGA.

PLGA microcapsule formation

Four microcapsule formulations with an oily inner core were produced. Two formulations were prepared with AngQb lyophilizates (formulation FA02, Table 5.2.2) and two with QbSAMSA, which is a fluorescein labeled Qb VLP (see chapter 2). Each drug was formulated with either Resomer® RG 502H or Resomer® RG 503. QbSAMSA was lyophilized according to freeze-drying protocol A (see chapter 2) in a formulation containing 1.0 mg / mL QbSAMSA, 2.5 % (w / v) trehalose dihydrate, 50 mM sodium chloride, 0.005 % (w / v) polysorbate 20 and 20 mM sodium phosphate

buffer (pH 7.2). For microcapsule preparation first lyophilizate / mineral oil suspensions with a weight ratio of 5 : 95 were produced as described on page 166. Then 50 mg of the respective suspensions were dispersed by vortexing for 3 s in 2 mL of an acetonitrile / ethyl acetate mixture (1 : 1) containing 10 % polymer. The consecutive processing steps were carried out as described in chapter 2. The microencapsulation process was examined by light microscopy. Furthermore, the structure of the microcapsules was analyzed by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

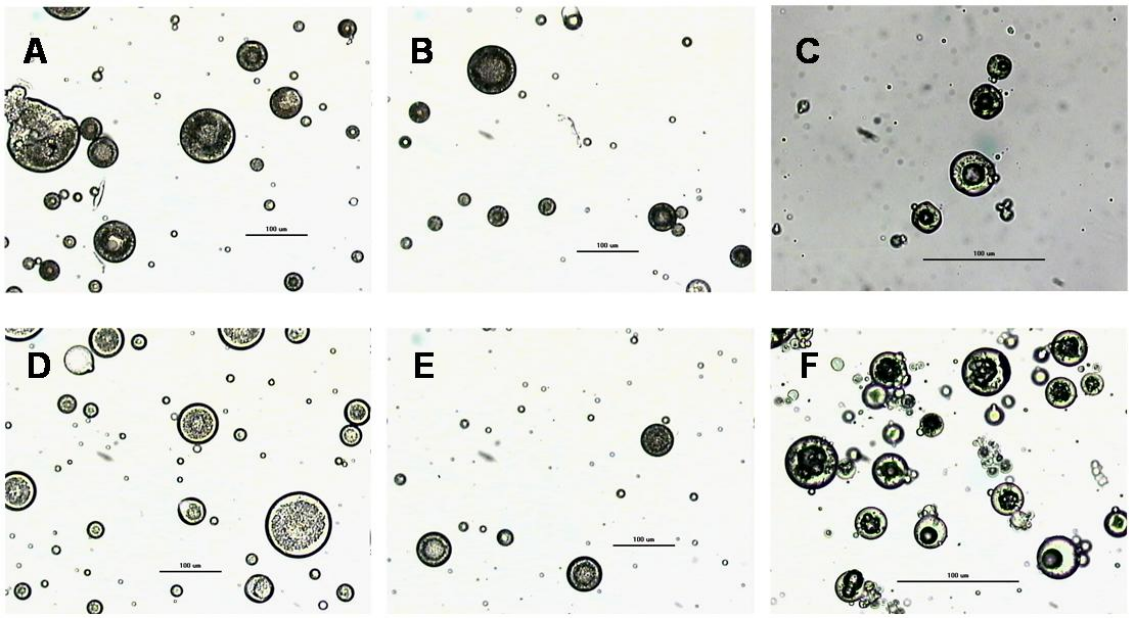


Figure 5.2.26 Photomicrographs of PLGA microcapsules with an oily inner core containing AngQb prepared with either Resomer® RG 502H (A-C) or Resomer® RG 503 (D-F) observed by light microscopy during the initial phase of the microencapsulation process (A / D), after solvent extraction (B / E) and after freeze-drying (C / F).

The photomicrographs in Figure 5.2.26 show the formation of PLGA microcapsules with an oily inner core during the various phases of the microencapsulation process. As described above intermediate microcapsules with a thin polymer film trapping the AngQb loaded oily cores were formed during the initial phase of the process. Upon solvent extraction / evaporation induced by further addition of aqueous PVA solution and water under continuous stirring the polymer shell solidified. After solvent extraction / evaporation the microparticles were first washed with hexane, to remove any residual mineral oil from the surface of the microparticles, then washed with purified water and lyophilized. The

photomicrographs C and F in Figure 5.2.26 indicated that the microcapsules remained intact during the washing steps and the subsequent freeze-drying process.

Further investigation of cross sections of the final microcapsules by SEM (Figure 5.2.27) showed that true core-wall structures with continuous polymer shells were formed. To examine whether the core really consists of mineral oil with entrapped VLP pictures of QbSAMSA microcapsules were taken by CLSM (Figure 5.2.28). The pictures showed bright spots inside the microcapsules which correspond to the fluorescence labeled VLP whereas the polymer shell showed no fluorescence.

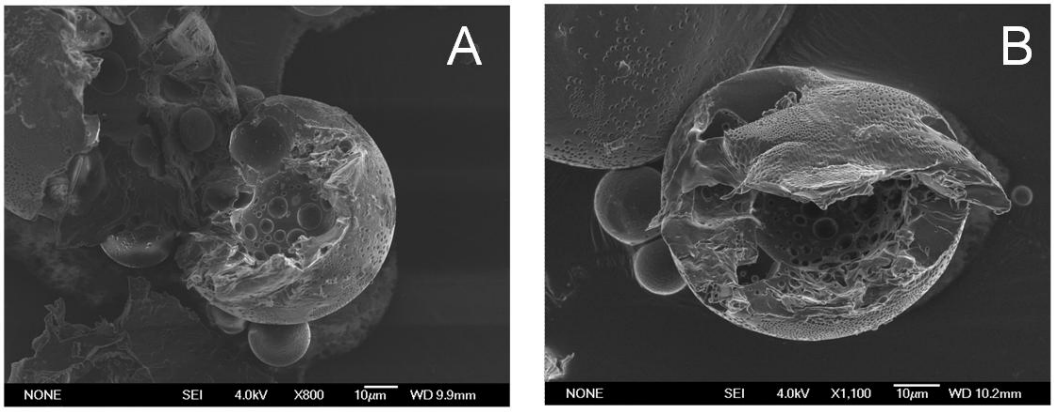


Figure 5.2.27 Scanning electron micrographs of a cross-sectional view of PLGA microcapsules with an oily inner core containing AngQb prepared with either Resomer® RG 502H (A) or Resomer® RG 503 (B).

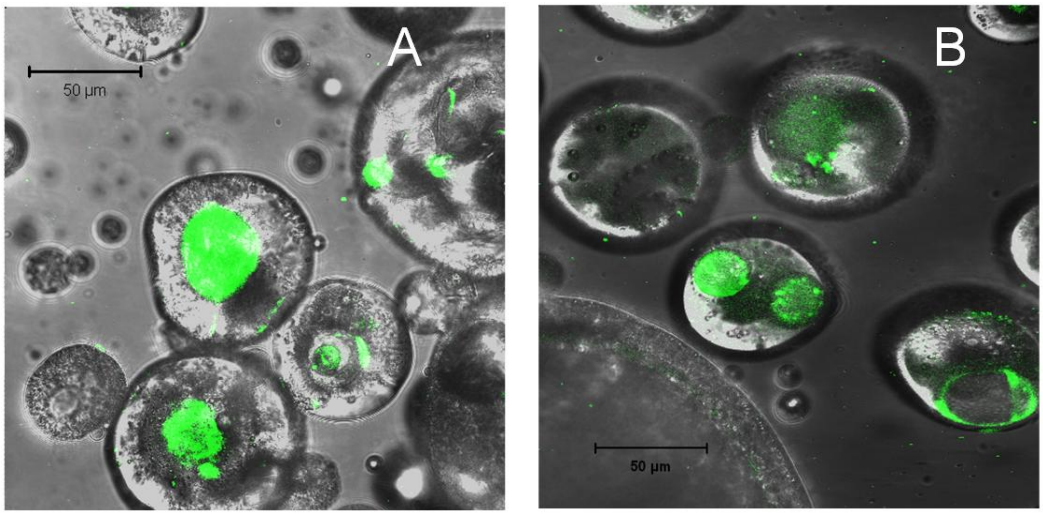


Figure 5.2.28 Confocal laser scanning micrographs of PLGA microcapsules with an oily inner core containing QbSAMSA prepared with either Resomer® RG 502H (A) or Resomer® RG 503 (B).

Thus, the hypothesis of microcapsule formation was corroborated. Furthermore, it was shown that microcapsules with similar appearance were formed for both PLGA materials Resomer® RG 502H and Resomer® RG 503.

Excursus – Evaluation of different techniques for the determination of the amount of VLP encapsulated in PLGA microcapsules

A critical issue during development of microparticle formulations is the accurate determination of the antigen amount encapsulated into the PLGA microspheres. These data are a prerequisite for studying the effects of different preparation techniques and formulations on the encapsulation efficiency. Furthermore, knowledge of the drug payload is important for calculating the cumulative amount of drug released during in vitro studies and for correct dosing of the respective formulation during in vivo studies.

Several techniques have been reported for the determination of antigens encapsulated in PLGA microparticles. One common method involves alkaline hydrolysis of PLGA microspheres followed by antigen quantification by protein assays like Lowry or BCA [Sanchez et al., 1999; Chattaraj et al., 1999; Feng et al., 2006]. However, as already highlighted in previous sections the correct determination of VLP content in a multicomponent sample by protein assays is very complicated because of several possible interferences between excipients and dyes. Hence, it was assumed that an extraction method might be more adequate for our purpose. For the extraction of antigens from PLGA microparticles acetonitrile [Katare et al., 2006] and methylene chloride [Li et al., 2002; Peyre et al., 2003] are commonly used solvents. Thereby, PLGA is dissolved in the organic solvent and the antigen is extracted with an aqueous phase. Another approach is e.g. to crush the particles and extract the encapsulated drug directly with an aqueous phase [Singh et al., 1991; Mohl, 2003].

For the evaluation of an appropriate extraction method for AngQb from PLGA microcapsules a microcapsule formulation was prepared according to the above described process (page 168) from 100 mg AngQb lyophilizate / mineral oil suspension (weight ratio 5 : 95) and 2 mL of a 1 : 1 mixture of ethyl acetate and acetonitrile containing 10 % Resomer® RG 503. Subsequently, samples of 50 mg each were extracted according to the following procedures:

(1) Microcapsules were ground in an agate mortar and VLP were extracted with PBST (phosphate buffered saline with 1 % polysorbate 20).

(2) Microcapsules were incubated with methylene chloride to dissolve PLGA and mineral oil. Then, PBS was added to extract the VLP.

(3) Microcapsules were incubated with acetonitrile to dissolve PLGA. Then, the suspension was centrifuged to precipitate the insoluble VLP lyophilizate / mineral oil suspension and the supernatant was discarded. The procedure was repeated three times. Finally, the VLP were dissolved in PBS.

(4) Microcapsules were processed as described in (3) with the exception that the VLP were finally dissolved in a reducing and denaturing aqueous solution containing 4 M guanidinium chloride (GuaHCl) and 0.1 M dithiothreitol (DTT).

The amount of AngQb in the different samples was determined by RP-HPLC as described in chapter 2.

In Table 5.2.9 the amounts of AngQb found when applying the above described extraction methods, calculated per 100 mg microcapsules, are summarized. The determined payload of AngQb varied strongly between the different methods used.

Table 5.2.9: Actual amounts of AngQb in a microcapsule formulation determined by various extraction methods followed by VLP quantification by RP-HPLC.

Extraction method	Amount VLP [µg / 100 mg microcapsules]
Grinding / PBST	10.3
Methylene chloride / PBS	13.4
Acetonitrile / PBS	17.4
Acetonitrile / GuaHCl and DTT	23.7

Extraction method 1 (crushing of the microcapsules and extraction with PBS) and extraction method 2 (extraction with methylene chloride / PBS) revealed the lowest amounts of AngQb. In the case of method 1 this might be related to an incomplete destruction of the microcapsules. The low amounts detected by method 2 can be a result of drug aggregation at the organic solvent / aqueous solution interface [Sah, 1999]. The extraction with acetonitrile / PBS or acetonitrile / GuaHCl and DTT revealed about 1.5 fold higher amounts of AngQb. This might be explained by the

absence of an organic solvent / water interface (acetonitrile is miscible with water) preventing VLP interfacial aggregation. The more complete extraction of AngQb achieved by method 4 in comparison to method 4 was possible due to the disassembling and the solubilization of the VLP by DTT (disrupture of disulfide bonds) and GuaHCl (disrupture of non-covalent interactions).

Thus, it was assumed that method 4 was the most appropriate method for the determination of the amount of AngQb encapsulated in PLGA microcapsules. However, as the evaluation was performed with a microcapsule formulation with an unknown AngQb payload this method was challenged in a further experiment. To investigate the effect of the presence of mineral oil and / or PLGA on the detectable amount of VLP, the following AngQb samples were analyzed by method 4: (a) Pure AngQb lyophilizate, (b) AngQb lyophilizate with mineral oil – weight ratio 1 : 19, (c) AngQb lyophilizate with mineral oil and Resomer® RG 502H – weight ratio 1 : 19 : 40, and (d) Temperature stressed AngQb lyophilizate (aggregation of AngQb) with mineral oil and Resomer® RG 502H – weight ratio 1 : 19 : 40. All samples were prepared in triplicate. The results are shown in Table 5.2.10.

Table 5.2.10 AngQb recovery determined by RP-HPCL for various AngQb samples after extraction with acetonitrile / GuaHCl and DTT.

Sample	Recovery AngQb [% of applied amount]
AngQb lyophilizate	96.4 ± 1.8
AngQb lyophilizate with mineral oil	94.0 ± 0.6
AngQb lyophilizate with mineral oil and PLGA	91.4 ± 0.6
Stressed AngQb lyophilizate with mineral oil and PLGA	92.4 ± 2.5

The experiment revealed that even for stressed AngQb samples in the presence of mineral oil and PLGA more than 90 % of the applied AngQb amount (calculated from the mass of accurately weight AngQb lyophilizate) could be extracted and quantified by the above described method. Thus, it was confirmed that the extraction procedure with acetonitrile / GuaHCl and DTT followed by AngQb quantification by

RP-HPLC is an accurate method for the determination of the AngQb payload in PLGA microcapsules with an oily inner core.

Formulation development studies

To examine the effect of various oil to PLGA ratios and various PLGA materials on the encapsulation efficiency of AngQb and the morphology and size distribution of the microcapsules six microcapsule formulations (see Table 5.2.11) were prepared as described in chapter 2. In brief, 50 to 200 mg AngQb lyophilizate / mineral oil suspension with a weight ratio of 5 : 95 were dispersed by vortexing in 2 mL of a 1 : 1 mixture of acetonitrile and ethyl acetate containing 200 mg of the specific PLGA. Then, the respective mixtures were emulsified in an aqueous solution containing 0.75 % PVA. For solvent extraction / evaporation further PVA solution and water was added. Finally, the microcapsules were collected, washed and lyophilized.

Table 5.2.11: Compositions PLGA microcapsule formulations containing an oily inner core with AngQb.

Formulation	AngQb lyophilizate [mg]*	Mineral oil [mg]	Resomer® RG 502H [mg]	Resomer® RG 503 [mg]
MCAng01	2.5	47.5	200.0	-
MCAng02	5.0	95.0	200.0	-
MCAng03	10.0	190.0	200.0	-
MCAng04	2.5	47.5	-	200.0
MCAng05	5.0	95.0	-	200.0
MCAng06	10.0	190.0	-	200.0

* 2.5 mg lyophilizate contain 300 µg AngQb

The payload of AngQb in the different formulations was determined by the above described extraction method. The encapsulation efficiency was calculated in difference of material weighed in and the payload determined after preparation. The size distribution and average size of the microcapsule formulations, expressed as volume mean diameter, was determined by laser diffraction. The stability of AngQb upon processing was investigated by SE-HPLC after extraction of the VLP from the

final microcapsule formulations with acetonitrile / PBS (compare, extraction method 3 described on page 171).

The influence of different polymers and varying oil suspension / PLGA ratios on the characteristics of microcapsule formulations is provided in Table 5.2.12.

Table 5.2.12: Characteristics of various AngQb microcapsule formulations.

Formulation	Loading [µg VLP / 100 mg MC]	Encapsulation efficiency [% of applied amount]	Mean diameter / diameter range *d*10 – *d*90 [nm]
MCAng01	9.6	8.0	80.1 27.3 – 146.3
MCAng02	15.8	7.9	88.3 20.6 – 183.2
MCAng03	31.0	10.3	124.4 28.8 – 333.7
MCAng04	9.0	7.5	99.0 28.7 – 196.9
MCAng05	23.7	11.8	135.1 45.3 – 243.3
MCAng06	43.2	14.4	523.4 53.5 – 1426.8

Microcapsules prepared from Resomer® RG 503 exhibited slightly higher encapsulation efficiencies and larger average microcapsule sizes as compared to microcapsules produced with Resomer® RG 502H. These differences can be explained by a higher viscosity of the organic phase for Resomer® RG 503 in comparison to Resomer® RG 502H. At higher viscosities of the organic phase migration of the AngQb loaded oily cores out of the embryonic microspheres might be slowed down leading to higher encapsulation efficiencies. Further, the more viscous organic phase formed larger microdroplets during the initial emulsification step resulting in larger microcapsules. Additionally, it was observed that with increasing ratios of oil suspension / PLGA increasing levels of AngQb loading were achieved. This might be due to the formation of larger microcapsules with larger oily cores and thinner PLGA shells as indicated by the increasing mean diameters. At an oil suspension / PLGA ratio of 1 : 1 for both PLGAs extremely large particles up to a few mm were detected. Here, probably for some microdroplets the formation of a coherent

PLGA shell was unfeasible, so that these large undefined PLGA structures were built. The presence of such clusters in formulations MCAng03 and MCAng06 was confirmed by light microscopy (photomicrographs not shown).

For all formulations only a poor encapsulation efficiency of 8 to 14 % was achieved. The most likely reasons seemed to be the excellent extractability of the hydrophilic VLP from the oily phase with water (see pages 164 - 165) enabling a fast leaching of the VLP from the oily microdroplets during the primary emulsification step during microcapsule preparation.

Concerning the stability of AngQb during processing SE-HPLC data revealed for all samples in comparison to the starting material higher amounts of VLP aggregates and fragments (Table 5.2.13). Interestingly, for the formulations prepared with Resomer® RG 502 H fragmentation was the bigger issue whereas in the formulations prepared with Resomer® RG 503 aggregation played the major role. The reason for VLP aggregation can be the exposure to interfaces between organic and aqueous phases and / or VLP-polymer interactions during processing. A reasonable cause for VLP fragmentation are shear forces occurring during emulsification [Yeo et al., 2001; Perez et al., 2002]. It was assumed that the faster hardening of Resomer® RG 503 in comparison to Resomer® RG 502 H was the reason for the divergent VLP stability problems. On the one hand the early hardening of the PLGA shell can have led to the inclusion of multiple small oil droplets per microcapsule, and thus, to the formation of larger interfaces and a more pronounced aggregation. On the other hand due to the fast hardening the VLP in the oily inner cores were more protected from mechanical stress leading to lower amounts of VLP fragments. Additionally to the preparation steps alteration of VLP integrity could have also been provoked by the extraction procedure. However, despite several deleterious conditions during processing and extraction the proportions of the main peak was for all formulations still 82 – 96 % indicating that the major part of the encapsulated VLP remained intact.

Table 5.2.13: Stability of AngQb in various microcapsule formulations, analyzed by SE-HPLC.

Formulation	Aggregates	Main peak	Fragments
	Amount [%]		
AngQb lyophilizate FA02	0.1	98.7	1.1
MCAng01	1.0	82.4	16.6
MCAng02	2.9	91.1	6.0
MCAng03	1.5	94.2	4.3
MCAng04	11.4	86.9	1.8
MCAng05	9.9	87.7	2.5
MCAng06	1.5	96.2	2.2

Finally, it was concluded that with respect to highest payload and acceptable size distribution the formulations MCAng02 (Resomer® RG 502H) and MCAng05 (Resomer® RG 503), prepared by applying an oil suspension / PLGA ratio of 1 : 2, were the most promising ones. Hence, in a next step it was investigated whether the preparation of these formulations is reproducible and the process to a certain extend upscalable.

Reproducibility of microcapsule preparation and upscalability

For the investigation of the reproducibility of the microencapsulation process the formulations MCAng02 and MCAng05 were prepared in triplicate with 100 mg AngQb lyophilizate / mineral oil suspension (weight ratio 5 : 95) and 200 mg PLGA dissolved in 2 mL in acetonitrile / ethyl acetate (initial batch size) as described in chapter 2. For upscalability testing the threefold amount e.g. 300 mg of the lyophilizate / mineral oil suspension and 600 mg PLGA dissolved in 6 mL acetonitrile / ethyl acetate were applied. Furthermore, also the threefold amount of PVA solution and water, respectively, was used. The resulting microcapsule formulations were examined for AngQb payload and size distribution. The results are summarized in Table 5.2.14.

The data revealed that the preparation of formulation MCAng02 was reproducible and upscalable, only marginal differences for the loading / encapsulation efficiency and the size distribution were observed. Concerning the loading / encapsulation efficiency of formulation MCAng05 it seemed that the process was also reproducible

and upscalable but with respect to the size distribution strong variations between the different batches were noticed. This might be related to the higher viscosity of formulation MCAng05 in comparison to formulation MCAng02 hindering a reproducible, more homogeneous emulsification of the organic phase in the PVA solution.

Table 5.2.14: Investigation of reproducibility and upscalability of the manufacturing process for the AngQb microcapsule formulations MCAng02 and MCAng05.

Formulation	Batch code / composition	Loading [µg VLP / 100 mg MC]	Encapsulation efficiency [% of total AngQb]	Mean diameter / diameter range *d*10 – *d*90 [µm]
MCAng02	A (100 mg oil susp. and 200 mg PLGA)	13.3	6.7	72.7 27.9 – 127.8
	B (100 mg oil susp. and 200 mg PLGA)	10.7	5.4	73.6 28.1 – 131.2
	C (100 mg oil susp. and 200 mg PLGA)	12.4	6.2	78.4 26.2 – 149.6
	D (300 mg oil susp. and 600 mg PLGA)	14.2	7.1	72.7 22.8 – 129.5
MCAng05	A (100 mg oil susp. and 200 mg PLGA)	21.1	10.5	328.6 39.5 – 888.9
	B (100 mg oil susp. and 200 mg PLGA)	26.7	13.4	405.2 49.4 – 1406.1
	C (100 mg oil susp. and 200 mg PLGA)	21.3	10.7	88.4 31.5 – 168.9
	D (300 mg oil susp. and 600 mg PLGA)	27.4	13.7	138.3 33.8 – 332.7

Summary and outlook

The aim of these experiments was to investigate the feasibility of PLGA microcapsules with an oily inner core as enhanced controlled release system for AngQb. Such a system was proposed to enable on the one hand a pulsed release of the incorporated drug, controlled by the degradation properties of the polymer, and on the other hand drug protection in the oily core until release.

It was shown that the preparation of such microcapsules with AngQb embedded in the oily core was possible. Furthermore, primary experiments indicated that the encapsulated material remained almost stable upon processing. However, by applying a solid / oil_1 / oil_2 / water emulsion technique the loading / encapsulation efficiency has not turned out satisfactory, and thus, the performance of in vitro release studies was not feasible. The most likely reason for the poor encapsulation efficiency was assumed to be the leaching of the VLP from the oily droplets during the emulsification step of the microencapsulation process. Thus, to achieve an adequate microcapsule loading, enabling in vitro and in vivo studies, the application of other preparation methods is necessary.

A possible approach might be the preparation of drug loaded lipidic microcores by spray-congealing as e.g. described by Maschke et al. [Maschke et al., 2007] which can subsequently be coated with PLGA. By using lipids with a melting temperature around 35 °C two requirements might be achieved: (1) In vivo release should be possible because the lipid cores are fluid at body temperature, and (2) Coating of the lipidic cores should be feasible by a simple oil / water emulsion technique without substantial loss of the incorporated drug when the coating procedure is performed e.g. at ambient temperature so that the lipid cores are solid, and thus, leaching of the incorporated drug is prevented.

5.3 Conclusion

In the present work primary experiments towards the development of single dose formulations for VLP vaccines were described.

First the release behavior of AngQb and QbG10p33, as model VLP, from PLGA and lipid based implants was examined. It was found that depending on the implant composition a cumulative release of 50 – 80 % VLP upon several weeks was possible. Lipid implants exhibited a biphasic release profile whereas for PLGA implants depending on the matrix composition both, triphasic and biphasic release profiles were achieved. Supplementary studies indicated that the stability of AngQb was affected by environmental conditions arising during incubation of lipid and PLGA based implants. In contrast it was shown that QbG10p33 was less susceptible to deleterious conditions associated with such devices during incubation.

Consequently, in vivo studies were carried out with PLGA and lipid based QbG10p33 implants. The induction of a long-lasting antibody and / or T cell response was examined over a time span of 3 months. It was found that all PLGA based implants led to the induction of high antibody titers during the first weeks. After the primary activation the antibody titers leveled off for PLGA implant formulations with a biphasic release profile whereas long-lasting high antibody levels were obtained for a PLGA formulation exhibiting a triphasic release profile. Further, for all formulations a primary induction of the T cell response was observed but here, no long-lasting effect was achieved for any composition. A meaningful interpretation of the results obtained for the lipid implant formulation was not possible due to an altered release profile as compared to previous studies. However, the in vivo study performed with the provisional implant formulations suggests that the development of single dose VLP based formulations aimed for the induction of antibody-induced immunity against is possible.

Next, feasibility studies with PLGA microcapsules with an oily inner core as enhanced controlled release system for AngQb were performed. With such a formulation two requirements might be achieved, stabilization of the drug in the oily core during incubation and pulsed release of the oily core controlled by the degradation properties of the PGLA shell. It was shown that the manufacture of such microcapsule formulation is possible by applying a solid / oil_1 / oil_2 / water emulsion

technique. However, with this method only poor payloads were achieved so that in vitro or in vivo studies could not be carried out. Consequently, further studies with enhanced preparation protocols need to be performed.

In summary, it can be stated that the preparation of controlled release formulations for VLP enabling the induction of long-lasting antibody responses by a single application seems to be possible. Due to the high potential of such improved vaccine formulations preventing the inconvenient, frequent dosing of a liquid vaccine formulation further investigations in this field are worthwhile.

5.4 References

Alonso, M. J., Gupta, R. K., Min, C., Siber, G. R., and Langer, R.; Biodegradable microspheres as controlled-release tetanus toxoid delivery systems; *Vaccine*, 1994, 12(4), 299-306

Ambuehl, P. M., Tissot, A. C., Fulurija, A., Maurer, P., Nussberger, J., Sabat, R., Nief, V., Schellekens, C., Sladko, K., Roubicek, K., Pfister, T., Rettenbacher, M., Volk, H. D., Wagner, F., Mueller, P., Jennings, G. T., and Bachmann, M. F.; A vaccine for hypertension based on virus-like particles: preclinical efficacy and phase I safety and immunogenicity; *Journal of Hypertension*, 2007, 25(1), 63-72

Amselem, S., Alving, C. R., and Domb, A. J.; Polymeric biodegradable lipospheres as vaccine delivery systems; *Polymers for Advanced Technologies*, 1992, 3(6), 351-357

Berkland, C., Pollauf, E., Varde, N., Pack, D. W., and Kim, K.; Monodisperse Liquid-filled Biodegradable Microcapsules; *Pharmaceutical Research*, 2007, 24(5), 1007-1013

Bilati, U., Allemann, E., and Doelker, E.; Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles; *European Journal of Pharmaceutics and Biopharmaceutics*, 2005, 59(3), 375-388

Bloom, B. R.; Vaccines for the Third World; *Nature*, 1989, 342(6246), 115-120

Boehm, G., Peyre, M., Sesardic, D., Huskisson, R. J., Mawas, F., Douglas, A., Xing, D., Merkle, H. P., Gander, B., and Johansen, P.; On Technological and Immunological Benefits of Multivalent Single-Injection Microsphere Vaccines; *Pharmaceutical Research*, 2002, 19(9), 1330-1336

Bradford, M. M.; A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding; *Analytical Biochemistry*, 1976, 72(1-2), 248-254

Breuer, U.; Bioanalytik, by F. Lottspeich, J. W. Engels (Eds.); *Engineering in Life Sciences*, 2007, 7(3), 283

Brunner, A., Mader, K., and Goepferich, A.; pH and osmotic pressure inside biodegradable microspheres during erosion; *Pharmaceutical Research*, 1999, 16(6), 847-853

Chang, A. C., and Gupta, R. K.; Stabilization of Tetanus Toxoid in Poly(DL-lactic-co-glycolic acid) Microspheres for the Controlled Release of Antigen; *Journal of Pharmaceutical Sciences*, 1996, 85(2), 129-132

Chattaraj, S. C., Rathinavelu, A., and Das, S. K.; Biodegradable microparticles of influenza viral vaccine: comparison of the effects of routes of administration on the in vivo immune response in mice; *Journal of Controlled Release*, 1999, 58(2), 223-232

Cleland, J. L.; Single-administration vaccines: controlled-release technology to mimic repeated immunizations; *Trends in Biotechnology*, 1999, 17(1), 25-29

Cleland, J. L., Barron, L., Berman, P. W., Daugherty, A., Gregory, T., Lim, A., Vennari, J., Wrin, T., and Powell, M. F.; Development of a Single-Shot Subunit Vaccine for HIV-1. 2. Defining Optimal Autoboost Characteristics To Maximize the Humoral Immune Response; *Journal of Pharmaceutical Sciences*, 1996a, 85(12), 1346-1349

Cleland, J. L., Barron, L., Daugherty, A., Eastman, D., Kensil, C., Lim, A., Weissburg, R. P., Wrin, T., Vennari, J., and Powell, M. F.; Development of a Single-Shot Subunit Vaccine for HIV-1. 3. Effect of Adjuvant and Immunization Schedule on the Duration of the Humoral Immune Response to Recombinant MN gp120; *Journal of Pharmaceutical Sciences*, 1996b, 85(12), 1350-1357

Cleland, J. L., Lim, A., Barron, L., Duenas, E. T., and Powell, M. F.; Development of a single-shot subunit vaccine for HIV-1: Part 4. Optimizing microencapsulation and pulsatile release of MN rgp120 from biodegradable microspheres; *Journal of Controlled Release*, 1997, 47(2), 135-150

Cleland, J. L., Lim, A., Daugherty, A., Barron, L., Desjardin, N., Duenas, E. T., Eastman, D. J., Vennari, J. C., Wrin, T., Berman, P., Murthy, K. K., and Powell, M. F.; Development of a single-shot subunit vaccine for HIV-1 with programmable in vivo autoboost and long-lasting neutralizing response; *Journal of Pharmaceutical Sciences*, 1998, 87(12), 1489-1495

Cleland, J. L., Powell, M. F., Lim, A., Barron, L., Berman, P. W., Eastman, D. J., Nunberg, J. H., Wrin, T., and Vennari, J. C.; Development of a single-shot subunit vaccine for HIV-1; *AIDS Research and Human Retroviruses*, 1994, 10(2), 21-26

Cleland, W. W.; Dithiothreitol, a new protective reagent for SH groups; *Biochemistry*, 1964, 3(4), 480-482

Compton, S. J., and Jones, C. G.; Mechanism of dye response and interference in the Bradford protein assay; *Analytical Biochemistry*, 1985, 151(2), 369-374

Coombes, A. G. A., Major, D., Wood, J. M., Hockley, D. J., Minor, P. D., and Davis, S. S.; Resorbable lamellar particles of polylactide as adjuvants for influenza virus vaccines; *Biomaterials*, 1998, 19(11-12), 1073-1081

Coombes, A. G. A., Major, D., Wood, J. M., Minor, P. D., and Davis, S. S.; Substrates of lactide polymers for influenza virus vaccines; *Proceedings of the International Symposium on Controlled Release of Bioactive Materials*, 1997, 24, 505-506

Costantino, H. R., Langer, R., and Klibanov, A. M.; Solid-Phase Aggregation of Proteins under Pharmaceutically Relevant Conditions; *Journal of Pharmaceutical Sciences*, 1994, 83(12), 1662-1669

Costantino, H. R., Langer, R., and Klibanov, A. M.; Aggregation of a lyophilized pharmaceutical protein, recombinant human albumin: effect of moisture and stabilization by excipients; *Nature Biotechnology*, 1995, 13(5), 493-496

Crotts, G., Sah, H., and Park, T. G.; Adsorption determines in-vitro protein release rate from biodegradable microspheres: quantitative analysis of surface area during degradation; *Journal of Controlled Release*, 1997, 47(1), 101-111

Da Silva, D. M., Pastrana, D. V., Schiller, J. T., and Kast, W. M.; Effect of Preexisting Neutralizing Antibodies on the Anti-tumor Immune Response Induced by Chimeric Human Papillomavirus Virus-like Particle Vaccines; *Virology*, 2001, 290(2), 350-360

Dewar, J. B., Hendry, D. A., and Newman, J. F. E.; Biodegradable serum albumin polymers for the sustained release of virus antigens in vaccines. A preliminary report; *South African Medical Journal*, 1984, 65(14), 564-565

Domb, A. J.; Lipospheres for controlled delivery of substances; *Drugs and the Pharmaceutical Sciences*, 2006, 158, 297-316

Dorta, M. J., Santovena, A., Llabres, M., and Farina, J. B.; Potential applications of PLGA film-implants in modulating in vitro drugs release; *International Journal of Pharmaceutics*, 2002, 248(1-2), 149-156

Eldridge, J. H., Staas, J. K., Meulbroek, J. A., McGhee, J. R., Tice, T. R., and Gilley, R. M.; Biodegradable microspheres as a vaccine delivery system; *Molecular Immunology*, 1991, 28(3), 287-294

Eliaz, R. E., and Szoka, F. C., Jr.; Robust and prolonged gene expression from injectable polymeric implants; *Gene Therapy*, 2002, 9(18), 1230-1237

Eliaz, R. E., Wallach, D., and Kost, J.; Delivery of soluble tumor necrosis factor receptor from in-situ forming PLGA implants: in-vivo; *Pharmaceutical Research*, 2000, 17(12), 1546-1550

Esparza, I., and Kissel, T.; Parameters affecting the immunogenicity of microencapsulated tetanus toxoid; *Vaccine*, 1992, 10(10), 714-720

Feng, L., Qi, X. R., Zhou, X. J., Maitani, Y., Wang, S. C., Jiang, Y., and Nagai, T.; Pharmaceutical and immunological evaluation of a single-dose hepatitis B vaccine using PLGA microspheres; *Journal of Controlled Release*, 2006, 112(1), 35-42

Freitas, S., Merkle, H. P., and Gander, B.; Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology; *Journal of Controlled Release*, 2005, 102(2), 313-332

Fu, K., Klibanov, A. M., and Langer, R.; Protein stability in controlled-release systems; *Nature Biotechnology*, 2000, 18(1), 24-25

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; *Pharmaceutical Research*, 2000, 17(1), 100-106

Gander, B., Meinel, L., Walter, E., and Merkle, H. P.; Polymers as a platform for drug delivery: reviewing our current portfolio on poly(lactide-co-glycolide) (PLGA) microspheres; *Chimia*, 2001, 55(3), 212-217

Garcia, J. T., Dorta, M. J., Munguia, O., Llabres, M., and Farina, J. B.; Biodegradable laminar implants for sustained release of recombinant human growth hormone; *Biomaterials*, 2002, 23(24), 4759-4764

Goepferich, A.; Mechanisms of polymer degradation and erosion; *Biomaterials*, 1996, 17(2), 103-114

Golmohammadi, R., Fridborg, K., Bundule, M., Valegaard, K., and Liljas, L.; The crystal structure of bacteriophage Q.beta. at 3.5 .ANG. resolution; *Structure (London)*, 1996, 4(5), 543-554

Gombotz, W. R., and Pettit, D. K.; Biodegradable Polymers for Protein and Peptide Drug Delivery; *Bioconjugate Chemistry*, 1995, 6(4), 332-351

Greenway, T. E., Eldridge, J. H., Ludwig, G., Staas, J. K., Smith, J. F., Gilley, R. M., and Michalek, S. M.; Enhancement of protective immune responses to Venezuelan equine encephalitis (VEE) virus with microencapsulated vaccine; *Vaccine*, 1995, 13(15), 1411-1420

Guse, C., Koennings, S., Maschke, A., Hacker, M., Becker, C., Schreiner, S., Blunk, T., Spruss, T., and Goepferich, A.; Biocompatibility and erosion behavior of implants made of triglycerides and blends with cholesterol and phospholipids; *International Journal of Pharmaceutics*, 2006, 314(2), 153-160

Hatefi, Y., and Hanstein, W. G.; Solubilization of particulate proteins and nonelectrolytes by chaotropic agents; *Proceedings of the National Academy of Sciences of the United States of America*, 1969, 62(4), 1129-1136

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; *Pharmaceutical Research*, 2007a, 24(8), 1527-1537

Herrmann, S., Winter, G., Mohl, S., Siepmann, F., and Siepmann, J.; Mechanisms controlling protein release from lipidic implants: Effects of PEG addition; *Journal of Controlled Release*, 2007b, 118(2), 161-168

Higaki, M., Azechi, Y., Takase, T., Igarashi, R., Nagahara, S., Sano, A., Fujioka, K., Nakagawa, N., Aizawa, C., and Mizushima, Y.; Collagen minipellet as a controlled release delivery system for tetanus and diphtheria toxoid; *Vaccine*, 2001, 19(23-24), 3091-3096

Hilbert, A. K., Fritzsche, U., and Kissel, T.; Biodegradable microspheres containing influenza A vaccine: immune response in mice; *Vaccine*, 1999, 17(9-10), 1065-1073

Iezzi, G., Karjalainen, K., and Lanzavecchia, A.; The duration of antigenic stimulation determines the fate of naive and effector T cells; *Immunity*, 1998, 8(1), 89-95

Jaganathan, K. S., Rao, Y. U. B., Singh, P., Prabakaran, D., Gupta, S., Jain, A., and Vyas, S. P.; Development of a single dose tetanus toxoid formulation based on polymeric microspheres: a comparative study of poly(,-lactic-co-glycolic acid) versus chitosan microspheres; *International Journal of Pharmaceutics*, 2005, 294(1-2), 23-32

Jain, R. A.; The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices; *Biomaterials*, 2000, 21(23), 2475-2490

Jain, R., Shah, N. H., Malick, A. W., and Rhodes, C. T.; Controlled drug delivery by biodegradable polyester devices: different preparative approaches; *Drug Development and Industrial Pharmacy*, 1998, 24(8), 703-727

Jiang, W., Costantino, H. R., and Schwendeman, S. P.; Stabilization of lyophilized proteins encapsulated in controlled-release polymers: Overview and case studies including formaldehyde-treated vaccine antigens; *Biotechnology: Pharmaceutical Aspects*, 2004, 2, 483-518

Jiang, W., Gupta, R. K., Deshpande, M. C., and Schwendeman, S. P.; Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens; *Advanced Drug Delivery Reviews*, 2005, 57(3), 391-410

Jiang, W., and Schwendeman, S. P.; Stabilization and controlled release of bovine serum albumin encapsulated in poly(D, L-lactide) and poly(ethylene glycol) microsphere blends; *Pharmaceutical Research*, 2001, 18(6), 878-885

Johansen, P., Gander, B., Merkle, H. P., and Sesardic, D.; Ambiguities in the preclinical quality assessment of microparticulate vaccines; *Trends in Biotechnology*, 2000a, 18(5), 203-211

Johansen, P., Men, Y., Merkle, H. P., and Gander, B.; Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination; *European Journal of Pharmaceutics and Biopharmaceutics*, 2000b, 50(1), 129-146

Johansen, P., Men, Y., Audran, R., Corradin, G., Merkle, H. P., and Gander, B.; Improving stability and release kinetics of microencapsulated tetanus toxoid by co-encapsulation of additives; *Pharmaceutical Research*, 1998, 15(7), 1103-1110

Johansen, P., Tamber, H., Merkle, H. P., and Gander, B.; Diphtheria and tetanus toxoid microencapsulation into conventional and end-group alkylated PLA/PLGAs; *European Journal of Pharmaceutics and Biopharmaceutics*, 1999, 47(3), 193-201

Jung, T., Koneberg, R., Hungerer, K. D., and Kissel, T.; Tetanus toxoid microspheres consisting of biodegradable poly(lactide-co-glycolide)- and ABA-triblock-copolymers: immune response in mice; *International Journal of Pharmaceutics*, 2002, 234(1-2), 75-90

Kaech, S. M., Wherry, E. J., and Ahmed, R.; Effector and memory T-cell differentiation: implications for vaccine development; *Nature Reviews Immunology*, 2002, 2(4), 251-262

Kaewvichit, S., and Tucker, I. G.; The release of macromolecules from fatty acid matrixes: complete factorial study of factors affecting release; *Journal of Pharmacy and Pharmacology*, 1994, 46(9), 708-713

Kang, F., and Singh, J.; Effect of additives on the release of a model protein from PLGA microspheres; *AAPS PharmSciTech*, 2001, 2(4), 30

Katare, Y. K., and Panda, A. K.; Influences of excipients on in vitro release and in vivo performance of tetanus toxoid loaded polymer particles; *European Journal of Pharmaceutical Sciences*, 2006, 28(3), 179-188

Kaufmann, S. H. E.; Novel Vaccination Strategies; *WILEY-VCH Verlag GmbH & Co.KGaA*, 2004

Kemp, J. M., Kajihara, M., Nagahara, S., Sano, A., Brandon, M., and Lofthouse, S.; Continuous antigen delivery from controlled release implants induces significant and anamnestic immune responses; *Vaccine*, 2002, 20(7-8), 1089-1098

Kent, J. S.; Cholesterol matrix delivery system for sustained release of macromolecules; US Patent 4452775 ,1984

Kessler, R. J., and Fanestil, D. D.; Interference by lipids in the determination of protein using bicinchoninic acid; *Analytical Biochemistry*, 1986, 159(1), 138-142

Khan, M. A., Healy, M. S., and Bernstein, H.; Low temperature fabrication of protein loaded microspheres; *Proceedings of the 19th International Symposium of Controlled Release of Bioactive Materials*, 1992, 518-519

Khan, M. Z., Tucker, I. G., and Opdebeeck, J. P.; Cholesterol and lecithin implants for sustained release of antigen: release and erosion in vitro, and antibody response in mice; *International Journal of Pharmaceutics*, 1991, 76(1-2), 161-170

Kim, H. K., and Park, T. G.; Microencapsulation of human growth hormone within biodegradable polyester microspheres: protein aggregation stability and incomplete release mechanism; *Biotechnology and Bioengineering*, 1999, 65(6), 659-667

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*, 2006, 314(2), 145-152

Koennings, S., and Goepferich, A.; Lipospheres as delivery systems for peptides and proteins; *Lipospheres in Drug Targets and Delivery*, 2005, 67-86

Kofler, N., Ruedl, C., Klima, J., Recheis, H., Boeck, G., Wick, G., and Wolf, H.; Preparation and characterization of poly(DL-lactide-co-glycolide) and poly(L-lactic acid) microspheres with entrapped pneumotropic bacterial antigens; *Journal of Immunological Methods*, 1996, 192(1-2), 25-35

Lee, H. K., Park, J. H., and Kwon, K. C.; Double-walled microparticles for single shot vaccine; *Journal of Controlled Release*, 1997, 44(2), 283-293

Li, X., Zhou, S., Wu, X., Yuan, M., Liu, L., Jia, W., Deng, X., and Huang, Z.; Poly-DL-lactide-poly(ethylene glycol) microspheres as oral and parenteral delivery systems for hepatitis B surface antigen; *Journal of Applied Polymer Science*, 2002, 83(4), 850-856

Lima, K. M., and Rodrigues Junior, J. M.; Poly-DL-lactide-co-glycolide microspheres as a controlled release antigen delivery system; *Brazilian Journal of Medical and Biological Research*, 1999, 32(2), 171-180

Liu, W. R., Langer, R., and Klibanov, A. M.; Moisture-induced aggregation of lyophilized proteins in the solid state; *Biotechnology and Bioengineering*, 1991, 37(2), 177-184

Lofthouse, S. A., Kajihara, M., Nagahara, S., Nash, A., Barcham, G. J., Sedgmen, B., Brandon, M. R., and Sano, A.; Injectable silicone implants as vaccine delivery vehicles; *Vaccine*, 2002, 20(13-14), 1725-1732

Lofthouse, S.; Immunological aspects of controlled antigen delivery; *Advanced Drug Delivery Reviews*, 2002, 54(6), 863-870

Lu, W., and Park, T. G.; Protein release from poly(lactic-co-glycolic acid) microspheres: Protein stability problems; *PDA Journal of Pharmaceutical Science and Technology*, 1995, 49(1), 13-19

Lucke, A., Kiermaier, J., and Goepferich, A.; Peptide acylation by poly(a-hydroxy esters); *Pharmaceutical Research*, 2002, 19(2), 175-181

Maschke, A., Lucke, A., Vogelhuber, W., Fischbach, C., Appel, B., Blunk, T., and Goepferich, A.; Lipids: An alternative material for protein and peptide release; *ACS Symposium Series*, 2004, 879, 176-196

Maschke, A., Becker, C., Eyrich, D., Kiermaier, J., Blunk, T., and Goepferich, A.; Development of a spray congealing process for the preparation of insulin-loaded lipid microparticles and characterization thereof; *European Journal of Pharmaceutics and Biopharmaceutics*, 2007, 65(2), 175-187

Matschke, C., Isele, U., van Hoogevest, P., and Fahr, A.; Sustained-release injectables formed in situ and their potential use for veterinary products; *Journal of Controlled Release*, 2002, 85(1-3), 1-15

Medlicott, N. J., and Tucker, I. G.; Pulsatile release from subcutaneous implants; *Advanced Drug Delivery Reviews*, 1999, 38(2), 139-149

Mohl, S.; The Development of a Sustained and Controlled Release Device for Pharmaceutical Proteins based on Lipid Implants; Dissertation, Ludwig-Maximilians-University Munich, 2003

Mohl, S., and Winter, G.; Continuous release of rh-interferon a-2a from triglyceride matrices; *Journal of Controlled Release*, 2004, 97(1), 67-78

Moore, A., McGuirk, P., Adams, S., Jones, W. C., McGee, J. P., O'Hagan, D. T., and Mills, K. H. G.; Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells; *Vaccine*, 1995, 13(18), 1741-1749

O'Hagan, D. T., and Singh, M.; Microparticles as vaccine adjuvants and delivery systems; *Expert Review of Vaccines*, 2003, 2(2), 269-283

Onishi, H., Takahashi, M., and Machida, Y.; PLGA implant tablet of ketoprofen: Comparison of in vitro and in vivo releases; *Biological & Pharmaceutical Bulletin*, 2005, 28(10), 2011-2015

Opdebeeck, J. P., and Tucker, I. G.; A cholesterol implant used as a delivery system to immunize mice with bovine serum albumin; *Journal of Controlled Release*, 1993, 23(3), 271-279

Packhaeuser, C. B., Schnieders, J., Oster, C. G., and Kissel, T.; In situ forming parenteral drug delivery systems: an overview; *European Journal of Pharmaceutics and Biopharmaceutics*, 2004, 58(2), 445-455

Palinko-Biro, E., Ronaszeki, G., Merkle, H. P., and Gander, B.; Release kinetics and immunogenicity of parvovirus microencapsulated in PLA/PLGA microspheres; *International Journal of Pharmaceutics*, 2001, 221(1-2), 153-157

Park, T. G.; Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition; *Biomaterials*, 1995, 16(15), 1123-1130

Park, T. G., Lu, W., and Crotts, G.; Importance of in vitro experimental conditions on protein release kinetics, stability and polymer degradation in protein-encapsulated poly(DL-lactic acid-co-glycolic acid) microspheres; *Journal of Controlled Release*, 1995, 33(2), 211-222

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*, 2002, 54(3), 301-313

Peyre, M., Sesardic, D., Merkle, H. P., Gander, B., and Johansen, P.; An experimental divalent vaccine based on biodegradable microspheres induces protective immunity against tetanus and diphtheria; *Journal of Pharmaceutical Sciences*, 2003, 92(5), 957-966

Pitt, C. G.; The controlled parenteral delivery of polypeptides and proteins; *International Journal of Pharmaceutics*, 1990, 59(3), 173-196

Preis, I., and Langer, R. S.; A single-step immunization by sustained antigen release; *Journal of Immunological Methods*, 1979, 28(1-2), 193-197

Ramchandani, M., Pankaskie, M., and Robinson, D.; The influence of manufacturing procedure on the degradation of poly(lactide-co-glycolide) 85:15 and 50:50 implants; *Journal of Controlled Release*, 1997, 43(2), 161-173

Ravivarapu, H. B., Moyer, K. L., and Dunn, R. L.; Sustained suppression of pituitary-gonadal axis with an injectable, in situ forming implant of leuprolide acetate; *Journal of Pharmaceutical Sciences*, 2000, 89(6), 732-741

Reid, R. E..; Peptide and Protein Drug Analysis; Drugs and the pharmaceutical sciences, *Marcel Dekker, New York*, 2000

Reithmeier, H., Herrmann, J., and Gopferich, A.; Lipid microparticles as a parenteral controlled release device for peptides; *Journal of Controlled Release*, 2001, 73(2-3), 339-350

Sah, H.; Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation; *Journal of Controlled Release*, 1999b, 58(2), 143-151

Sah, H.; Protein Behavior at the Water/Methylene Chloride Interface; *Journal of Pharmaceutical Sciences*, 1999a, 88(12), 1320-1325

Sanchez, A., Villamayor, B., Guo, Y., McIver, J., and Alonso, M. J.; Formulation strategies for the stabilization of tetanus toxoid in poly(lactide-co-glycolide) microspheres; *International Journal of Pharmaceutics*, 1999, 185(2), 255-266

Sanchez, A., Gupta, R. K., Alonso, M. J., Siber, G. R., and Langer, R.; Pulsed controlled-release system for potential use in vaccine delivery; *Journal of Pharmaceutical Sciences*, 1996, 85(6), 547-552

Sandor, M., Enscore, D., Weston, P., and Mathiowitz, E.; Effect of protein molecular weight on release from micron-sized PLGA microspheres; *Journal of Controlled Release*, 2001, 76(3), 297-311

Santovena, A., Garcia, J. T., Oliva, A., Llabres, M., and Farina, J. B.; A mathematical model for interpreting in vitro rhGH release from laminar implants; *International Journal of Pharmaceutics*, 2006, 309(1-2), 38-43

Schwarz, K., Storni, T., Manolova, V., Didierlaurent, A., Sirard, J. C., Roethlisberger, P., and Bachmann, M. F.; Role of Toll-like receptors in costimulating cytotoxic T cell responses; *European Journal of Immunology*, 2003, 33(6), 1465-1470

Schwendeman, S. P.; Recent advances in the stabilization of proteins encapsulated in injectable PLGA delivery systems; *Critical Reviews in Therapeutic Drug Carrier Systems*, 2002, 19(1), 73-98

Schwendeman, S. P., Cardamone, M., Klibanov, A., Langer, R., and Brandon, M. R.; Stability of proteins and their delivery from biodegradable polymer microspheres; *Drugs and the Pharmaceutical Sciences*, 1996, 77, 1-49

Schwendeman, S. P., Costantino, H. R., Gupta, R. K., and Langer, R.; Peptide, protein, and vaccine delivery from implantable polymeric systems. Progress and challenges; *Controlled Drug Delivery*, 1997, 229-267

Schwendeman, S. P., Costantino, H. R., Gupta, R. K., Siber, G. R., Klibanov, A. M., and Langer, R.; Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation; *Proceedings of the National Academy of Sciences of the United States of America*, 1995, 92(24), 11234-11238

Shi, L., Caulfield, M. J., Chern, R. T., Wilson, R. A., Sanyal, G., and Volkin, D. B.; Pharmaceutical and immunological evaluation of a single-shot hepatitis B vaccine formulated with PLGA microspheres; *Journal of Pharmaceutical Sciences*, 2002, 91(4), 1019-1035

Singh, M., Li, X. M., McGee, J. P., Zamb, T., Koff, W., Wang, C. Y., and O'Hagan, D. T.; Controlled release microparticles as a single dose hepatitis B vaccine: evaluation of immunogenicity in mice; *Vaccine*, 1997, 15(5), 475-481

Singh, M., Singh, A., and Talwar, G. P.; Controlled delivery of diphtheria toxoid using biodegradable poly(D,L-lactide) microcapsules; *Pharmaceutical Research*, 1991, 8(7), 958-961

Sinha, V. R., and Trehan, A.; Biodegradable microspheres for protein delivery; *Journal of Controlled Release*, 2003, 90(3), 261-280

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; *Analytical Biochemistry*, 1985, 150(1), 76-85

Storni, T., Ruedl, C., Schwarz, K., Schwendener, R. A., Renner, W. A., and Bachmann, M. F.; Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects; *Journal of Immunology*, 2004, 172(3), 1777-1785

Sturesson, C., and Degling, W. L.; Comparison of poly(acryl starch) and poly(lactide-co-glycolide) microspheres as drug delivery system for a rotavirus vaccine; *Journal of Controlled Release*, 2000, 68(3), 441-450

Takahashi, M., Onishi, H., and Machida, Y.; Development of implant tablet for a week-long sustained release; *Journal of Controlled Release*, 2004, 100(1), 63-74

Tamber, H., Johansen, P., Merkle, H. P., and Gander, B.; Formulation aspects of biodegradable polymeric microspheres for antigen delivery; *Advanced Drug Delivery Reviews*, 2005, 57(3), 357-376

Thomasin, C., Ho, N. T., Merkle, H. P., and Gander, B.; Drug microencapsulation by PLA/PLGA coacervation in the light of thermodynamics. 1. Overview and theoretical considerations; *Journal of Pharmaceutical Sciences*, 1998, 87(3), 259-268

Thomasin, C., Corradin, G., Men, Y., Merkle, H. P., and Gander, B.; Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response; *Journal of Controlled Release*, 1996, 41, 131-145

Tobio, M., Schwendeman, S. P., Guo, Y., McIver, J., Langer, R., and Alonso, M. J.; Improved immunogenicity of a core-coated tetanus toxoid delivery system; *Vaccine*, 1999a, 18(7-8), 618-622

Tobio, M., Nolley, J., Guo, Y., McIver, J., and Alonso, M. J.; A novel system based on a Poloxamer/PLGA blend as a tetanus toxoid delivery vehicle; *Pharmaceutical Research*, 1999b, 16(5), 682-688

Toussaint, J. F., Dubois, A., Dispas, M., Paquet, D., Letellier, C., and Kerkhofs, P.; Delivery of DNA vaccines by agarose hydrogel implants facilitates genetic immunization in cattle; *Vaccine*, 2007, 25(7), 1167-1174

van de Weert, M., Hoechstetter, J., Hennink, W. E., and Crommelin, D. J. A.; The effect of a water/organic solvent interface on the structural stability of lysozyme; *Journal of Controlled Release*, 2000, 68(3), 351-359

van de Weert, M., Hennink, W. E., and Jiskoot, W.; Protein instability in poly(lactic-co-glycolic acid) microparticles; *Pharmaceutical Research*, 2000, 17(10), 1159-1167

Vogelhuber, W., Magni, E., Mouro, M., Spruss, T., Guse, C., Gazzaniga, A., and Goepferich, A.; Monolithic triglyceride matrices: a controlled-release system for proteins; *Pharm Dev Technol*, 2003, 8(1), 71-79

von Burkersroda, F., Schedl, L., and Goepferich, A.; Why degradable polymers undergo surface erosion or bulk erosion; *Biomaterials*, 2002, 23(21), 4221-4231

Walduck, A. K., and Opdebeeck, J. P.; Effect of the profile of antigen delivery on antibody responses in mice; *Journal of Controlled Release*, 1997, 43(1), 75-80

Walduck, A. K., Opdebeeck, J. P., Benson, H. E., and Prankerd, R.; Biodegradable implants for the delivery of veterinary vaccines: design, manufacture and antibody responses in sheep; *Journal of Controlled Release*, 1998, 51(2-3), 269-280

Wang, F., Lee, T., and Wang, C. H.; PEG modulated release of etanidazole from implantable PLGA/PDLA discs; *Biomaterials*, 2002, 23(17), 3555-3566

Wang, P. Y.; Lipids as excipient in sustained release insulin implants; *International Journal of Pharmaceutics*, 1989, 54(3), 223-230

Wiechelman, K. J., Braun, R. D., and Fitzpatrick, J. D.; Investigation of bicinchoninic acid protein assay: identification of the groups responsible for color formation; *Analytical Biochemistry*, 1988, 175(1), 231-237

Witt, C., Mader, K., and Kissel, T.; The degradation, swelling and erosion properties of biodegradable implants prepared by extrusion or compression moulding of poly(lactide-co-glycolide) and ABA triblock copolymers; *Biomaterials*, 2000, 21(9), 931-938

Yeo, Y., Baek, N., and Park, K.; Microencapsulation methods for delivery of protein drugs; *Biotechnology and Bioprocess Engineering*, 2001, 6(4), 213-230

Yeo, Y., and Park, K.; Control of encapsulation efficiency and initial burst in polymeric microparticle systems; *Archives of Pharmacal Research*, 2004, 27(1), 1-12

Youan, B. B. C., Jackson, T. L., Dickens, L., Hernandez, C., and Owusu-Ababio, G.; Protein release profiles and morphology of biodegradable microcapsules containing an oily core; *Journal of Controlled Release*, 2001, 76(3), 313-326

Zhou, S., Liao, X., Li, X., Deng, X., and Li, H.; Poly-D,L-lactide-co-poly(ethylene glycol) microspheres as potential vaccine delivery systems; *Journal of Controlled Release*, 2003, 86(2-3), 195-205

Zhou, X., Liu, B., Yu, X., Zha, X., Zhang, X., Chen, Y., Wang, X., Jin, Y., Wu, Y., Chen, Y., Shan, Y., Chen, Y., Liu, J., Kong, W., and Shen, J.; Controlled release of polyethylenimine/DNA complexes from mannose-bearing chitosan microspheres as a potent delivery system to enhance immune response to HBV DNA vaccine; *Journal of Controlled Release*, 2007, 121(3), 200-207

Zhu, G., Mallery, S. R., and Schwendeman, S. P.; Stabilization of proteins encapsulated in injectable poly(lactide-co-glycolide); *Nature Biotechnology*, 2000, 18(1), 52-57

Zhu, G., and Schwendeman, S. P.; Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives; *Pharmaceutical Research*, 2000, 17(3), 351-357

Zhuang, F. F., Liang, R., Zou, C. T., Ma, H., Zheng, C. X., and Duan, M. X.; High efficient encapsulation of plasmid DNA in PLGA microparticles by organic phase self-emulsification; *Journal of Biochemical and Biophysical Methods*, 2002, 52(3), 169-178

6 FINAL CONCLUSION

The present work addresses the development of stable, freeze-dried formulations for a specific VLP based vaccine, NicQb, meeting the requirements for large scale clinical studies and commercial use, and in vitro as well as in vivo feasibility studies for single dose sustained release systems for VLP vaccines.

For the development of VLP based pharmaceutical products the application of reliable analytical tools is of great importance. For the assessment of physical properties of virus-like particles up to now three main techniques, i.e. TEM, DLS and SE-HPLC are established. As all of these techniques have some inherent drawbacks there is the strong need for additional, enhanced analytical tools.

In **Chapter 3** asymmetrical flow field-flow fractionation (AF4) is introduced as a new, versatile analytical tool for the assessment of VLP compositions. The rational development of reliable AF4 methods is presented. It is shown that VLP compositions can be classified by AF4 into different fractions, i.e. VLP fragments, monomers, dimers, oligomers and aggregates. Furthermore, by coupling AF4 to UV and MALLS detectors accurate quantification of the specific fractions and the determination of the molecular weight distributions are possible. Comparative experiments revealed that AF4 exceeds the capabilities of DLS and SE-HPLC with regard to the analysis of the physical properties of VLP compositions, and thus, it can be stated that AF4 is a valuable analytical method for the characterization of VLP formulations.

Recently, the VLP based vaccine NicQb was introduced by Cytos Biotechnology AG as a new drug for the treatment of nicotine addiction. For large scale clinical trials and commercialization the development of stable formulations enabling storage for prolonged periods is required. In **Chapter 4** the development of stable, freeze-dried NicQb formulations is described. Due to the well structured study setup from pH, freeze-thaw, freeze-drying and finally long-term stability studies a fast progress towards a commercializable product was feasible.

The primary conducted pH stability study revealed two divergent trends, for the preservation of chemical stability (nicotine binding and integrity of VLP shell) pH

values below 6.6 were favourable whereas pH values higher than 6.2 were necessary to prevent NicQb aggregation. Hence, as a compromise the optimum pH range was assessed between 6.2 and 6.6.

In freeze-thaw studies the effect of sodium chloride, trehalose and polysorbate 20 on the stability of NicQb was investigated. The study indicated that polysorbate 20 prevents NicQb aggregation during freeze-thawing whereas trehalose had no protective effect. Sodium chloride induced NicQb aggregation but up to a sodium chloride concentration of 90 mM NicQb aggregation could be prevented in the presence of polysorbate 20.

In the next step the development of a gentle but robust freeze-drying cycle for trehalose based NicQb formulations is described which is on the one hand concerning the process parameters easily transferable to large-scale freeze-driers, and on the other hand short enough (2 days) to meet the criteria for economically acceptable processes. The effect of polysorbate 20, trehalose, different buffer systems, sodium chloride and varying NicQb concentrations on the stability of NicQb during freeze-drying was investigated. It was found that a formulation composed of trehalose as lyoprotectant, polysorbate 20 as cryoprotectant and either sodium or potassium phosphate as buffer agents was highly beneficial to preserve the stability of NicQb during freeze-drying. Lyophilizates with excellent appearance, low residual moisture contents and high glass transition temperatures were achieved.

The stability of NicQb in four lyophilized formulations was tested up to six months at 2-8 °C, 25 °C / 60 % RH and 40 °C / 75 % RH storage temperature. Thereby, a formulation containing trehalose as lyoprotectant and bulking agent is compared to a formulation containing mannitol as bulking agent and trehalose as lyoprotectant in a weight ratio of 4 : 1. Additionally, the influence of potassium phosphate vs. sodium phosphate as buffer agents and the effect of formulation pH values of 5.8 vs. 6.2 on the stability of NicQb were tested. All dried formulations showed excellent appearance and were easily reconstituted to parenteral applicable liquids. It was shown that all formulations were capable of stabilizing NicQb during freeze-drying and upon storage at 2-8 °C and 25 °C, physical as well as chemical stability of NicQb was maintained. Upon storage at 40 °C a marginal increase of the amount of nicotine derivatives, cleaved from the VLP surface, was observed for all formulations, which was related to the slightly increasing residual moisture contents upon storage at 40 °C. Concerning

the lyophilizate morphology it was found that the trehalose based lyophilizates remained amorphous whereas for the formulations containing trehalose and mannitol an uncontrolled crystallization of mannitol was observed upon storage at 40 °C. In vivo experiments demonstrated that even after long-term storage at accelerated temperatures comparable antibody titers to the bulk material and the lyophilized starting material were attainable.

In the last part the optimization of the freeze-drying process applied for the preparation of the mannitol / trehalose based formulations and the effect of a pure crystalline mannitol on the stability of NicQb is described. The experiments showed that by applying an annealing step complete crystallization of mannitol during the freeze-drying process could be achieved preventing uncontrolled crystallization of mannitol upon storage. Furthermore, it was found that a completely crystalline matrix was not capable of stabilizing NicQb during freeze-drying and upon storage.

Finally, it was concluded that the addition of trehalose as lyoprotectant, leading to an amorphous matrix, and polysorbate 20, preventing NicQb aggregation was very beneficial for stabilizing NicQb during freeze-drying and upon storage.

Vaccines typically require multiple applications to achieve the desired immune response. The frequent dosing is inconvenient with respect to patient's compliance and treatment costs, and thus, the development of single injection formulations is highly desirable. In **Chapter 5** primary steps towards the development of single dose formulations for VLP vaccines are described. For this purpose two VLP vaccines, AngQb and QbG10p33 were used.

First, as provisional sustained release devices, implants composed of PLGA or tristearin as matrix materials, lyophilized VLP, and optionally PEG as pore forming substance and $Mg(OH)_2$ as basic additive were prepared by simple compression. The in vitro release behavior of the two VLP vaccines from different implant compositions was studied. Liberation of the VLP from PLGA or lipid implants was observed for several weeks. For PLGA implants both, triphasic (initial burst release, lag phase, second release period) and biphasic (initial burst release followed a further continuous release period) could be achieved by variations of the matrix composition. Lipid implants exhibited a biphasic release profile. The cumulative release of VLP ranged from 50 % to 80 %, depending on the implant composition. The incomplete release

was ascribed for both VLP to moisture induced aggregation, occurring during implant incubation and adsorption to the matrix materials. In the case of AngQb PLGA implants VLP aggregation induced by an acidic microenvironment arising during matrix degradation was deemed to be a further parameter for incomplete release. Supplementary experiments revealed that QbG10p33 was less susceptible than AngQb to potentially deleterious environmental conditions developing in the matrices upon incubation.

In vivo experiments were performed with PLGA and lipid based QbG10p33 implants. It was shown that all PLGA based implant formulations induced initially high antibody titers. Long-lasting high antibody levels were achieved by a PLGA implant formulation which exhibited a triphasic release profile whereas for PLGA formulations with a continuous release profile the antibody titers leveled off. Concerning the induction of p33-specific $CD8^+$ T cells it was found that all formulations led to a primary activation but no further stimulation of the specific T cells was achieved. A meaningful discussion of the effect of tristearin implants on antibody and T cell response was not possible because of an inexplicably change of the release profile as compared to previous in vitro studies. Finally, it was reasoned that the development of single dose formulation for VLP based vaccines aimed for the induction of antibody-mediated immune response is possible.

In the last part of this chapter basic experiments towards the preparation of an enhanced sustained release device, PLGA microcapsules with an oily inner core, are described. This formulation was supposed to protect the drug in the oily core and to offer, depending on the degradation behavior of PLGA applied, a pulsed release of the drug. In preliminary experiments it was shown that the manufacture of such microcapsules is feasible and that the integrity of the VLP within the microcapsules was almost preserved upon processing, but with the currently used solid / oil_1 / oil_2 / water emulsion only poor encapsulation efficiencies were achieved. Hence, in further studies alternate preparation methods e.g. formation of solid lipid cores by spray congealing followed by PLGA coating by oil in water emulsion, need to be evaluated.

In conclusion, these preliminary experiments indicated that the preparation of single dose formulations for VLP is feasible and that with such devices a long-lasting antibody response can be achieved in vivo.

In summary, stable freeze-dried NicQb formulations possible for large scale clinical trials and commercialization were developed. The fast progress was promoted by the early establishment of AF4, as a sensitive analytical tool for the investigation of the physical stability of VLP. Additionally, it was shown that basically the preparation of sustained release systems for VLP is possible, and that with such devices long-lasting antibody responses can be induced. Based on these preliminary, promising results further studies in this field seem to be worthwhile.

This Thesis has been Presented and Published in Parts in:

Original Research Article

R. Lang, L. Vogt, A. Zürcher, B. Dorigo, B. Schimmele, and G. Winter

Rational Design of a Stable, Freeze-Dried Virus-Like Particle Based Vaccine Formulation

(accepted by Drug Development and Industrial Pharmacy, May 2008)

Patent Application

R. Lang, L. Vogt, A. Zürcher, and G. Winter

Nicotine-Carrier Vaccine Formulation

EP1854478 / WO 2007/131972 (2006)

Short Communications

R. Lang, G. Winter

Virus-Like Particle Characterization Using New AF4 Channel Technology

Application Note published online, www.wyatt.com (2007)

T. Serno, R. Lang, S. Gottschalk, and G. Winter

Convenient Separation of GCSF Using Asymmetrical Flow Field-Flow Fractionation

Application Note published online, www.wyatt.com (2007)

R. Lang, G. Winter

Characterization of Virus Like Particles by Asymmetrical Flow Field Flow Fractionation

Application Note published online, www.wyatt.com (2006)

G. Gottschalk, R. Lang, and G. Winter

Quantification of Insoluble Monoclonal Antibody Aggregates

Application Note published online, www.wyatt.com (2006)

Poster Presentations

R. Lang, S. Gottschalk, and G. Winter

Comparison of Asymmetrical Flow Field-Flow Fractionation to Various Commonly Used Analytical Tools for Biopharmaceuticals

AAPS Annual Meeting and Exposition, San Diego, CA, USA (2007)

R. Lang, L. Vogt, A.. Zürcher, and G. Winter

Stabilization of a Virus-Like Particle Based Vaccine by Freeze-Drying

AAPS Annual Meeting and Exposition, San Diego, CA, USA (2007)

W. Hild, R. Lang, G. Winter, and A. Goepferich

Towards the Characterization of Quantum Dots by Asymmetrical Flow Field-Flow Fractionation

DPhG Jahrestagung, Erlangen, Germany (2007)

Curriculum Vitae

Rainer Albert Lang

Personal Data

Date and Place of Birth	May 29th 1976, Medias (Romania)
Nationality	German
Marital Status / Children	Unmarried, three children

Work Experience and Academic Training

Since 05 / 2005	Assistant Head of Production, Intervet / Schering-Plough Animal Health, Unterschleissheim, Germany
10 / 2004 - 04 / 2008	PhD studies under supervision of Prof. Dr. Gerhard Winter Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-University Munich (Cooperation project with Cytos Biotechnology AG, Schlieren, Switzerland)
01 / 2004 - 03 / 2008	Retail pharmacist, Munich

Education

05 / 2003 - 10 / 2003	Practical education in pharmacy (industry) IDEA AG, Munich
11 / 2002 - 04 / 2003	Practical education in pharmacy (retail) Iris Apotheke, Munich
10 / 1998 - 09 / 2002	Study of pharmacy Ludwig Maximilians University, Munich University of Regensburg
09 / 1996 - 07 / 1999	Professional education as Pharmaceutical-Technical Assistant (PTA) Rosen-Apotheke, Friedberg Berufsfachschule für PTA, Augsburg
09 / 1995 - 09 / 1996	Community service Lebenshilfe für Behinderte e.V., Aichach
1986 - 1995	High School, Graduation with Abitur Deutschherren-Gymnasium Aichach