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Twin-Screw Extruded Lipid Implants for Vaccine Delivery

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Abstract

In recent years there has been a considerable focus on the development of subunit vaccines, preferred over traditional vaccines for reasons of safety and purity. However, subunit vaccines are less immunogenic than attenuated vaccines and need therefor multiple administrations in combination with immunostimulatory adjuvants, in order to induce immunity. The sustained release of a vaccine together with the release of an adjuvant is a potential alternative to giving multiple doses. The aim of this thesis was to manufacture lipid implants for vaccine delivery by twin-screw (tsc) extrusion and evaluate the potency of these lipid systems to stimulate an immune response *in vivo*.

To accomplish this, lipid implants consisting of cholesterol, soybean lecithin, and Dynasan 114 (D114) were prepared. Different formulations were evaluated for their extrudability before adding the model antigen ovalbumin (OVA) and the adjuvant Quil-A (QA) to the formulation. Investigating the release behaviour of OVA and QA showed that mainly cholesterol influences the release behaviour of OVA, increasing the fraction of cholesterol slows down the release of OVA. To further slow down the release of OVA from the implants, they were cured at different temperature resulting in an even longer OVA release. Furthermore, the addition of QA to the implants influenced the release behaviour of OVA and vice versa. The investigation of the implant polymorphism after the extrusion process as well as during storage showed good stability. To combine the advantage of particulate delivery and sustained release, preformed liposomes were incorporated into the implants prior to extrusion. For the analysis of the immune response, two sets of animal experiments in mice were performed, one evaluating the kinetics of the release of the model antigen *in vivo*, a second one to evaluate the immune response *in vivo*. Evaluation of these data indicated a correlation between the *in vitro* and *in vivo* release behaviour of OVA. Furthermore, immune responses similar to those induced by two booster injections, consisting of OVA and alum could be achieved using implant formulations containing QA. These results further emphasized the importance of adjuvant in the formulation. The incorporation of preformed liposomes into the implants on the other hand did not lead to an improved outcome. In a second part of this work, an *in vivo* tumour study was

prepared, using the TRP2 peptide as active ingredient. Due to the use of this expensive peptide, a transfer to a different extruder was necessary. The influence that a change of the production device has on the implants characteristics was investigated. Once the formulation was adapted to the new extruder, implants containing TRP2 and QA were produced. The *in vitro* release of TRP2 proved to be very slow, much different from the OVA release. Furthermore, the preparation of vesicular phospholipid gels (VPGs) as an alternative lipid delivery system for TRP2 was investigated. The TRP2 release from the VPGs was also slow and incomplete. Both formulations were used in an *in vivo* tumour growth study. Mice were injected with B16F10luc2 melanoma cells, 6 days later formulations were administered. VPGs showed adverse reactions in the mouse and are therefore not s suitable delivery system. TRP2 implants showed a slow delay in the start of tumour growth, but were not more potent that TRP2 in PBS injections given to the mice. The very slow *in vitro* release data of TRP2 brought up the question about interactions between the lipid implants and the peptide influencing the release. Choosing peptides of different size and hydropathy, an investigation of their release behaviour and interaction with the implants was conducted.

In conclusion, lipid implants were well tolerated and offer a great potential as sustained release delivery system for vaccines. They allow releasing the active component and the adjuvant together, enabling to achieve a strong immune response.

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Publications arising from this thesis

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Marie-Paule Even, Gerhard Winter, Sarah Hook and Julia Engert. Sustained delivery systems for vaccines and adjuvants. *Manuscript in preparation for Expert Opinion*

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List of abbreviations

APC	antigen presenting cell
BSA	bovine serum albumin
CFSE	5, 6-carboxy-fluoresceine diacetate succinimidyl ester
CHOL	cholesterol
CMC	carboxymethylcellulose
CNT	chitosan nanoparticle
D114	Dynasan 114
DC	dendritic cell
FACS	fluorescence-activated cell sorting
FCA	Freunds' complete adjuvant
HBsAg	Hepatitis B surface antigen
IEP	Isoelectric point
IFA	Incomplete Freund's adjuvant
INF-ϒ	Interferon-ϒ
MHC	major histocompatibility complex
MPL	monophosphoryl lipid A
MW	molecular weight
OVA	Ovalbumin
PAMPS	pathogen associated antigen patterns
PBS	phosphate buffered saline
PC	phosphaditylcholine
PLA	poly(d,l-lactide)
PLCG	poly(d,l-lactide-co-glycolide)
PLGA	poly(lactide-co-glycolide) acid
PRR	pattern recognition pattern

QA	Quil-A
rGP120	recombinant glycoprotein 120
RP-HPLC	reversed phase high-pressure liquid chromatography
SD	standard deviation
SEM	scanning electron microscopy
SLN	solid lipid nanoparticle
SNP	silica nanoparticle
TEM	transmission electron microscopy
TT	tetanus toxoid
TRP2	tyrosinase-related protein 2
tsc	twin-screw
VPG	vesicular phospholipid gel

Chapter One

General Introduction

Parts of this chapter will be submitted as review article:

Marie-Paule Even, Gerhard Winter, Sarah Hook, Julia Engert

1 General Introduction

1.1 Introduction

The world's first vaccination was performed by Edward Jenner in 1796. Vaccination represents the most effective approach to prevent diseases [1]. According to Louis Pasteur, a vaccine is defined as a "suspension of live (usually attenuated) or inactivated microorganisms (e.g. viruses or bacteria) or fractions thereof administered to induce immunity and prevent infectious disease" [2]. Spreading of infectious diseases such as diphtheria, measles, mumps, pertussis and smallpox have been reduced due to the development of safe and effective vaccines and their widespread distribution in many countries. Even though a lot of vaccines are commercially available, vaccines for many diseases, including two of the world´s leading killers, Malaria and HIV, remain elusive [2,3]. In addition, there is an increasing awareness that vaccines might also be considered as therapies against chronic infections or cancer [4]. Conventional vaccines are based on entire live attenuated or inactivated pathogens, or their inactivated toxins, that do not lead to an infection, but are capable of inducing protective immunity. These vaccines are very effective in terms of protection, but there are several drawbacks arising from their preparation. On the one hand, there are safety considerations, namely the difficulty of being able to ensure adequate attenuation or killing of the pathogen [5]. This possible risk can be of particular consequences in the case of fatal incurable diseases such as AIDS [5]. Conventional vaccines can additionally induce vaccine related diseases in people with immune deficiencies. Between 1969 and 1982, 94 cases of paralytic poliomyelitis were reported in the US due to the use of live, oral polio vaccines [6,7]. Apart from safety considerations, it is sometimes difficult to prepare sufficient material for vaccine production, for example for viruses that cannot be cultivated *in vitro*. Therefore, new approaches for vaccine development, not based on the entire organism, are being considered.

To overcome the negative side effects associated with the use of whole microorganisms, purified antigens instead of whole pathogens are used for vaccination. The identification and production of the antigens of pathogens able to induce protective immune response is made possible by new techniques allowing the

identification, production, isolation and characterization of relevant antigens. These non-replicating subunit vaccines are based on the targeted delivery of specific antigens (proteins, peptides or DNA) to cells of the immune system [8]. Even though subunit vaccines are safer, they pose new challenges, because the immune response against purified antigens alone is often insufficient to stimulate protective immunity [6,9]. This is because subunit antigens do not provide any of the necessary signals to activate innate immunity [8] and are often degraded before activation of the immune system can occur. Therefore, subunit vaccines are often given together with immunostimulatory adjuvants to enhance their immunogenicity [10] and multiple doses of subunit vaccines are given in order to stimulate protective immunity. This renders subunit vaccines unattractive in terms of cost and patient compliance as well as from a logistics point of view especially, in developing countries [4].

Ramon was the first to describe vaccine adjuvants about 80 years ago [4,11]. According to the definition, an adjuvant (Latin "adjuvare" which means to help) is any material that helps to increase the humoral or cellular immune response to an antigen [12]. The use of an appropriate adjuvant component in subunit vaccines can turn an ineffective vaccine into an effective vaccine [13].

Single shot formulations, able to release the antigen in a sustained manner or able to mimic giving two or several booster injections, would have a major impact on vaccine compliance [14] and would reduce costs. A huge number of different delivery systems for vaccination, made of different materials, have been tested, such as particles [15-19], liposomes [20-22], ISCOMs [6,23], gels [24] and implants [25-29]. This review will focus on sustained release systems (Figure 1-1). Different release profiles will be compared and the impact of sustained antigen release on immune responses explored (Figure 1-2).

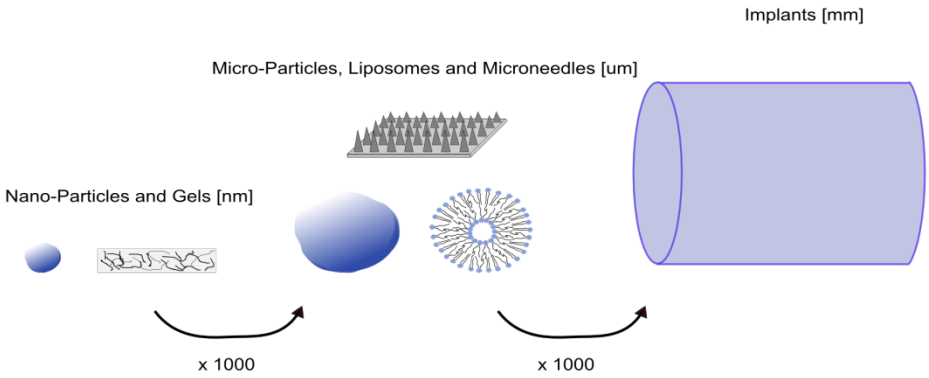

Figure 1-1: Different release systems described in this review. Nanoparticles and gels in the nm range, microparticles, liposomes and microneedles in the μm range and implants in the mm range. Between each group is a difference in size of the order of magnitude of 1000.

1.2 Sustained release delivery systems for vaccination

The goal of research in this area is to improve existing vaccines and to allow the use of novel vaccines by presenting the antigens to the immune system in a way that induces strong, long-lasting immunity. Delivery systems should closely imitate the composition and characteristics of actual pathogens and should protect the antigens from degradation upon delivery. Different release kinetics have been evaluated, to determine whether continuous or pulse release is preferable (Figure 1-2), since antigen release kinetics have a clear impact on immunogenicity [30].

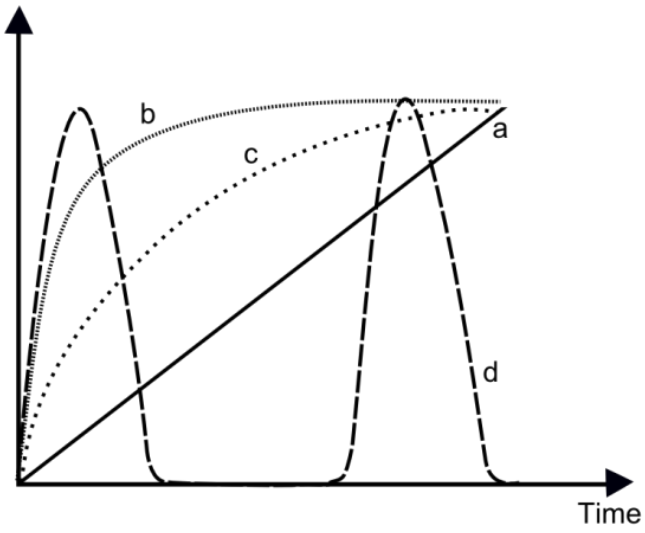

Figure 1-2: Release profile: (a) zero-order release profile, (b) first-order release profile with burst release during the beginning of the release, (c) first-order release profile, (d) dual-pulsed release profile with two pulses. Schematic representation modified after Engert [31].

1.2.1 Particles

There has been a major focus on the development of particulate vaccines and adjuvants over the past years [32]. An antigen-loaded particle may act as an antigen depot, slowly releasing the antigen to prolong its availability. But compared to other sustained release systems, particles are particularly interesting as it is known that the uptake of vaccine antigen by antigen presenting cells (APC's) is enhanced if the antigen is presented in particulate rather than its soluble form [33]. A vast number of studies investigating particles for vaccine delivery have been conducted.

1.2.1.1 PLGA particles

The most commonly described material for particles is poly(lactide-co-glycolide) acid (PLGA). The immune response to ovalbumin (OVA) entrapped in PLGA microparticles (5.32 μm) was compared to that stimulated by emulsified OVA in Freunds' complete adjuvant (FCA) by O'Hagan *et al* (1991) [34]. The immune response to the OVA-loaded particles was higher than to OVA in FCA. The PLGA microparticles have potential as a system for controlled vaccine delivery due to their ability to slowly degrade and release the entrapped antigen. In a second study, O' Hagan *et al* (1993) [35] showed that mice immunized with OVA-loaded PLGA particles are able to induce strong IgG antibody responses and maintain them for a full year. Furthermore, they studied the

effect of particle size on immunogenicity and found that smaller particles (1.5 μm) were more immunogenic than microparticles of 72.6 μm. Joshi *et al* (2013) also investigated the influence of particle size on immune responses and confirmed the previous results [17]. PLGA particles loaded with OVA and CpG oligodeoxynucleotides sized between 17 μm and 200 nm were administered to C57BL/6 mice. The particles showed a release between 48 h (300 nm particles) and 350 h in PBS at 37 °C. *In vivo* the highest antigen-specific cytotoxic T cell responses as well as the highest OVA-specific antibody titres were found in mice immunized with the 300 nm sized particles. These results suggest that smaller particles induce and stimulate a stronger immune response. But not only is the size of the particles important, but also the duration during which the antigen is presented to the immune system. OVA-loaded PLGA particles showing a slower OVA release, resulted in higher and constant antibody levels over the duration of a year, compared to faster releasing particles [36]. These results imply that the particulate nature alone is not enough, but that also a sustained presentation of the antigen is necessary to induce a long-term immunity. These results were confirmed by investigating the immune response of lactic/glycolic acid polymer microcapsules in mice using bovine serum albumin (BSA) as a model antigen [37] where a higher antibody titre and persisting immune response were measured up to 142 days. The adjuvant effect was comparable to that of FCA and stronger than that of aluminum hydroxide. To investigate if the presence of particles alone was enough to stimulate an immune response, one group was injected with blank microcapsules and antigen. The immune response from this group was no greater than that induced by BSA in saline. These results show that blank particles do not possess adjuvanticity, but that the sustained release of the model protein is necessary [37]. In a different study Sah *et al* (1996) [38] confirmed this by testing BSA-loaded poly(d,l-lactide-co-glycolide) (PLCG) and poly(d,l-lactide) (PLA) microparticles (with an *in vitro* release of up to 18 days) in mice where immune responses up to 27 weeks were measured. BSA-loaded particles stimulated a better immune response than BSA dissolved in saline or adsorbed to alum. All these studies show the importance of a slow sustained antigen release.

Many other groups investigated particles for tetanus toxoid (TT) vaccines. Sasiak *et al* (2001) [39] investigated the stability of experimental vaccines containing TT within PLGA microspheres by incubating them at 37 °C. Changes in structure of TT could be detected and were related to the breakdown of the encapsulating polymers into their acid components. This polymer breakdown can lead to an increased acidity of the vaccine surroundings once released from the particles. Similar changes occurred when incubating un-encapsulated TT in low pH solutions. Protective immunity, equal to freshly prepared vaccines, was induced by microparticles that retained their spherical shape after incubation. This indicates that as long as the particles stay intact, TT remains stable and immunogenic. Raghuvanshi *et al* (1993) [40] successfully immunized rats with TT-loaded PLGA microparticles, finding a comparable immune response over 5 month compared to alum TT injections. Singh *et al* (1997) [41] used TT-loaded PLCG microparticles to immunize Sprague-Dawley rats. The antibody responses were monitored for 1 year and compared to rats that were immunized with TT adsorbed to alum at 0, 1 and 2 months. The best antibody responses were achieved by TT adsorbed to alum and also entrapped in microparticles, indicating the importance of the adjuvant. Esparza and Kissel (1992) [42] also studied the parameters that affect the immunogenicity of microencapsulated TT. TT-loaded PLA:PGA particles in water and TT-microparticles in an water-in-oil emulsion, using either peanut oil or IFA, were compared to alum-TT. Whereas similar primary IgG responses were observed for the different formulations, TT-loaded microparticles induced a stronger and longer lasting secondary antibody response than alum-TT, proving that TT antigenicity is maintained after microencapsulation. Furthermore, it was shown that the choice of carrier is important. The strongest secondary antibody response was obtained when TT microparticles were given in water-in-oil emulsions, where IFA proved to be more potent adjuvant than peanut oil. All these particles released the antigen in a sustained manner.

1.2.1.2 Particles releasing antigen pulses

Sanchez *et al* (1996) [43] tried to develop a release system that mimicked a conventional course of immunization requiring several injections. Therefore, they designed particles

that release the antigen in a pulsed manner. TT was entrapped in an oil-based core and the outer shell was formed by PLCG. These particles release TT after 21 days and 49 days, which was made possible by carefully selecting the copolymer composition of these two types of particles. Cleland *et al* (1998) [44] investigated the use of pulsatile release of a subunit vaccine for HIV-1, recombinant glycoprotein 120 (rGP120), from PLGA microspheres. Depending on the polymer rGP120 was released at 1 and 6 month after administration. In guinea pigs, neutralizing antibody titres that were comparable to titers obtained from two immunizations of rGP120 and QS-21 were induced by a single immunization with rGP 120-loaded PLGA microspheres resuspended in soluble rgp120 and QS-21. In baboons, immunization with rGP120-loaded microspheres resulted in long-lasting neutralizing antibody titres that were greater than repeated immunizations with soluble rgp120 and QS-21. Continuous release of rGP120 from PLGA microspheres induced a lower humoral response than the repeated pulses. This is in contrast to the previously mentioned TT studies. These results show that either continuous or pulsatile release might lead to a stronger immune response depending on the vaccine in questions.

1.2.1.3 Particles for influenza vaccination

Sustained release microparticles are an interesting alternative to current vaccines to enhance antibody titres against the major surface glycoprotein hemagglutinin. To this end, PLGA particles loaded with influenza vaccine were investigated [45]. *In vitro* this system released the vaccine in a pulsatile manner and in mice a higher primary IgG antibody response was stimulated by the PLGA-microsphere vaccine. These findings show the potential for single dose influenza vaccination using particles as the delivery system. Oral and subcutaneous administration routes of the influenza PLG and poly(isobutylcyanoacrylate) microparticles were compared by Chattaraj *et al* (1998) [46]. The best results were obtained when the vaccine was administered subcutaneously follow by oral boosting. These are very promising results, making vaccination more effective and easier accessible when only one visit to a medical facility is necessary.

1.2.1.4 Microparticles for hepatitis B vaccines

Microparticles were also studied for the controlled release of a single dose hepatitis B vaccine. CD1 mice were immunized with 30 μg of Hepatits B surface antigen (HBsAg) in PLG and PLA particles [47]. Control mice received three injections of HBsAg in alum at 0, 1 and 6 months. Antibody levels stimulated by a single microparticle injection appeared comparable to the three alum injections for at least 1 year.

1.2.1.5 Solid lipid nanoparticles for vaccine delivery

Mishra *et al* (2010) [48] used solid lipids nanoparticles (SLN,) made of tristearin, as a potential delivery system for HBsAg. They explored the effect of surface modifications of the SLN on loading efficiency as well as cellular uptake. A greater cellular uptake and a greater Th1 immune response was induced by SLN particles compared to soluble HBsAg. Particularly, mannosylated formulations showed great potential by producing sustained antibody titers.

1.2.1.6 Chitosan particles

Jaganathan *et al* (2004) [49] compared TT-loaded PLGA particles with chitosan particles. Both particles provided sustained antigen release *in vitro* (TT release up to 35 days) and in guinea pigs PLGA and chitosan TT-loaded particles stimulated equivalent immune responses, comparable to a prime-boost alum-TT vaccine. This indicates that the expensive PLGA polymer might be replaced by other, cheaper materials.

1.2.1.7 Liposomes

Another well-studied particulate carrier for vaccines are liposomes. Liposomal drug formulations are already being used in the clinic (Doxil®, Ambisome®), making this formulation even more attractive. As early as 1974, liposomes were investigated as antigen carriers, using Diphtheria toxoid, and were found to increase antibody response [20]. Ever since, they have been widely studied to increase vaccine efficiency [21,50-52]. Demento *et al* (2012) [53] compared the efficacy of OVA- loaded liposomes and PLGA particles in inducing long-term immunity in mice. The *in vitro* release indicated a difference between the two carrier systems, with OVA being released much slower from particles than from liposomes, and PLGA vaccinated mice had improved

immune responses. This study showed that the difference in performance was not due to the different materials, but due to the release kinetics of the antigen, indicating once more the importance of a sustained antigen release.

In order to develop a sustained release liposome, Tiwari *et al* (2009) [54] manufactured gel core liposomes. BSA-loaded gel core liposomes were compared to conventional liposomes as well as alum absorbed BSA and BSA alone given by intramuscular injection to Balb/c mice. Gel core liposomes induced efficient systemic antibody responses, justifying the potential use for vaccine delivery. In a second paper Tawari *et al* (2009) [55] used the gel core liposomes as a delivery system for Pfs25 malaria antigen with or without the CpG ODN adjuvant. The authors showed that *in vitro* antigen is released from gel core liposomes for up to 20 days, whereas for conventional liposomes the release stopped after 5 days. Immunizations with gel core liposomes induced a significant and durable immune response compared to conventional liposomes. Moreover including the CpG ODN adjuvant further enhanced the immune response to the vaccine.

1.2.2 Gels, patches and microneedles

1.2.2.1 Thermosensitive gels

Gordon *et al* (2008) [56] investigated the use of thermosensitive chitosan hydrogels as sustained vaccine delivery devices for chitosan nanoparticles (CNPs). By adding polyol salts to chitosan solutions, gels are formed upon increasing temperature [57,58]. Nanoparticles and gels were loaded with OVA and the immune response was examined in mice. Chitosan hydrogels loaded with OVA were able to induce both a cell-mediated and humoral immunity, whereas OVA-loaded CNPs did not exhibit any significant immunogenicity. *In vitro* it could be shown that the release of FITC-OVA from the gels was more sustained than from CNPs, with less than 10% released OVA after 10 days. CNPs on the other hand, released over 50% FITC-OVA within the same time. In a following study, silica nanoparticles (SNPs), of a size of approximately 300 nm, were included into thermosensitive chitosan hydrogels as a particulate sustained release vaccine delivery system [59]. Gel-based systems containing SNP-associated stimulate both cell mediated and humoral immunity *in vivo*. A higher CD4+ T cell

proliferation was induced by chitosan gels containing OVA-loaded SNP and Quil-A (QA) than chitosan gels containing soluble QA and OVA, indicating the importance of the SNP in this system.

Kojarunchitt *et al.* (2011 and 2014) [60,61] investigated thermoresponsive Poloxamer 407 (P407) – Pluronic-R (25R4) gels chitosan-methyl cellulose (MC) formulations as single-dose, sustained release vaccines. The gels were liquids at room temperature and formed stable gels at physiological temperatures. The model antigen OVA was used and Quil A and monophosphoryl lipid A as adjuvants. Chitosan-MC gels showed a sustained antigen release of at least up to 14 days in mice, whereas the release of antigen was not sustained from the P407–25R4 gels. Both cellular and humoral responses were stimulated by the chitosan-MC gels. It appeared that the incorporation of a particulate vaccine (cubosomes) did not facilitate synchronous vaccine release [60]. The chitosan-MC gels though showed great potential as sustained release delivery systems.

1.2.2.2 Microneedle patches

Other interesting systems for sustained transdermal antigen delivery are microneedles. Lee *et al* (2008) [62] achieved a sustained delivery of sulforhodamine over hours to days out of dissolving microneedles made of carboxymethylcellulose (CMC). To quantify the release, microneedle patches were inserted into human cadaver skin and the transdermal flux was measured. In 2010 Raphael *et al* [63] introduced a densely packed dissolving microprojection array made out of CMC for vaccine delivery. The authors reported stimulation of a systemic immune response when administering the dissolving patches containing either OVA or Fluvax2008 (a commercial trivalent influenza vaccine) to a mouse model.

Chen *et al* (2012) [64] reported that chitosan microneedles loaded with BSA, could achieve an *in vitro* drug release of up to 8 days. The gentle fabrication process did not alter the secondary structure of BSA. *In vivo*, when applying Alexa Fluor 488 labelled BSA-loaded microneedles to rat skin, it could be shown that the BSA diffuses gradually through to the dermal layer. In a second study Chen *et al* (2013) [65] reported on a new

chitosan microneedle array whereby the needles were attached to a mechanically strong support made of poly(L-lactide-co-D,L-lactide). The microneedles successful separated from the supporting array when inserted into rat skin, penetrating approximately 600 nm into the skin and gradually delivering the antigen (OVA) for up to 14 days. OVA immunization in rats using these microneedles resulted in significantly higher antibody responses than those stimulated by traditional intramuscular immunization. Subsequent studies found that the immune response was mostly dependent on the dose, rather than on the depth of delivery, microneedle density or the area of application [66].

Numerous studies have been conducted to investigate the use of microneedles for influenza vaccination. Immunogenic responses using low-dose influenza vaccines delivered intradermally by microneedles where found to be similar to those induced by the full-dose intramuscular vaccination [67]. The trial was conducted in 180 healthy adults and a marketed influenza vaccine for the 2006/2007 influenza season (RIX ® by GSK Biologicals) was used for all injections. Protective efficacy and long-term sustained immunogenicity were the result of using microneedle patches for vaccination [68]. Patches were left on the back of the mice for 10 minutes. A caveat to all the mouse studies is that the differences between rodent and human skin (thickness, number of hairs) likely over estimates the efficacy of the transdermal formulations.

Vaccine stability is a problem when using microneedles. Studies have been conducted to determine the effects of drying and of storage time on antigen stability and *in vivo* immunogenicity of influenza microneedle vaccines [69,70]. Choi *et al* (2012) [70] were interested in long-term stability of microneedles coated with whole inactivated influenza vaccine. They were guided by the hypothesis that damage can occur to the influenza vaccine coated onto microneedles due to crystallization or phase separation of the microneedle coating matrix. Vaccine stability was measured *in vitro* by hemagglutination activity. Studies showed that the vaccine lost stability and had reduced immunogenicity in proportion to the degree of phase separation and coating matrix crystallization [70]. Kim *et al* (2011) [69] showed that the *in vivo* immunogenicity

as well as the hemagglutination activity could be improved after storage when the vaccine was coated on microneedles together with trehalose [69].

1.2.3 Implants

A very interesting and not yet fully investigated system for vaccine delivery are implants. Different types of implants for vaccine delivery have been investigated, including both continuous as well as pulsatile release systems [71-73].

1.2.3.1 Implants made from polymers

Preis and Langer (1979) described the use of an inert pellet, less than 1 mm in diameter, made out of ethylene-vinyl acetate copolymer for vaccine delivery. Antigens over a wide range of molecular weights were tested (ribonuclease MW 14'000, BSA MW 68'000, and gamma-globulin MW 158'000) and released continuously from the pellets. The implants were administered subcutaneously to mice and were able to stimulate an immune response comparable those induced by two injections [71]. These results show that implants are a promising system for single-step immunization. However, these implants had to be surgically removed once the antigen was released, which limits their use in terms of patience acceptability. Furthermore, their manufacturing requires the use of organic solvents and exposure to heat [74] which will likely compromise the integrity of protein antigen, limiting the practical application of these systems.

Kohn *et al* (1986) investigated the use of a biodegradable polymer for antigen delivery based on poly(CTTH-iminocarbonate) [75]. This polymer's primary degradation product, N-benzyloxycarbonyl-L-tyrosyl-L-tyrosine hexyl ester was found to be as potent an adjuvant as FCA. The produced implants, transparent, slightly brittle films containing 10% w/w of BSA, were implanted subcutaneously in the back of mice. BSA released from these implants induced significant levels of anti-BSA antibodies over 56 weeks. Even though these implants were made of a biodegradable material, the manufacturing process still required the use of organic solvents.

1.2.3.2 Implants made from lipids

Lipids such as triglycerides [44], mixtures of triglycerides with cholesterol (CHOL) and phospholipids [30], and phospholipids or blends of phospholipids and CHOL, [45] have been investigated as interesting alternatives to polymers for the development of controlled-release systems. Lipids are generally biocompatible and biodegradable [28,76-78]. In addition, harsh condition during manufacturing, such as heat or the use of organic solvents, can be avoided.

Khan (1991) [79] studied implantable matrix systems prepared from CHOL and lecithin using BSA as antigen. Antibody responses to BSA in mice implanted with these matrixes were studied, as well as the effects of the CHOL-lecithin ratio on the erosion and the release of BSA *in vitro*. It was shown that the release rate, as well as the erosion of the pellets, was dependent on the CHOL-lecithin ratio. In addition, gelatin proved to be useful as filler and release modifier. Antibody levels were higher in mice immunized with implants compared to mice immunized with a single or three BSA injections given in phosphate-buffered saline (PBS). An immune response could be induced and maintained in mice for at least 10 month following implant administration [80].

Opdebeeck *et al* (1993) also produced CHOL pellets by direct compression for the delivery of BSA. Pellets were implanted under the skin of mice and released BSA, after an initial burst, slowly over 77 days. Comparing to mice receiving a single or 3 injections of the same dose of BSA, mice immunized with pellets had higher antibody levels. A control group receiving injections as well as blank implants demonstrated that the slow release of the BSA was responsible for the higher antibody titers rather than the presence of the pellet itself [81].

Walduck *et al* (1998) [82] tested CHOL and lecithin implants delivering a recombinant antigen (recombinant Dichelobacter nodosus pili) and the adjuvant Quil A (QA) in sheep. *In vitro,* the presence of QA in the implants enhanced antigen release and QA was required *in vivo* to induce immune responses, however minor skin irritation was observed. Antibody responses to the s.c. placed implants were compared to those

induced by two injections given 4 weeks apart. Even though sheep immunized with implants produced antibodies, significantly higher levels of antibodies were observed in sheep immunized with two injections. In order to delay antigen release, implants were coated either with CHOL and lecithin using a manually operated single punch tablet press or with an enteric coating polymer (hydroxyl propyl methylcellulose phthalate) by spraying onto the implants using compressed air. Also double coated implants were tested, being first coated with polymer and then with CHOL and lecithin. Coated implants were given together with simple implants to deliver a prime and a boosting dose of antigen. The double implant system was able to achieve equivalent antibody titres to those detected in animals receiving injections. However antibody levels were not sustained past 6 weeks. Walduck further reported that the period of delivery rather than the delivery profile was important. This was confirmed by another study performed by Walduck and Opdebeeck (1997) [83] investigating the effect of antigen delivery profiles on antibody responses in mice. They showed that continuous BSA delivery is as effective as giving injections over the same period of time, showing how promising sustained release devices might be for single-step vaccination programs.

Myschik *et al* (2007) [28] investigated the release kinetics, the morphology of structures released from implants as well as the morphology of the implant itself for different formulations consisting of varying ratios of QA:CHOL:L-α-Phosphaditylcholine (PC). It was shown *in vitro*, that the formulation of lipid implants for sustained release delivery of subunit antigens in combination with colloidal particles is possible. In a second study Myschik *et al* (2008) [29] demonstrated *in vivo* that lipid implants containing the adjuvant QA were able to stimulate immune responses comparable to two immunizations with an immediate-release vaccine, containing equivalent amounts of QA and antigen, administrated by injection. It was also shown that stronger cell-mediated and humoral immunity was induced by QA-containing sustained-release implants as compared to lipid implants without adjuvant. This result emphasizes the importance of the adjuvant in this kind of implants. Tantipolphan (2009) [84] investigated lecithin:CHOL implants for the controlled release of proteins and

suggested that the incorporation of salts into the lecithin system could delay the initial release.

In spite of all the enumerated advantages, using lipids in pharmaceutical formulations holds a few challenges to overcome. The major problem is the instability of lipids during storage. An increase of melting ranges or of melting enthalpy, the formation of pores in the surface, changes in rheological properties or a decrease in tensile strength might result from aging of lipids [48]. These changes associated with the aging of lipids are of great significance for sustained release dosage forms. Storage and long term stability are therefore issues that must be kept in mind.

1.2.3.3 Implants made from silicone

Kemp *et al* (2002) [73] manufactured injectable implants for single-shot vaccine delivery consisting of mannitol, sodium citrate and non-biodegradable silicon. The production process of these implants did not require the use of organic solvents or high temperatures, rendering it attractive for antigen incorporation. Two different types of implants were produced, releasing the antigen avidin over 1 month or over several months. Because of its lipophilic nature, silicone is impermeable to water and bodily fluids, allowing the possibility of such a release system. Immune responses induced by these two types of implants were compared to AlzetTM mini-osmotic pumps and conventional antigen delivery in sheep. IL-1 or alum were used as adjuvants. The authors showed that the presence of an adjuvant was important and that implants releasing the antigen together with adjuvant over several months were able to result in higher antibody titers than the injections and the other type of implants.

Lofthouse *et al* (2002) [27] investigated a similar system for vaccination in sheep using either the model antigen avidin or *Clostridium tetani* and *Clostridium novyi toxoids*. A matrix type implant, delivering antigen *in vitro* over approximately 1 month, and a coated rod type, that delivers antigen for several months, were compared. Implants were produced by extrusion and cured for 3-4 days at 25 °C. Coated rod implants were co-extruded with an outer covering of silicone. The antibody response to the matrix implants was comparable to conventional vaccination with aluminium hydroxide

adjuvant (alum). The slow releasing rod implants stimulated higher antibody titres than the alum injection group. Moreover, following vaccination with coated rod implants, a prolonged antibody response was observed.

1.3 Adjuvants

A number of the studies reviewed above have demonstrated the crucial role adjuvants play in sustained release vaccine formulations. Adjuvants (Table 1-1) can satisfy several purposes: 1) increase the immunogenicity of highly purified or recombinant antigens; 2) minimize the number of immunizations or the amount of antigen needed to acquire protective immunity; 3) improve the efficacy of vaccines; or 4) operate as antigen delivery system [13]. An ideal adjuvant should be non-toxic, provide good immunological memory, not induce autoimmunity, be stable under a broad range of storage conditions (time, temperature and pH) and should stimulate a strong humoral and/or T cell immune response [85]. The immune system is activated by adjuvants because they represent pathogen associated molecular patterns (PAMPS). PAMPS assist the immune system to differentiate between self and foreign substance [86]. The growing understanding that adjuvants represent PAMPS, which are recognized by pathogen recognition receptors constitutes the bone of contention for the research of optimal synthetic adjuvants from diverse sources, including small molecules [4,32].

There are different classifications for adjuvants. Moyle and Toth [9] distinguished two classes of adjuvants, immunopotentiators and delivery systems. Immunopotentiators, as indicated by their name, activate the innate immune system. Antigen uptake and presentation can be improved by particulate vaccine delivery systems [9], modulating or enhancing immune responses [87]. The effect of adjuvants on the nature of the immune response can be profound. Adjuvants can influence the immune system toward either a Th1 or Th2 type response [88]. Protective immunity against intracellular infection agents, as for example bacteria or certain viruses, and presumably against cancer cells, requires a Th1 response. Contrariwise, Th2 immunity is efficient for protection against certain viral infections as well as most bacterial infections [89]. According to Marciani (2000) [89], a Th1 immune response, mediated by Th1 helper cells, is a necessity for cytotoxic T lymphocyte production. A Th1 response

produces the cytokines interleukin-2 (IL-2), tumour necrosis factor-β and interferon-ϒ. In mice a Th1 immune response is characterized by enhanced production of IgG2a, IgG2b and IgG3, whereas a Th2 response is identified by an enhanced production of IgG1 and secretory IgA as well as by the production of cytokines IL-4, IL-5 and IL-10. Water/oil emulsions and alum represent the most commonly used adjuvants, but they are only capable of inducing a Th2 immune response. Most of the currently available adjuvants (water/oil emulsions and alum) mainly stimulate a Th2 type immune response which is most of the time ineffective against intracellular pathogens [89]. There is a wide range of different adjuvants such as monophosphoryl lipid A (MPL), liposomes, ISCOMs, saponins, and many more [1]. The classical, and for many years only FDA-approved, adjuvant for vaccines is alum, which provides a depot releasing the vaccine in a sustained manner [3,88]. Aluminium based adjuvants induce a Th2-type antibody dominated response. However vaccines for intracellular infections or cancer, require cellular immunity and need both a CD8 as well as CD4 T cell response [12]. In recent years, much research has been conducted to identify and develop new adjuvants for use in humans [90,91]. Monophosphoryl lipid A (MPL) is for example of a newer Toll-like receptor (TLR)-dependent adjuvant that is approved for use in human vaccines [3]. Adjuvants must fulfil three requirements to be licensed for use in humans: safety, immunogenicity and clinical efficacy [12]. Many have failed to satisfy all three conditions.

Table 1-1: Overview of the adjuvants used in studies described in this review

Adjuvant	Source	Systems
Alum	Aluminium slats	• PLGC particles (Singh et al 1997) • Silicon implants (Lofthouse et al 2002)
CpG ODN	Synthetic oligodeoxynucleotides containing unmethylated CpG motifs	• Liposomes (Tawari et al 2009)
Quil-A	Quillaja saponaria, Molina Tree	• Chitosan hydrogels (Gordon et al 2010) • CHOL/Lecithin implants (Walduck et al 1998) • CHOL/PC pellets (Myschik et al 2008) • CHOL/Lecithin/D114 implants (Even et al 2014)
QS-21	Purified fraction of Quil-A	• PLGA microparticles (Tawari et al 1998)

1.3.1 Adjuvants approved for human use in sustained vaccine delivery

The challenge is to identify an adjuvant suitable for human use that is capable of inducing cellular and antibody immune responses. For a long time aluminium salt based adjuvants were the only ones approved for human use (diphtheria-pertussis-tetanus, dipheteria-tetanus, Hepatitis A) [92]. Oil-in-water emulsions have also been successfully used in different vaccines. MF59™ is an oil-in-water nano-emulsion that is used in Europe as an adjuvant for influenza vaccines [93], and was the first adjuvant to receive approval for human use after alum [94]. But like alum, MF59™ cannot induce a Th1 immune response and also acts through a depot effect [94,95]. AS03 is another oil-in-water emulsion that is used in influenza vaccines [91]. Another licensed adjuvant is AS04, an aqueous formulation of MPL and alum [96]. Able to induce a Th1 response, MPL is a non-toxic derivative from lipopolysaccharide of *Salmonella Minnesota* and a component of a licensed HBV vaccine [97]. Another licensed adjuvant are virosomes, used in influenza (Inflexal) and HBV (Epaxal) vaccination, they are composed of liposomes and hemagglutinin [91].

1.3.2 Adjuvants in research for sustained vaccine delivery

Many other adjuvants are not approved for human use yet, but have been investigated in preclinical research and in human clinical trials, among them the saponins. In the 1930s *Quillaja saponaria* extracts were first identified to have adjuvant properties [98]. Dalsgaard (1974) [99] used a defined *Quillaja* saponin, QA, showed an increase in the immune response in cattle to a food-and-mouth disease (FMD) vaccine. QA as well as QS21, a purified fraction of QA, are widely used in research, but are relatively toxic. Incorporation of QA into liposomes is order to reduce toxicity [90]. QA and QS21 can perforate lipid membranes by binding to CHOL. The CHOL present in liposomes or ISCOMs interacts with the saponin, thereby blocking it from interacting with cholesterol in cell membranes. Different studies have been conducted on this adjuvant [6,20,21,51,100-102] but so far it has not been licensed for human use.

1.4 Immune System

The activity of white blood cells called leukocytes play a crucial role in both innate and adaptive immunity. All cellular elements of the blood are derived from hematopoietic stem cells of the bone marrow. Once mature they circulate in the bloodstream and in the lymphatic system [86]. The lymphatic system is a key component of the immune system and is composed of lymph vessels and lymphoid organs. Lymphoid organs are divided into primary and secondary lymphoid organs. Lymphocytes are generated in the primary lymphoid organs, the bone marrow and the thymus. Mature lymphocytes then circulate through and reside in the secondary lymphoid organs (lymph nodes, spleen and mucosal lymphoid tissues) and adaptive immune responses are initiated here. A system of lymphatic vessels drains the extracellular fluid from tissue through the lymph nodes. In this manner macrophages as well as mature dendritic cells migrate to the lymph nodes and lymphocytes residing in the lymph nodes are taken to the blood [86].

1.4.1 Immune response

The immune response is divided into two different parts differing by the specificity and speed of reaction, namely the innate and the adaptive immunity [103]. Although

we can divide the immune response into two parts, both types of immune functions interact closely.

1.4.2 Innate immune response

The innate immunity is the first line of defence, responding quickly and non-specifically. The innate immune response is responsible for the recruitment and activation of neutrophils at the site of infection to kill pathogens [103]. Neutrophils are the most numerous and important cells of the innate immune response. Although the innate immune response is not antigen-specific, pattern recognition receptors (PRRs) allow discrimination of self from non-self. Pathogen-associated molecular patterns (PAMPs) are molecular structures in pathogens that are recognised by PRRs found on macrophages, neutrophils and dendritic cells [104]. Macrophages also provide a first line of defence. Once activated by the cytokine interferon-ɣ (IFN-ɣ), they engulf and kill invading microorganisms and dispose of pathogens (such as mycobacteria, protozoa and fungi) and infected cells [86,103]. Absence or mutation of the IFN-ɣ receptor leads to severe mycobacterial infections [105]. A cytokine is a general name for any protein secreted by a cell that affects the behaviour of nearby cells bearing appropriate receptors [86].

Dendritic cells (DCs) are the main class of innate antigen presenting cells (APCs) and act as sentinel cells of the innate immune system and as the link between the innate and acquired immune systems. DCs are widely distributed in the body and are the most capable of initiating acquired immune responses [106]. They do this though the cell surface expression of peptide- major histocompatibility complex (MHC) or lipid-CD1 complexes [107,108]. Additional stimulatory signals produced by DC are required for full activation of the acquired immune response and these are produced following binding of PRR to PAMPs [86].

1.4.3 Adaptive immune response

The adaptive immune response is antigen specific and less rapid than innate immunity. The reason for this is that the pathogen first needs to be taken up by DC and then processed into small polypeptides known as antigens. Only when antigen is

bound to proteins of the MHC T cell activation can take place, resulting in the generation of effector T cells. This process occurs over 2-3 days once the T cell meets an antigen-presenting cell bearing its specific antigen [86,103]. Dendritic cells possess pathways to process non-protein antigens (such as lipids) which are loaded onto CD1 molecules [108]. The adaptive immune response, through the processes of clonal expansion and affinity maturation [86], is able to develop populations of memory lymphocytes so that in case of a secondary infection responses occur much faster and more efficiently. The development of populations of memory cells is what many vaccines aim to achieve.

The hematopoietic stem cells of the bone marrow amongst others give rise to two different categories of white blood cells, the myeloid and the lymphoid lineages. The later one compromises the lymphocytes of the adaptive immune response. There are two types of lymphocytes, T and B lymphocytes (or cells).

1.4.3.1 T Lymphocytes

T cells mature and differentiate in the thymus and have antigen receptors on their surface (T-cell receptors). There are two different types of effector T cells, one carries the cell-surface protein CD8 on its surface, the other one the cell-surface protein CD4 [86]. T-cells are only able to recognize antigens that have been processed and are displayed as peptides or lipids bound to particular cell surface glycoproteins called MHC [86]. CD4+ lymphocytes only recognize antigen presented by MHC class II and CD8+ cells by MHC I class molecules [103]. MHC I class molecules complex with antigens derived from proteins synthesized within the cell, and can stimulate responses to viruses, intracellular pathogens or abnormal tumour antigens. MHC II class molecules binds to antigens derived from extracellular pathogens.

CD8+ T cells

Upon activation, CD8+ T cells become cytotoxic effector or memory T cells. They are able to destroy cancer cells and cells infected by intracellular pathogens through the release of three types of cytotoxic proteins; granzymes, perforin and granulysin [86]. Perforin delivers the granzymes into target cells, where the latter are able to induce

apoptosis. Furthermore, CD8 cytotoxic T cells contribute to the host defence by releasing different cytokines (IFN- ɣ, TNF-α and LT-α), inducing increased expression of MHC class I molecules in infected cells and directly inhibiting viral replication [86].

CD4+ T cells

CD4+ T cells can differentiate into a number of different T helper cell subsets, Th1, Th2, Th9, Th17, Th22 and follicular helper T cells (Tfh cells)[86,108-110]. Dependent on the type of infection, these subsets promote different types of responses. Th1 and Th2 cells have been investigated most fully and are distinguished by the cytokines they secrete [33] and the types of responses they induce. Upon activation, Th1 cells secrete interferon-ɣ, IL-2 and the tumour-necrosis factor-β. IL-2 induces T-cell proliferation, stimulates not only CD4+ production but also increases CD8+ T-cell proliferation and cytotoxicity. T_H2 cells on the other hand favour antibody production by secreting IL-4, IL-5, and IL-6. Furthermore they inhibit macrophage function by secreting IL-10. Additionally IL-4 down-regulates T_H1 on responses and induces further Th2 responses [103].

1.4.3.2 B Lymphocytes

B-lymphocytes mature in the bone marrow and utilise membrane-bound antibody as antigen receptors. Around 10^5 antibodies are attached to the surface of each B-lymphocyte [111]. After an antigen binds to a B-cell receptor, the lymphocyte differentiates into an antibody-secreting plasma cell or memory B cell [86]. In contrast to T cells, naïve B cells are able to recognize native antigen. Upon activation B cells can differentiate into plasma cells which secrete large amounts of antibody [111].

1.4.3.3 Antigen presenting cells

B and T lymphocytes are the mediators of immunity, but the presence of these two parties does not always lead to immunity. In addition, the presence of antigen presenting cells (APCs) is necessary [106,112].

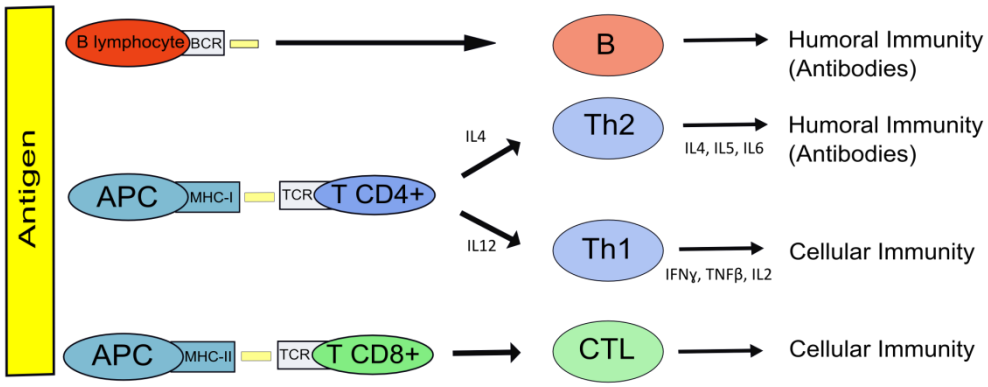

Figure 1-3: Antigens, APCs, different MHC molecules and resulting immune responses. Schematic representation modified after Beck et al [113].

1.5 Conclusion

All the mentioned studies clearly show that there has been a focus on the development of sustained release vaccine delivery systems over the last 30 years. There is no doubt that new delivery strategies are required to advance the field and to facilitate the development of new vaccines to treat both communicable and non-communicable diseases. All the studies agree on the fact that adjuvants are needed to induce a strong and durable immune response. The search for and investigation of new adjuvants is as important as the development of new delivery systems. Most studies investigating antigen release systems confirm that slow, sustained antigen release is able to induce a stronger immune response than repeated injections. However, there are a few studies that show that pulsatile administration achieves better immunity. This suggests that the optimal type of delivery may be antigen and adjuvant dependent.

Substantial progress has been made, especially in the field of polymeric particles for vaccine delivery. But unfortunately the manufacture of most of these particles requires harsh conditions [17,34,36]. Heating, high shear forces, exposure to organic solvents, increased osmotic pressure as well as acylating degradation products can lead to irreversible changes in structure and activity of proteins [114]. Furthermore, polymer degradation *in vivo* can cause changes in the local pH that can lead to activity loss for

protein based drugs [26] [115]. Even though some promising results have been reported, more research has to be performed before these systems are ready for human use. Microneedle delivery of vaccines is a particularly interesting area showing much promise, although the issue of antigen stability must be addressed. Stability is also a major challenge with lipid implants or particles, as upon storage the lipids themselves might be subject to polymorphic changes. Review of all the literature in this area does not point to any particular optimal system or material. There have been major achievements made in the development of sustained release vaccine delivery systems as well as in the development of new adjuvants. However there are still many barriers to be overcome before such systems are widely used in man.

1.6 Aim of the Thesis

From a review of the literature it becomes clear that there is a need for new delivery systems to improve existing vaccines and to allow the use of novel vaccines. Sustained vaccine release seems to be the key to achieve strong, long lasting immune responses. Lipid implants are a promising alternative to other sustained deliver systems described in literature. Since lipids are biodegradable as well as biocompatible they provide an interesting platform. Some research on lipid implants for sustained vaccine delivery has been carried out and published. However, the production of such lipid vaccine delivery systems by twin-screw extrusion has not previously been investigated and is therefore the subject of this thesis.

The aim was to produce lipid implants by twin-screw extrusion and investigate the sustained release from these implants *in vitro* and *in vivo*. In a first step, the model antigen ovalbumin (OVA) was incorporated and the immune response was tested *in vivo* using murine models. In a second step, the long peptide TRP2 was incorporated into the implants and an *in vivo* tumour growth study was performed.

Chapter Two describes the preparation and characterisation of the release behaviour of OVA and Quil A (QA) from the implants. The influence of the formulation and adjuvant on the release of OVA is investigated. Furthermore the influence on the OVA release of post-treating the lipid implants by curing is described.

Chapter Three investigates the occurrence of unstable polymorphs due to the extrusion process as well as during storage. Furthermore the OVA release *in vitro* was compared to the OVA release *in vivo* in a murine model. The ability of the implants to induce an immune response was tested *in vivo* and the importance of QA in the formulation was studied.

Chapter Four describes the production of twin-screw extruded lipid implants containing the TRP-2 peptide for tumour therapy. The consequences of a change of the production device on the implant characteristics are shown. Furthermore, the preparation of vesicular phospholipid gels (VPGs) as an alternative lipid delivery system is described. VPGs as well as implants containing TRP-2 were analysed in an *in vivo* tumour study.

Chapter Five examines the interactions between peptides and the lipid implants. Chapter Four suggested that the release of the TRP-2 peptide from the implants is very slow. The reason for this behaviour was investigated by choosing molecules of different size and hydropathy, and investigating their release behaviour and interaction with the implants.

Chapter Two

Impact of implant composition of twin-screw extruded lipid implants on the release behaviour

Parts of this chapter have been submitted as a manuscript to the *International Journal of Pharmaceutics* and are in revision:

Even MP, Bobbala S, Kok Liang Kooi, Hook S, Winter G, Engert J. Impact of implant composition of twin screw extruded lipid implants on the release behaviour.

2 Impact of implant composition of twin-screw extruded lipid implants on the release behaviour

The following chapter deals with the production of lipid implants for vaccine delivery produced by twin-screw extrusion. The general introduction highlights the fact that there is a need for novel vaccine delivery systems in order to optimize the use of existing vaccines and make the use of new vaccines possible. Lipid implants have shown great prospect as sustained release vaccine delivery devices. However, so far they have always been produced by direct compression or similar procedures, making the production of big batch sizes a time consuming act. The use of a twin-screw extruder as production device is investigated. The use of twin-screw extrusion would be a process that is easy to scale up. The impact of the formulation on the release behaviour of the model antigen is discussed, as well as its stability after release. An adjuvant is incorporated into the implants. The influence the adjuvant had on the antigen release is investigated as well as the release behaviour of the adjuvant itself. Furthermore, the possibility to influence the antigen release by curing the implants after extrusion is examined.

In the following, the text of the manuscript as submitted is reprinted. Sharan Bobbala performed the Quil A release study and Christian Minke helped with the SEM. All other work was done by myself.

Abstract

The development of vaccine delivery systems that will remove or reduce the need for repeated dosing has led to the investigation of sustained release systems. In this context, the duration of antigen release is of great importance as is the requirement for concomitant adjuvant release. In this work, lipid implants consisting of cholesterol (CHOL), soybean lecithin, Dynasan 114 (D114), the model antigen ovalbumin (OVA) and the adjuvant Quil-A (QA) were produced by twin-screw extrusion. The release of antigen and adjuvant was investigated *in vitro* and we observed complete OVA release over a period of 7 days while QA was released in a linear fashion over a period of up to 12 days. In order to extend the OVA release, lipid implants were subjected to post-extrusion curing at 45-55°C. The OVA release could be extended to up to 14 days. Furthermore, the influence of the implant composition on the release of the model antigen was investigated. It was shown, that the percentage of cholesterol in particular plays an important role in modulating release.

Key words: lipid implants, twin-screw extrusion, vaccine delivery, sustained release of antigen, ovalbumin, Quil-A

2.1 Introduction

Vaccination often requires the administration of a prime and booster immunisation to induce a strong memory immune response and long-term protection. Often subunit antigens such as proteins or peptides are used in modern vaccines, replacing whole pathogens, for purity and safety considerations [5]. In order to induce immunity, multiple doses of these subunit antigens, in combination with immunostimulatory adjuvants, are required as they are less immunogenic than whole-pathogen vaccines [6]. However, repeated administration of vaccines often compromises patient compliance. Therefore, a delivery system, which is able to release antigen in a sustained manner, could be a major advance in the development of vaccines. The general concept of sustained release of vaccines has already been investigated by Preis and Langer [71] using ethylene-vinyl acetate copolymer pellets and by Lofthouse et al. [27] using silicone based implants. The sustained antigen from both types of implants proved effective in eliciting prolonged antibody formation. It should be noted that such systems made from non-biodegradable polymers would require surgical removal [116].

For this reason biodegradable polymers such as polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA), which can release drug continuously after parenteral administration [114,117,118], are increasingly being investigated today for drug and vaccine delivery as injectable or implantable depot formulations. However, an issue with these formulations is polymer degradation to smaller chain acids upon contact with water, leading to significant drops in the micro-environmental pH, which can result in a loss of activity with protein-based drugs [26,119]. The use of synthetic polymer matrix materials has the additional problem that during manufacture irreversible changes in structure and activity of proteins can be induced by heating, high shear forces, exposure to organic solvents and increased osmotic pressure [114].

An interesting alternative is to utilize lipid implants as parenteral controlled delivery systems. Lipids are considered to be safe for diverse types of applications and are widely used in the food and cosmetic industry. Lipids such as triglycerides [114], mixtures of triglycerides with cholesterol (CHOL) and phospholipids [28],

phospholipids or blends of phospholipids and CHOL [120] have been considered as alternatives to polymers for the development of controlled-release systems. Lipids are generally biocompatible and biodegradable and are normally not inherently immunogenic [76,121,122]. Lipid implants have been thoroughly investigated as sustained delivery systems for protein and to a lesser extent for vaccine delivery [26,29,78,123-127].

Recently, we reported that lipid implants for vaccine delivery can be prepared by twin-screw extrusion [127]. Administration of these implants to mice resulted in enhanced antigen-specific IgG titers when both an adjuvant (Quil A, QA) and antigen (ovalbumin, OVA) were present in the implants. The release behavior of adjuvant used in sustained release implants will be an important aspect to consider as it has been reported that antigen and adjuvant must be released synchronously in order to obtain an optimal immune response [128]. Therefore the release of QA, a saponin derived from the tree Quillaia saponaria, and OVA from different implant formulations was examined. Both release behaviors were compared to ensure the release of adjuvant and antigen was simultaneous.

It has been reported that implants releasing OVA over a period of 7 days could induce immune responses similar in magnitude to two injections [127], this led to the hypothesis that longer antigen release may induce even stronger immune responses. In order to tune the release of antigen and adjuvant the effect of curing implants post-production was examined. Kreye et al [129] had reported that curing lipid implants composed of Dynasan 120 sustained the release of propranolol hydrochloride. We wanted to investigate if similar results could be achieved for antigen release. Curing temperatures ranging from 45°C to 55°C were chosen for our implants. The curing temperatures were slightly below the melting temperature of the lipids in order to melt the outer surface of the implants and change the size of pores at the surface of the implant therefore influencing antigen release [129].

2.2 Materials and methods

2.2.1 Materials

Ovalbumin from chicken egg white (OVA) grade V was purchased from Sigma-Aldrich. Cholesterol (CHOL), purity 95%, was purchased from AlfaAesar (Karlsruhe, Germany). Soybean Lecithin (approx. 90% phosphatidylcholin) was purchased from APPLICHEM LIFESCIENCE (Darmstadt, Germany). PBS tablets from Oxoid Limited (Basingstoke, England). Purified Quil-A (QA) was sourced from Brenntag Biosector (Frederikssund, Denmark) as a lyophilised powder and used as supplied. Dynasan 114 (D114) was kindly provided by SASOL Germany GmbH (Witten, Germany).Ultrapure deionized water having a conductivity of less than 0.055 μS/cm (Milli-Q Water systems, Millipore, MA, USA) was used throughout the study. All other chemicals were of analytical grade.

2.2.2 Preparation of lipid implants by twin-screw extrusion

Mixtures of soybean lecithin, CHOL, D114, with and without OVA and/or QA were used to prepare the implants. High grade stainless steel beakers for milling in a swing mill, Retsch® CryoMill (Retsch Technology, Haan, Germany) were filled with soybean lecithin and D114. The system was precooled with liquid nitrogen for 10 minutes at 5 Hz, the mixture was then ground for 1 minute at 25 Hz. A plastic mortar and pestle were used to mix the obtained powder by hand with the remaining components. QA and OVA were then gradually blended with the lipid mixture and subsequently fed into a twin-screw extruder (Haake MiniLab® Micro Rheology Compounder, Thermo Haake, Germany). The implants were extruded at a rotation speed of 40 rpm at an extrusion temperature of 45°C with closed bypass channel using an outlet of 2 mm diameter. The resulting implants had a diameter of 2 mm and were subsequently cut into lengths of 2.5 cm, resulting in an implant mass of about 0.08 g.

2.2.3 Curing of lipid implants prepared by twin-screw extrusion

Implants were cured using an oven (UM 400, Memmert GmbH + Co.KG, Schwabach, Germany) at 55°C for 15 minutes (min), or at 40, 45 or 50°C for 60 min, respectively. Implants were placed into Eppendorf tubes leaving the cap open. The tubes were then

horizontally placed into the heating cupboard. To ensure that the complete surface of the implants was heated equally, implants were turned by turning the Eppendorf tube (every 5 minutes for incubation time of 15 min, every 15 min for 60 min incubation time). At 55°C a curing time of 15 min instead of 60 min was chosen to avoid implant deformation.

2.2.4 Differential scanning calorimetry (DSC)

A DSC 204 Phoenix (Netsch, Selb, Germany) was used to analyse each lipid before extrusion. Thermograms of implants were recorded directly after the extrusion as well as after the post-treatment. Samples of about 4 mg were each weighed into aluminium crucibles. A heating and cooling rate of 5 K/min was used as between 20°C and 160°C. An empty crucible served as reference.

2.2.5 *In vitro* release of OVA from implants

The release of the model antigen OVA was investigated over a period of up to 15 days. Lipid implants of a length of 2.5 cm (n=3) were incubated at 37°C in a Heidolph 1000 Incubator in vials containing 1.8 ml phosphate buffered saline (PBS) (pH 7.4, 0.01 M, 0.05% NaN_3). At defined time points samples were taken and the release medium was exchanged completely. All samples were centrifuged at 14000 rpm (Mikroliterzentrifuge Z 160 M, Hermle Labortechnik, Wehingen, Germany) for 5 minutes to remove lipid particulates. OVA was measured in the supernatant by UV (Agilent Technologies 8453) at a wavelengths of 280 nm. For each tested mixture an implant containing neither OVA nor QA was used as a blank for the UV measurements. Measurements were performed as long as OVA was released from the implants. Each implant was weighted before the release and the total amount of protein present in each implant was calculated individually using a standard curve prepared by an 11-fold 1:1 dilution starting from a sample of 3 mg OVA in 1 mL PBS. All measured samples lay within the linear part of the standard curve (3 mg/mL – 5 μg/mL).

2.2.6 *In vitro* release of Quil A from implants

Implants were cut in 3.5 cm lengths and weighed. All implants contained 55% cholesterol, 340 – 750 μg of QA and 145 – 2240 μg of OVA. Implants were placed into 5 mL tubes filled with 1.8 mL PBS buffer (pH 7.3) and incubated at 37°C (Clayson incubator, New Zealand). At defined time points samples were taken and the release medium was exchanged completely. Samples were centrifuged for 30 minutes at 14000 rpm in a bench top centrifuge (Prism R, Labnet International Inc., Edison, USA) to pellet the lipid fractions. The supernatant was transferred into an Eppendorf tube and stored at -20°C until high performance liquid chromatography with evaporating light scattering detector (HPLC-ELSD) analysis was carried out.

2.2.7 Size exclusion chromatography

Protein integrity was determined by size exclusion chromatography (SEC) using a Dinoex HPLC system (Dionex, Softron GmbH, Germering, Germany). A TSKgel G300 SWXL size-exclusion column (7.8 mm x 30.0 mm, Tosoh Bioscience, Stuttgart, Germany) was kept at 25°C and an injection volume of 100 μL of each sample were used. The running buffer consisted of 50 mM PBS (pH 7.0, 0.05% NaN_3), with a flow rate set to 0.5 mL/min.

2.2.8 Scanning electron microscopy (SEM)

A Jeol JSM-6500 F (Jeol JSM-6500F, Tokyao, Japan) was used at an acceleration voltage of 2 kV and a magnification of 150 was used. After release, implants were dried in a vacuum dryer (Memmert, Schwabach, Germany) at 20°C and 50 mbar for 24 hours. Implants were cut and attached to aluminum blocks with double adhesive tape and were analyzed without further treatment.

2.2.9 High performance liquid chromatography with evaporating light scattering detector (HPLC-ELSD)

Analysis was carried out as described by Bobbala *et al.* [130]. The HPLC system consisted of 1200 Series evaporating light scattering detector (ELSD) system from Agilent Technologies (Santa Clara, USA) equipped with a ZORBAX Eclipse XDB-C8 Column (2.1 x 50 mm; 3.5 μm, Agilent Technologies). The column was maintained at a

temperature of 25°C. A guard column (2.1 x 12.5 mm, Agilent Technologies) was used to prevent contamination of the column and was also maintained at 25°C. The injection volume was 20 μL. The mobile phase consisted of water/acetonitrile (75:25% v/v) containing 0.01% v/v formic acid. A flow rate of 0.25 mL per minute was used. The ELSD settings were as follows: nebulizing temperature of 30°C, nitrogen gas pressure at 3.5 bar, gain at 10.

2.3 Results and discussion

2.3.1 Preparation of implants

Compared to lipid implant preparation methods often seen in the literature, for example compression or melting methods, twin-screw extrusion is a faster and more easily scaled up production procedure. However, the extrusion conditions as well as the lipid composition need to be chosen with great care. After an extrudable formulation has been identified, the extrusion temperature has to be optimized so as to allow the lipid mass to be uniformly extruded through the outlet die, which is not possible if the temperature is either too high or too low. In our study, a number of different formulations were tested applying different extrusion temperatures. All formulations consisted of a mixture of CHOL, soybean lecithin and D114 with or without antigen (OVA) and/or adjuvant (QA) (Table 2-1). The percentage of CHOL was varied from 40% to 60% while at the same time the percentage of D114 (the low melting point lipid) was decreased from 45% to 25%. An increase of CHOL to more than 60% resulted in blockage of the extruder, indicating the importance of the low to high melting lipid ratio for the extrusion process. The percentage of soybean lecithin was kept constant at 15% in all formulations as increasing it above 15% resulted in lipid mixtures that were too soft for extrusion.

The amount of protein antigen (0.13% OVA) and adjuvant (0.66% QA) included in the implants, was based on the amounts required to induce immune responses [127]. For the measurement of the quantity of released antigen, 2% of OVA were loaded into the implants to be able to detect OVA release. Two concentrations of QA were examined (0.3% and 0.66% QA).

Table 2-1: Different formulations extruded in this study

Formulation	Cholesterol [%]	D114 [%]	Soybean lecithin [%]	OVA [%]	QA [%]
1	40	45	15	2	-
2	50	35	15	2	-
3	55	30	15	-	-
4	55	30	15	2	-
5	55	30	15	2	0.3
6	60	25	15	2	-
7	60	25	15	2	0.3
8	55	30	15	-	0.3
9	55	30	15	-	0.66
10	55	30	15	0.13	0.66
11	55	30	15	2	0.66

2.3.2 Antigen and adjuvant release from tsc-extruded lipid implants

Formulations consisting of different ratios of CHOL: D114: soybean lecithin (Table 2-1) were prepared and the release of OVA was investigated *in vitro*. Each sample was loaded with on average 1.5 mg of OVA, corresponding to 2% OVA in the formulation. A comparison of the different formulations (Table 2-1, formulations 1, 2, 4 and 6) revealed that increasing the percentage of CHOL from 40 to 60% retarded the release of OVA from the implant (Figure 2-1). This behaviour is in accordance with previous reports by Demana et al. [120] that showed that the release of the model antigen PE-FITC-OVA could be slowed down by over 50% by adding an additional 72% of cholesterol to the formulation. Furthermore we can see that release discontinues after 100 hours, except for the formulation containing 60% of cholesterol. The release is either too low to be detected or the remaining OVA stays inside the lipid matrix. A difference in release between implants containing 40%, 50%, 55%, or 60% CHOL respectively, can already be observed at 6 hours. Roughly 20% of OVA were released from all the formulations, however implants containing 60% CHOL released approximately only 10% OVA after 6 hours (Figure 2-1).

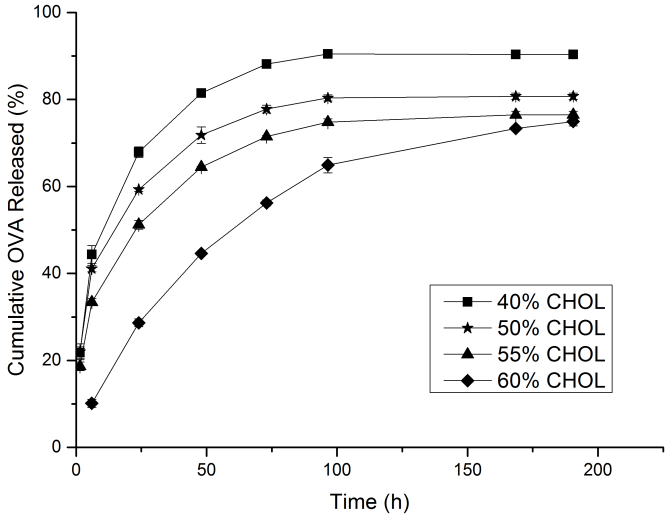

Figure 2-1: Cumulative release of OVA from implants containing 40%, 50%, 55%, or 60% CHOL (Table 2-1, formulations 1, 2, 4 and 6). Data are the mean and SD of 3 independent replicates.

The effect of QA in the implant formulations was then investigated. Implants contained 0.3% or 0.66% QA, and 2% of OVA, respectively. The amount of adjuvant found to induce immune responses corresponds to 0.66% [127]. The addition of QA may impact on OVA release as QA is a water-soluble saponin and upon incubation QA may be released into the media. This could enhance the formation of pores in the implant matrix and as a consequence diffusion of the antigen may be accelerated. In Figure 2-2 release of OVA in presence of QA in the formulation is shown. For the two different percentages of QA tested in this experiment (0.3% and 0.66%), the OVA release was independent of the amount of QA incorporated in the implants (Figure 2-2 A). Therefore, in the following experiments we investigated the influence of QA on the OVA release in implants containing 0.3% QA and either 55% and 60% CHOL and compared them to formulations without QA (Figure 2-2 B). Formulations containing QA showed a faster release of antigen compared to the same formulations without adjuvant. After 48 hours approximately 20% more OVA were released from the formulations containing QA. Formulations with QA containing 55% CHOL showed a difference in the release of OVA after 48 hours opposed to the formulations containing 60% CHOL indicating a difference in the release profile already at the first time point (6

hours). This confirms previous findings of Myschik et al. [28] who showed that the presence of QA in the formulation enhances the release of OVA through the creation of pores in the lipid matrix.

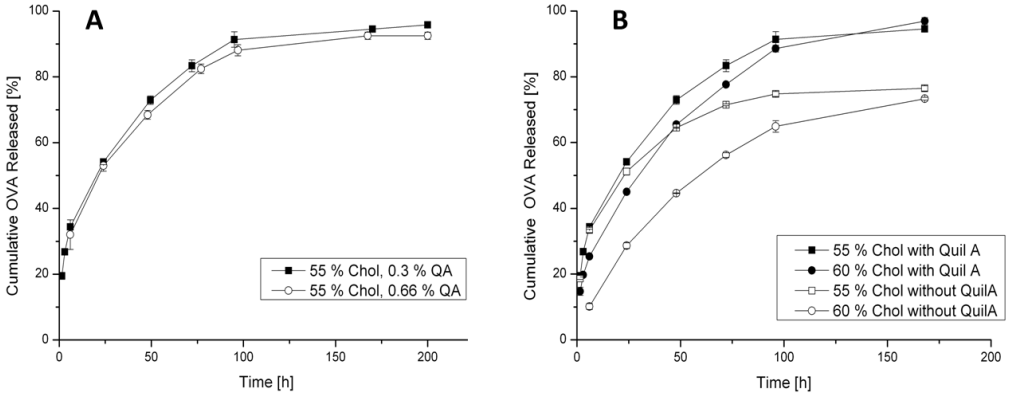

Figure 2-2: (A) Cumulative release of OVA from implants containing 55% CHOL, 0.3% respectively 0.66% of QA and 2% of OVA (Table 2-1, formulations 5 and 11). **(B)** Cumulative release of OVA from 55% CHOL and 60% CHOL implants with and without QA (Table 2-1, formulations 4-7). Data are the mean and SD of 3 independent replicates.

The release of QA from implants was quantified using HPLC with an ELSD. The implants were all composed of 55% CHOL, but contained different amounts of OVA and QA (Table 2-1, formulations 5 and 8-11). It was observed that in the presence of OVA the release of QA was faster for both QA concentrations (0.66% and 0.3% QA) (Figure 2-3). This is likely due to pore formation facilitating the release of QA from the lipid matrix. Interestingly, in contrast to antigen release, there was no initial burst release of the adjuvant and instead linear release was observed for all formulations tested (Figure 2-3). QA is released over a longer period of time (1-3 extra days) compared to the model antigen (see Figure 2-2 and 2-3), meaning that QA should always be present when OVA is being released, increasing the likelihood that an effective immune response will be generated. After analysing both, antigen and adjuvant release, it can be concluded that both are released faster in the presence of the other one, but importantly adjuvant is always released concomitantly with antigen.

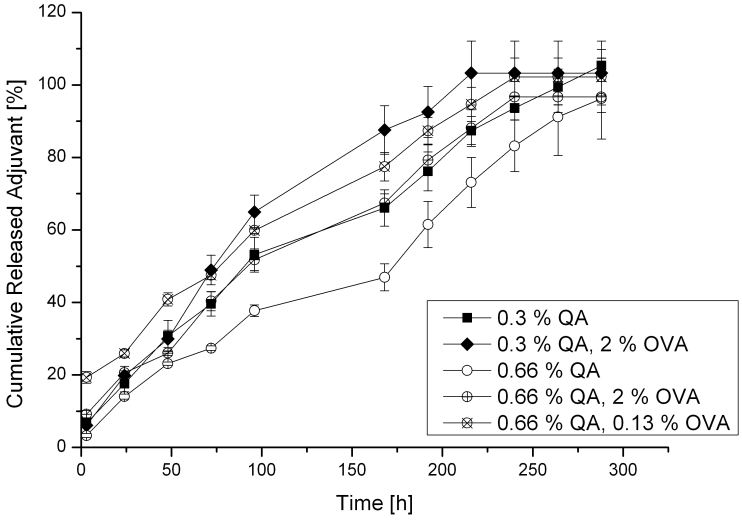

Figure 2-3: Cumulative release of QA from implants (55% CHOL) with different ratios of OVA and QA (Table 2-1, formulations 5 and 8-11). Data are the mean and SD of 3 independent replicates.

2.3.3 Integrity of OVA after extrusion and upon release

As well as investigating the overall release of the model antigen, the integrity of OVA after extrusion and release was investigated using SEC-HPLC. This technique allows quantification of the amount of monomeric protein as well as dimers or oligomers. In general, protein aggregation can occur due to different stress factors (pH, temperature, shear or mechanical stress) during manufacturing but also upon incubation [131]. Changes in the tertiary and quaternary structure of the protein should be investigated, as these may cause problems in terms of product quality such as efficacy or safety [132]. On the other hand, protein aggregates are able, in some cases, to enhance the immune response [133]. As a reference, OVA was incubated in PBS at 37°C with and without QA over a period of 15 days to ensure that the incubation does not compromise the integrity of OVA. The OVA used was composed of 90% monomer as raw material. The percentage of monomer present during the incubation in PBS with and without QA was around 90% and remained constant over 15 days (Figure 2-4). Figure 2-4 shows the percentage of OVA monomer and dimer present after it was released from the implants. It can therefore be concluded that the extrusion process itself did not cause any protein denaturation. After one week the amount of protein released as dimer increased suggesting that some kind of degradation and dimer

formation took place during the incubation. As this increase in dimer is only observed in the OVA released from the implants, it is likely that while incorporated in the lipid matrix, changes in protein structure occur. In general Van der Waals and hydrophobic attractions between side chains and backbone atoms are responsible for folding of proteins [132]. The inclusion of QA into the PBS had no effect on OVA stability and only a minor effect on protein stability was detectable when QA was included in the implants.

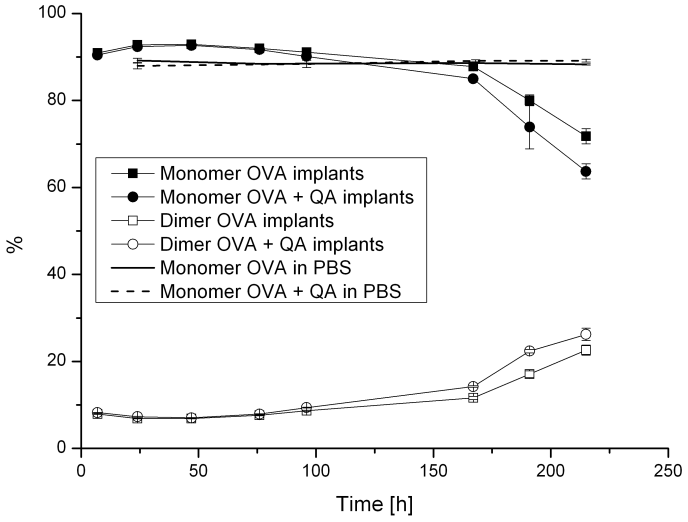

Figure 2-4: Percentage of OVA monomer and dimer released over a period of 9 days from implants with and without QA (Table 1, formulations 4 and 5). Percentage of OVA monomer for OVA and OVA+QA incubated in PBS at 37°C. Data are the mean and SD of 3 independent replicates.

2.3.4 Surface characteristics of implants before and after 72 hours of release

The surface of the implants after the extrusion process and following an incubation of 72 hours in media at 37°C was investigated using SEM (Figure 2-5). Blank implants, implants without OVA and QA (Table 2-1, formulation 3), were compared to implants containing OVA only or a combination of OVA and QA (Table 2-1, formulations 4 and 5). Directly after extrusion, there were no visible differences in the appearance of the three formulations with the implant surfaces appearing relatively smooth. After 72 hours incubation in media, differences in the surface appearance were visible and were more pronounced for implants containing OVA or OVA and QA. A few very small

pores were observed for the blank implants (Figure 2-5 B) while the surface of OVA implants contained some larger pores (20-30 μm) and the entire implant surface appeared rougher (Figure 2-5 D). Implants containing OVA and QA also had a rough surface with many small pores (less than 10 μm in size) and a few larger ones (Figure 2-5 F). This high number of small pores on the surface might explain the difference in release between implants with and without QA.

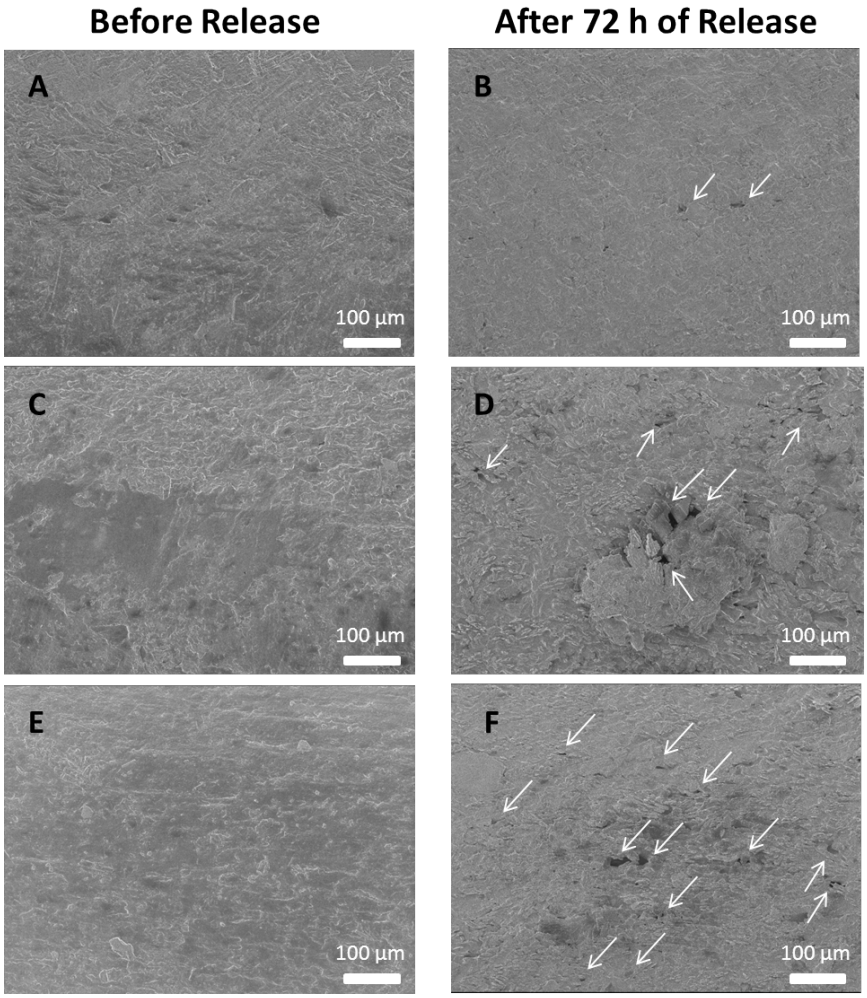

Figure 2-5: Scanning electron micrographs obtained from lipid implants after extrusion and after the 72 hours in PBS buffer (pH 7.4) at 37°C. Formulations contained 55% CHOL, 15% soybean lecithin and 30% D114 (Table 1, formulation 3-5). **(A)** and **(B)** blank implant. (**C)** and **(D)** contained 2% OVA. **(E)** and **(F)** 2% OVA and 0.3% QA. White arrows indicating the pores. Scale bar = 100 μm. Magnification 150 x.

2.3.5 Release of OVA from cured implants

The extruded implants described above released antigen and adjuvant over a 7 to 12 day period. Little information is available on the optimal period of immune

stimulation required to induce protective effector and memory immune responses, therefore studies were carried out to determine if antigen release could be sustained longer. One potential mechanism for achieving a more sustained release is to cure the implants, thereby creating a denser lipid matrix on the outer surface and as a consequence slowing antigen release [129]. Curing temperatures between 40 and 55°C were used as these are below the melting temperature of the low melting lipid Dynasan 114 (55-58°C). Implants were cured for 60 min except for curing at 55°C. At 55°C a curing time of 15 min instead of 60 min was chosen to avoid implant deformation. The melting temperature of D114 is close to 55°C and the implants lost their shape when curing them longer than 15 min at this temperature.

Curing of the implants (Table 2-1, formulations 4 and 5) extended release of antigen to 14 days, which was twice as long as release from the non-cured implants (Figure 2-6 A and B). Incomplete OVA release was again observed from implants without QA and this was in most cases further reduced (to less than 60%) by curing compared to non-cured implants. Also for implants containing QA, curing slowed the release (around 60% at 4 days as compared to 90% release for the non-cured implants), extending the release to 14 days (Figure 2-6 B). Interestingly, from day seven on increased release was observed from implants cured at higher temperatures. This is in agreement with studies reporting that the use of curing temperatures closer to the melting temperature of the lipid results in the formation of larger pores and channels compared to curing at lower temperatures [129].

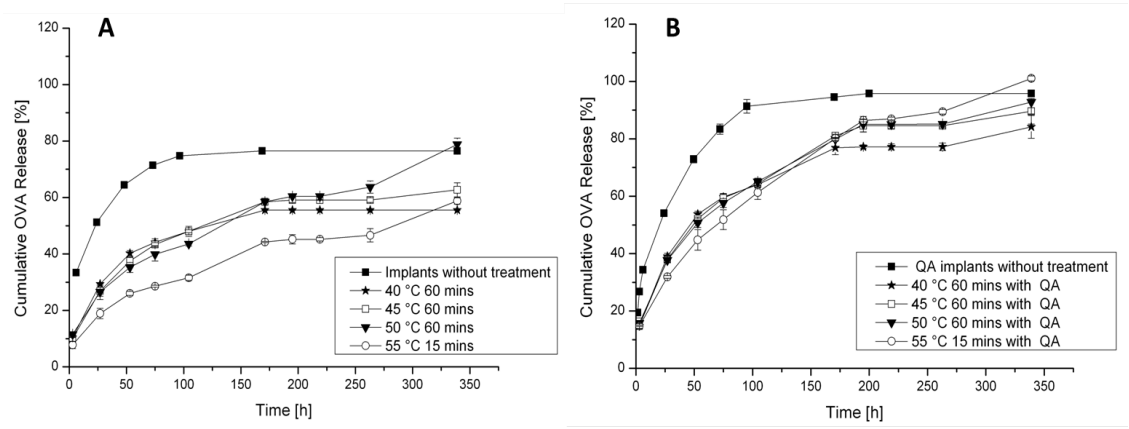

Figure 2-6: Cumulative release of OVA from cured implants containing 55% CHOL. **(A)** Without QA. **(B)** With QA. Data are the mean and SD of 3 independent replicates.

These results show that curing does slow down antigen release, but among cured implants there are differences in release depending on the curing temperature. Depending on the curing temperature, pores of different sizes could have been formed on the implant surface. If there are larger or more pores it is easier for the release buffer to penetrate into the implants and higher amounts of drug can be dissolved in buffer and become available for diffusion. Buffer movement into implants has an important influence on the release duration [134]. To confirm these assumptions, scanning electron micrographs of implant surfaces were recorded after curing and after 14 days of release. After curing no difference in surface structure resulting from the different curing temperatures could be observed (data not shown). Figure 2-7 shows the micrographs taken for implants containing OVA after 14 days incubation in release buffer. The micrographs show that implants cured at temperature higher than 40°C have pores on their surface after 14 days of incubation. The same was observed for implants containing QA+OVA. On the other hand, blank implants did not show any pores on their surface (data not shown).

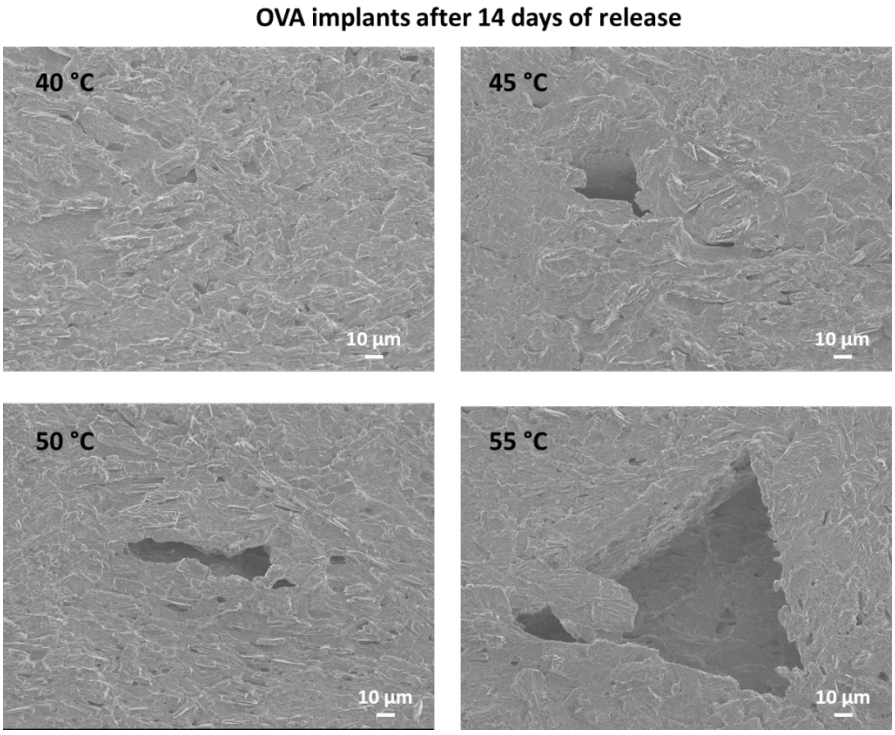

Figure 2-7: Scanning electron micrographs obtained from lipid implants after 14 days in PBS buffer (pH 7.4) at 37°C. Implants were cured at 40°C, 45°C, 50°C respectively 55°C. Scale bar = 10 µm. Magnification 500 x.

2.3.6 Investigation of lipid stability using differential scanning calorimetry (DSC)

The thermal stress induced by the curing step may lead to transitions to unstable polymorphs which subsequently, upon storage, may transform to a more stable polymorph [135]. Changes in the matrix structure of implants can be the consequence of such rearrangements and might have an influence on release behaviour. Therefore, polymorphic modifications were investigated directly after the curing process by differential scanning calorimetry. Prior to thermal analysis, melting points and polymorphic behaviour of the individual lipids (D114, CHOL and soybean lecithin) were measured as reference values (Figure 2-8 A). One single endothermic event at 59°C (n=3, SD=0.8) can be identified in the thermogram of D114. This is characteristic for the melting of the stable β-modification of pure D114 at around 56°C [136]. CHOL showed a sharp melting peak at 149°C (n=3, SD=0.5) as well as a small endothermic event at 38.4°C (n=3, SD=0.2) characteristic for impurities in cholesterol. Soybean lecithin did not show a distinct melting peak but the thermogram indicated that an endothermic event occurred between 40 and 60°C. During the extrusion process the

implants undergo thermal and mechanical stress, therefore a thermogram of the extrudates immediately after the extrusion was recorded to investigate if any changes to the polymorphism of the lipids took place (Figure 2-8 B no curing). The extrusion process did not affect the crystalline state of the material as no re-crystallization events or unstable modifications after extrusion could be identified. Two endothermic peaks were detected in the thermograms of the extrudates (Figure 2-8 B no post-treatment) corresponding to the melting endotherms of D114 and CHOL. It can be assumed that D114 crystallized in the stable β-modification, as the first peak is observed at 59°C, corresponding to the melting endotherm of D114 also observed for the pure D114 (Figure 2-8 A). In contrast to the endotherm observed for pure CHOL, the intensity and shape of the endothermic event associated with melting of CHOL changed. Decreased peak intensity as well as a shift from 149°C to 117°C was apparent. Vogelhuber *et al.* (2003) [115] described the same behaviour when studying glyceryl trimyristate/cholesterol mixtures and this was interpreted as a melting point depression. These results let to the assumption that small amounts of triglycerides dissolve in the cholesterol phase.

Thermograms of cured implants are shown in Figure 2-8 B. No re-crystallizations took place and no unstable modifications appeared except for the implants cured at 55°C. For these implants the thermogram showed two smaller peaks and one major peak. The temperatures of the two smaller peaks, 38.9°C (n=3, SD = 0.19) and 47.7°C (n=3, SD = 0.05) correspond to the values found in literature for the unstable α and β' modifications of D114 [137]. The major peak at 58.1°C (n=3, SD=0.92) corresponds to the stable β modification. In conclusion, curing too close to the melting temperature of D114 is not recommended as unstable polymorphs occurred due to this curing step. However, storing these implants for 1 week at 25°C was enough time for the instable form to shift back into the stable β modification (Figure 2-8 B). This shows that higher curing temperatures can be used, if tempering the implants post-manufacture allows for the transformation of unstable modifications back to stable forms.

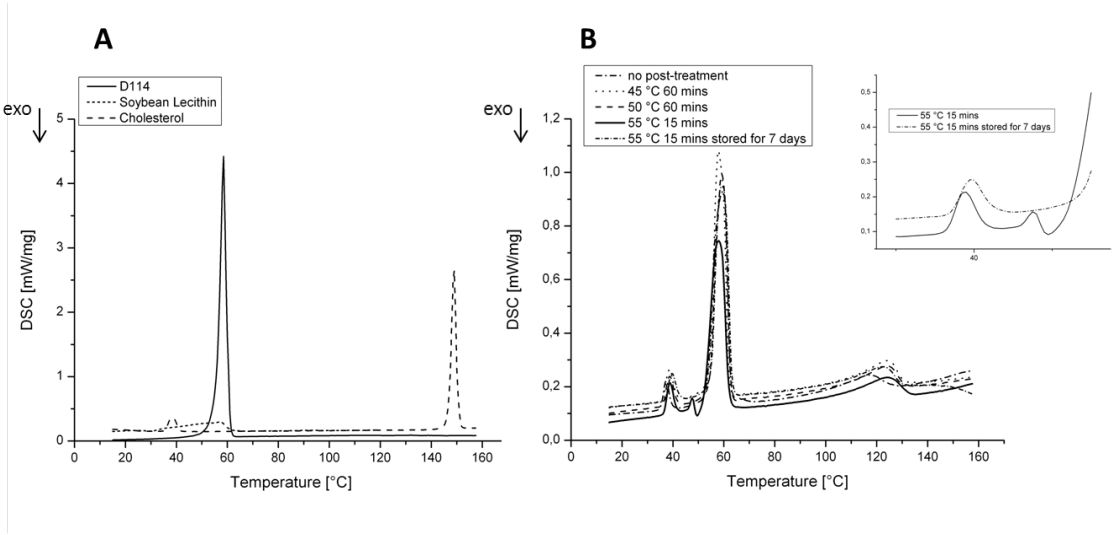

Figure 2-8: DSC thermograms of: **(A)** pure components, D114 (—), CHOL (- - -) and soybean lecithin (···), **(B)** implants right after extrusion (-·-·) and after post treatment at 45°C (···), 50°C (- - -) ,55°C (—) and 55°C (---) after 1 week storage at room temperature (n = 3). (Formulations 4 and 5 Table 2-1)

2.4 Conclusion

This study showed that the formulation of the implant as well as the addition of actives to the formulation have an impact on release behaviour of the model antigen. Importantly the adjuvant QA was released over approximately the same period of time as the model antigen, which is an important feature for vaccine delivery. Furthermore, post-extrusion curing of the implants slowed the release of the model antigen. However the curing temperature itself is of great importance as it influences the antigen release and can lead to unstable polymorphs and therefore must be chosen carefully.

2.5 Data additional to the publication

Data not shown in the publication can be found in **Appendix B.**

Chapter Three

In vivo investigation of twin-screw extruded lipid implants for vaccine delivery

Parts of this chapter have been published as peer-reviewed article:

Even MP, Young K, Winter G, Hook S, Engert J. In vivo investigation of twin-screw extruded lipid implants for vaccine delivery. Eur J Pharm Biopharm. 2014 Jul;87(2):338-46

3 In vivo investigation of twin-screw extruded lipid implants for vaccine delivery

In the last chapter the use of twin-screw extrusion as possible production device for lipid sustained vaccine delivery systems was investigated. It was shown that implants were successfully produced by tsc extrusion. A simultaneous adjuvant and antigen release behaviour was observed. Now the stability of the implants was analysed. First the effect the mechanical and thermal stress that the extrusion process itself has on the lipids was examined. As they easily undergo polymorph changes thermograms of the lipids before and after extrusion were recorded. The influence storage might have on the mechanical properties and polymorph state of implants was investigated. Furthermore, the potential advantage of particulate delivery was studied. Preformed liposomes were incorporated into the lipid implants during the extrusion process. The main part of this chapter revolves around an *in vivo* study in a mouse model. Implants with and without preformed liposomes were compared to an antigen + adjuvant injection. In a first study, the *in vivo* release of the model antigen was investigated and compared to the *in vitro* release. In a second study, the induced immune response was analysed. Cytokine secretion was measured to identify if a Th1 or Th2 type response was triggered. Moreover, CD4 and CD8 OVA-specific T-cell proliferation was measured as well as antibody titres. These studies should evaluate if the lipid implant systems are well tolerated *in vivo* and are able induce an immune answer *in vivo*.

In the following parts of this chapter the published text is reprinted. All the work was conducted by me. The two *in vivo* studies were led with the help of Katie Young and Julia Engert under the supervision of Sarah Hook at the School of Pharmacy, University of Otago. Richard Easingwood, Otago Centre for Electron Microscopy, for assisted with TEM.

Abstract

Sustained release systems have become the focus of attention in vaccine delivery as they may reduce or prevent the need for repeated dosing. In this work, lipid implants were prepared by twin-screw extrusion and investigated as vaccine delivery systems in vivo. The lipid implants consisted of cholesterol, soybean lecithin, and Dynasan 114. Ovalbumin (OVA) was employed as a model antigen and Quil-A (QA) as an adjuvant. In addition, OVA and QA loaded liposomes were prepared by the lipid-film hydration method, freeze-dried and then added to the lipid matrix prior to extrusion. Implants were administered subcutaneously and the kinetics of antigen release as well as the overall immune response stimulated were analysed by measuring CD4+ and CD8+ T cell proliferation, OVA-specific IgG production as well as cytokine (IFN-γ and IL4) secretion. Vaccine release from the implants was completed by 14 days. Inclusion of adjuvant into the implants was required for the generation of cellular and humoral immune responses. Inclusion of liposomes into the implant did not enhance the resulting immune responses generated

Key words: lipid implants, twin-screw extrusion, vaccine delivery, sustained release of antigen, ovalbumin, Quil-A

3.1 Introduction

Modern vaccines often utilise subunit antigens such as peptides or proteins instead of the whole pathogen for safety and purity reasons [5]. However, subunit vaccines need to be administered multiple times in combination with immunostimulatory adjuvants in order to induce immunity [5]. This is because subunit vaccines are less immunogenic than whole-pathogen vaccines as they lack secondary signals required for the stimulation of immune responses [6].

The most commonly used and licensed adjuvant is alum. While the exact mode of action of alum is still point of discussion in the research community, a prolonged release from an antigen depot has been proposed to be important [138,139]. Thus, single-shot administration of a vaccine with sustained (7-10 days) and synchronised release of the antigen and adjuvant is a potential alternative to giving multiple vaccine doses and one which will potentially induce superior immune responses.

Different systems for the controlled release of vaccines have been evaluated, including controlled release particles, implants and depot systems. A variety of materials have been analysed, with early studies using non-degradable polymers, requiring surgical removal of the system after the drug has been released, and more recent studies utilising biodegradable systems [116]. Biodegradable PLGA nanoparticles have been investigated for the controlled release of protein antigens such as OVA and have shown great potential [34]. Systems can be developed to release antigen for a range of times from days up to months [34].

Implants as vaccine delivery systems have been investigated since the early 1970s. These implants consisted either of silicone, ethylene-vinyl-acetate copolymer, or collagen.

Later, lipid implant systems for sustained release of drug or antigen were investigated [28,79,126]. The majority of the implants were prepared by direct compression, a technique that can be applied in the laboratory setting, but is challenging for scale-up to larger batch sizes and the resulting disk-shaped implants are not feasible for human use. Using cholesterol and L-α-phosphatidylcholine in combination with an adjuvant,

Myschik (2008) [28] was able to show that lipid implants manufactured by direct compression were able to stimulate immune responses comparable to two immunizations with an equivalent liquid vaccine [29]. Importantly, these implants stimulated CD8 immune responses – the type required for therapeutic cancer vaccines. Unfortunately, efficacy of cancer vaccines is low even with multiple doses of vaccine being given, demonstrating the need for new vaccine formulations [140].

The aim of the current work was to manufacture lipid implants for vaccine delivery by twin-screw extrusion and evaluate the efficacy of this implant system in stimulating immune responses *in vivo*. Twin-screw extrusion has been used successfully for the preparation of implants from triglycerides and mixtures of triglycerides for the sustained delivery of proteins [124], however, it has not been used to produce implants for vaccine delivery where the amount of active ingredient included is much lower. The extrusion process easily converts the raw materials into a product of uniform density and shape in a one-step production process by pushing it through a die under controlled conditions. Lipid implants consisting of cholesterol, soybean lecithin, and Dynasan 114 were prepared with a load of 20 μg antigen and 100 μg adjuvant. To combine the advantages of particulate delivery and sustained release, preformed liposomes were incorporated into the implants prior to extrusion.

After preparation, the implants were administered through a trocar into the subcutaneous tissue. For the analysis of the immune response, two sets of experiments were performed, one evaluating the kinetics of the release of the model antigen *in vivo*, a second one to evaluate the immune response *in vivo*. Ovalbumin in a liquid alum dispersion served as control.

3.2 Materials and methods

3.2.1 Materials

Ovalbumin from chicken egg white (OVA) grade V was purchased from Sigma-Aldrich. Ovalbumin, Fluorescein CO (FITC-OVA) from Life Technologies (Darmstadt, Germany). Cholesterol (CHOL), purity 95%, was purchased from AlfaAesar (Karlsruhe, Germany). Soybean Lecithin (approx. 90% phosphatidylcholine) was

purchased from APPLICHEM LIFESCIENCE (Darmstadt, Germany). Purified Quil-A (QA) was sourced from Brenntag Biosector (Frederikssund, Denmark), as a lyophilised, powder, and was used as supplied. Dynasan 114 (D114) was kindly provided by SASOL Germany GmbH (Witten, Germany). Chloroform (HPLC grade) was purchased from Fisher Scientific. Ultrapure deionised water having a conductivity of less than 0.055 μS/cm (Milli-Q Water systems, Millipore, MA, USA) was used throughout the study. All other chemicals were of analytical grade.

The following agents were used for the immunological study: anti-CD16/CD32 antibody (2.4G2 Fc block), CD4-FITC, CD4-biotin, CD4 V500, CD8-APC, CD8-PE, CD8 PE-Cy 7, Va2-PE, Vb5.1-biotin, PI, CD122 FITC, CD44 APC, CD127 V450 and anti-CD3e (BD Biosciences). 5, 6-carboxy-fluoresceine diacetate succinimidyl ester was purchased Molecular Probes. All single-cell suspensions were prepared in sterile complete Iscove's Modified Dulbecco's Medium [cIMDM; IMDM supplemented with 5% foetal bovine serum, 1% penicillin/streptomycin, 1% glutamax and 0.01% 2-mercaptoethanol all from Gibco Life Technologies (New-York, USA)].

3.2.2 Preparation of lipid implants by twin-screw (tsc) extrusion

Implants were prepared from mixtures of soybean lecithin, CHOL, D114, with and without OVA and/or QA. Soybean lecithin and D114 were transferred into high grade stainless steel beakers for milling in a swing mill Retsch® CryoMill (Retsch Technology, Haan, Germany). After precooling the system with liquid nitrogen for 10 minutes at 5 Hz, soybean lecithin and D114 were ground for 1 minute at 25 Hz. The obtained powder was mixed by hand, using a plastic mortar and pestle, with the remaining ingredients. The final mixture was then gradually blended with a mix of OVA and QA and subsequently fed into a twin-screw extruder (Haake MiniLab® Micro Rheology Compounder, Thermo Haake, Germany). The implants were extruded with closed bypass channel and a rotation speed of 40 rpm at an extrusion temperature of 45°C. The resulting implants had a diameter of 2 mm and were subsequently cut into lengths of 0.5 cm, resulting in an implant mass of approximately 16 mg.

3.2.3 Preparation of implants by twin-screw extrusion containing preformed liposomes

Liposomes consisting of soybean lecithin and cholesterol were prepared using the lipid film hydration method as described previously [141]. Briefly, 0.9 mg of soybean lecithin and 0.23 mg of cholesterol were dissolved in 70 mL of chloroform [28]. The organic solvent was evaporated under reduced pressure in a water bath at 45°C for approximately 1 hour using a rotary evaporator (Laborota 4001, Heidolph, Germany). Residual chloroform was removed by flushing of the flasks with nitrogen. The thin lipid film was rehydrated by an aqueous solution (70 mL) containing OVA with or without QA. Glass beads were added to the flasks that were rotated for 1 h and an additional rehydration time of approximately 3 h was given for the samples to equilibrate. The liposome size and size distribution were determined using a Laser Scattering Particle Size Distribution Analyzer (LA-950, HORIBA Scientific). The obtained dispersion was then freeze dried in a Christ Epsilon 2-6D freeze-drier (Christ, Germany) for a total duration of 44 hours employing a conventional freeze-drying protocol.

Lyophilized powders were mixed to D114 and additional CHOL in a plastic mortar and subsequently fed into the twin-screw extruder. The extrusion was performed at 45°C.

3.2.4 Applicability of tsc extrudates through trocar into cadaver pig skin

Extrudates of a length of 1 cm and a diameter of 2 mm were introduced into cadaver pig skin using a trocar with an inner diameter of 2 mm. After application into the pig skin, the skin was carefully cut open and the extrudates were retrieved. Pictures of the implants were taken before and after the application.

3.2.5 Texture analysis of implants

The mechanical stability, the hardness/softness of the lipid implants, was tested using a TA.XT *Plus* Texture Analyser (Stable Micro Systems). A stainless steel cylinder with a diameter of 5 mm was attached to the machine and used to compress the implants and thereby determine their hardness. For each measurement, n=3 implants were analysed.

3.2.6 Differential scanning calorimetry (DSC)

All lipids were analysed by DSC (204 Phoenix, Netsch, Selb, Germany) prior to their use, to obtain a reference to identify eventual polymorph changes induced by processing or subsequent storage. Samples of approximately 4 mg were weighed into aluminium crucibles. Heating and cooling rates were set to 5 K/min between 20°C and 160°C. An empty crucible was used as the reference.

3.2.7 In vitro release of FITC-OVA from implants

FITC-OVA was incorporated into the implants to investigate the *in vitro* release of the model antigen. The extruded strand was cut into pieces of a length of 2.5 cm. The implants (n=3) were placed into vials containing 1.8 mL PBS (pH 7.4, 0.01M, 0.05% NaN_3) and incubated at 37°C in a Heidolph Inkubator 1000. At defined time intervals samples were taken and the release medium was exchanged completely. The taken samples were centrifuged for 5 minutes at 14000 rpm to remove potential lipid pieces. Protein content was determined via size exclusion chromatography (SEC)(Dionex GmbH, Idstein, Germany) The flow rate was 0.5 mL/min and 100 µL of each sample were injected onto a TSKgel G3000 SWXL size-exclusion column (7.8 mm x 30.0 mm, Tosoh Bioscience, Stuttgart, Germany). The running buffer consisted of 50 mM PBS (pH 7.0, 0.05% NaN_3).

3.2.8 Animals

Female C57Bl/6 and OT-I and OT-II transgenic mice were bred and maintained under specific pathogen-free (SPF) conditions at the HTRU, Dunedin, New Zealand. Mice were between 6 and 8 weeks of age and had access to water and food available *ad libitum*. All experiments were approved by the University of Otago Animal Ethics Committee, AEC-Code: D64/12.

3.2.9 Preparation of OVA-specific TCR transgenic T cells

Spleen, axial and brachial lymph nodes were harvested from OT-I and OT-II transgenic mice. These mice contain high proportions of CD4 (OT-I) and CD8 (OT-II) T cells that specifically recognise peptides derived from chicken ovalbumin [142]. Red blood cells were lysed using lysis buffer (0.16M ammonium chloride, pH 7.4 and 0.17M Tris–HCl

(ratio 9:1), pH 7.65) (5 mL/spleen). Single-cell suspensions were prepared in sterile complete IMDM. After centrifugation (1100 rpm, 8 min), the cells were resuspended and counted by phase-contrast microscopy using trypan blue stain to exclude dead cells. The cells were then either stained with 5, 6-carboxy-fluoresceine diacetate succinimidyl ester (CFSE) [143,144] or left unstained. After several washing steps the cells were resuspended in sterile PBS at required concentrations, and were injected via the tail vein into C57Bl/6.

3.2.10 Immunisation protocol to determine the kinetics of antigen release

This set of experiments determined the duration of antigen release from the implants. Six different formulations were analysed (Table 3-1).

On day 0 the C57Bl/6 mice were immunised with the vaccines. The vaccines (implants or aqueous control vaccine) were given subcutaneously into the neck of the mice. Aqueous vaccines were given in a volume of 200 µl. For insertion of implants mice were anaesthetized with isoflurane, and an injection of carprofen (5 mg/kg) was given before the surgery for post-operative analgesia. Once a surgical level of anaesthesia was reached (measured by lack of pedal withdrawal) a small incision was made at the base of the back and a trocar was used to insert the implant to the dorsal skinfold under aseptic conditions. The incision was closed with a Michel clip. The cages were placed on heating pads until all mice were recovered fully from anaesthesia.

On day 1, 7, 14 or 21, 4 $x10^6$ CFSE stained CD4 and CD8 OVA transgenic T cells from OT-I and OT-II transgenic mice were injected intravenously in 200 µl sterile PBS into the immunized C57Bl/6 mice. Three days later the mice were sacrificed by cervical dislocation and the spleens were removed. Antigen-specific proliferation of CD4 and CD8 cells was measured by flow cytometry as described below.

Table 3-1: Composition of the different formulations used for the in vivo experiments and the corresponding immunisation protocol.

Group	Formulation	Adjuvant	Immunisation time point: Immunology experiments
1	Blank implant	No	Day 0
2	OVA implant	No	Day 0
3	OVA/QA implant	Yes	Day 0
4	OVA implant liposomes	No	Day 0
5	OVA/QA implant liposomes	Yes	Day 0
6	Alum + OVA	Yes	Days 0, 14

3.2.11 Immunisation protocol to determine the quality of immune response

On day -1 transgenic OT-I and OT-II cells were obtained from the spleens and lymph nodes of transgenic mice as described in the previous section. Transgenic cells were injected via the tail vein into C57Bl/6 mice at a concentration of 2 $x10^5$ transgenic cells/mL in 200 μL sterile PBS. On day 0 the C57Bl/6 mice were immunised with the vaccines as described in the kinetics experiments. On day 14, mice receiving alum formulations were boosted. On day 28, mice were sacrificed using a lethal overdose of ketamine/xylazine and blood was collected from the distal aorta. The axial and brachial lymph nodes draining the site of vaccination were collected to assess the local immune response to the vaccination. The spleens were also harvested to allow for the assessment of the systemic immune response to the vaccine.

3.2.12 Flow cytometry

Aliquots of cells were washed in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% BSA and 0.01% sodium azide) and were incubated with anti-CD16/CD32 antibody (2.4G2 Fc block) to block non-specific binding. A combination of mAb was added to the samples and incubated on ice in the dark for 10 min. Samples were then stained with: anti-CD16/CD32 antibody (2.4G2 Fc block), CD4-FITC, CD8-PE-Cy 7, Vβ5-biotin and Vα2-PE. To visualise the biotinylated antibodies, SA-APC was

used. A BD FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA) was used for the acquisition of flow cytometry data. Data analysis was carried out using FlowJo (Tree Star, Inc., Ashland, USA).

3.2.13 OVA-specific IgG response in serum of vaccinated mice

Blood samples were collected from all animals and spun for 10 minutes at 5000 rpm to separate the serum. The serum was stored at -20°C until analysis as described previously.

3.2.14 Measurement of OVA-specific Interferon-ϒ

Single cell suspensions (2 x 10^6 cells/mL) prepared from the lymph nodes and spleens of vaccinated mice were restimulated with α-CD3 (10 μg/mL) plus IL-2 (2 ng/mL), Ova (200 μg/mL) plus IL-2 (2 ng/mL), or IL-2 (2 ng/mL) in triplicate. Plates were incubated at 37°C and 5% CO_2 for three days. A 100 μL aliquot of supernatant was removed from each well for analysis of IFN-γ production using the BD Cytometric Bead Array (BD CBA) according to the manufacturer's instructions.

3.2.15 Transmission electron microscopy (TEM)

Implants of a length of 1.5 cm were placed into vials containing 1.5 mL MilliQ water in a shaking water bath at 37°C. Samples were taken after 1 and 8 days by complete exchange of the release media. Carbon-coated copper grids were glow discharged (Edwards E306A Vacuum Coater, England). 10 μL of each sample was adsorbed onto a grid and the excess sample was removed using a filter paper. The grids were negatively stained with 10 μl of 1% phosphotungstic acid (pH 6.8).

3.2.16 Statistical analysis

Statistical analysis was carried out using Prism (GraphPad Software Inc. La Jolla, USA).

3.3 Results and discussion

3.3.1 Preparation of implants

The mixtures of excipients utilised resulted in the successful preparation of lipid implants using tsc-extrusion. The selected temperature of 45°C allowed the lipid mass

to be softened and then uniformly extruded through the outlet die. Extrudates were not pasty or brittle and showed no deliquescence during handling. The inclusion of the preformed liposomes in the lipid mix had no impact on extrudate production.

The release of liposomes from the implants was investigated as the particulate nature of the vaccine is crucial for the induction of an immune response [145]. Implants were incubated in water and samples of water were taken after one day and eight days. All implants released large multilamillar liposomes, examples of which are shown in Figure 3-1. The liposomes had a diameter between 125 nm and 1000 nm. These images showed that the lipids spontaneously form liposomes upon release independent of their formulation or the presence of OVA or QA. This formation of liposomes can be explained by the fact that liposomes form when lipid cakes or thin lipid films are hydrated and stacks of lipid bilayers become fluid and swell [22].

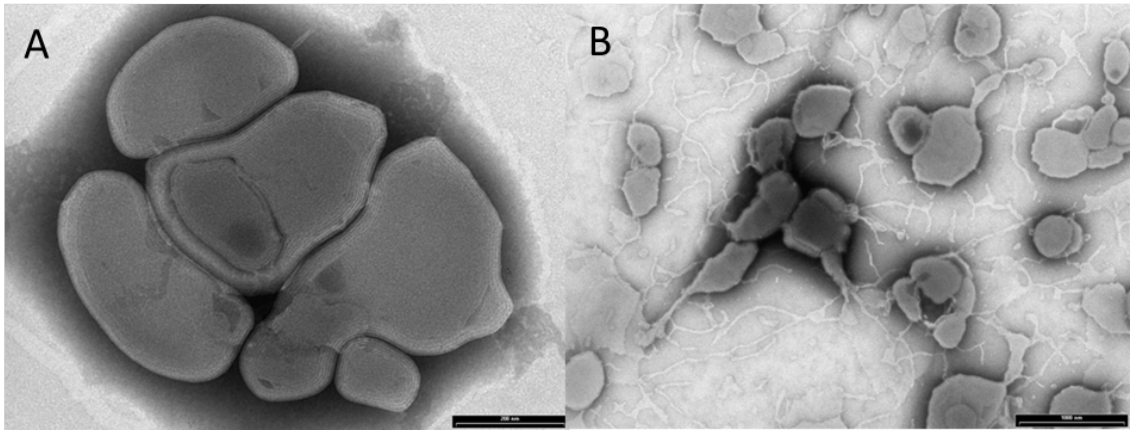

Figure 3-1: TEM images of the release in purified water after 24 h of **(A)** blank implant (55% Chol, 30% D114, 15% soybean lecithin). **(B)** OVA + QA Lip implant (55% CHOL, 30% D114, 15% soybean lecithin containing 0.13% OVA and 0.66% QA).

3.3.2 Lipid modification after extrusion and during storage

The extrusion process necessitates the melting of the low melting lipids during the process to enable the mass to be moved forward by the screws and be pushed through the outlet die. Therefore during extrusion the lipids are exposed to mechanical as well as thermal stress. These stresses may induce transitions to unstable polymorphs which subsequently upon storage may transform to a more stable polymorph [135]. Such rearrangements can be associated with changes in the matrix structure of the lipid implants and might have an influence on release behaviour as well as on mechanical properties. Therefore, polymorphic modifications and the mechanical stability of implants, formulated using CHOL:D114:soybean lecithin at ratio 50:35:15, were investigated directly after extrusion and over a storage period of 3 months and at storage temperatures of 25°C and 4°C.

Firstly the polymorphic behaviour and melting points of the individual lipids (D114, CHOL, and soybean lecithin) were determined as references (Figure 3-2, Figure 2-8 A).

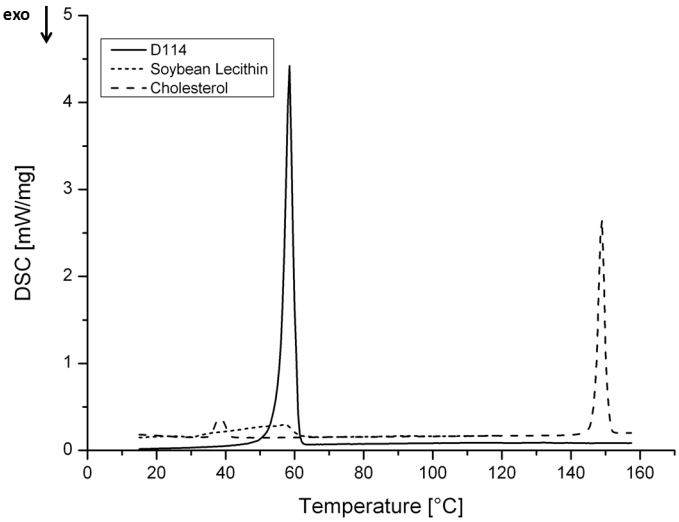

Figure 3-2: DSC thermograms of implants pure components, D114, CHOL and soybean lecithin.

The thermogram of D114 shows one single endothermic event at 59°C (n=3, SD=0.8) which is characteristic for the melting of the stable β-modification of pure Dynasan 114 at around 56°C [136]. The melting behaviour of CHOL shows a small melting peak at 38.4°C (n=3, SD=0.2) characteristic for impurities in cholesterol with the main melting

point of cholesterol at 149°C (n=3, SD=0.5). The DSC measurement of soybean lecithin showed no distinct melting peak but melting occurred between 40 and 60°C.

Next, the thermal behaviour of the lipid blend after the cryomilling step was analysed, no unstable polymorph transformation could be observed. Thermograms of the extrudates immediately after extrusion were recorded to investigate if the thermal or mechanical stress caused by the extrusion induced any changes to the polymorphism of the lipids (Figure 3-3 Day 0). No re-crystallization events or instable modifications after extrusion could be detected indicating that the crystalline state of the material was not affected by the extrusion process. The thermogram (Figure 3-3) of the extrudates shows two endothermic peaks corresponding to the melting endotherm of cholesterol and D114. The location of the melting endotherm of D114 remained constant at 59°C therefore it can be assumed that D114 crystallized in the stable β-modification. Interestingly, the shape and intensity of the CHOL endotherm changed with a shift from 149°C to 117°C and a decrease in the intensity of the signal. The same behaviour was observed by Vogelhuber *et al.* (2003) [115] when studying glyceryl trimyristate/cholesterol mixtures and this was interpreted as a melting point depression. Vogelhuber *et al.* furthermore state that these results are indicative of the dissolution of small amounts of triglyceride in the cholesterol phase.

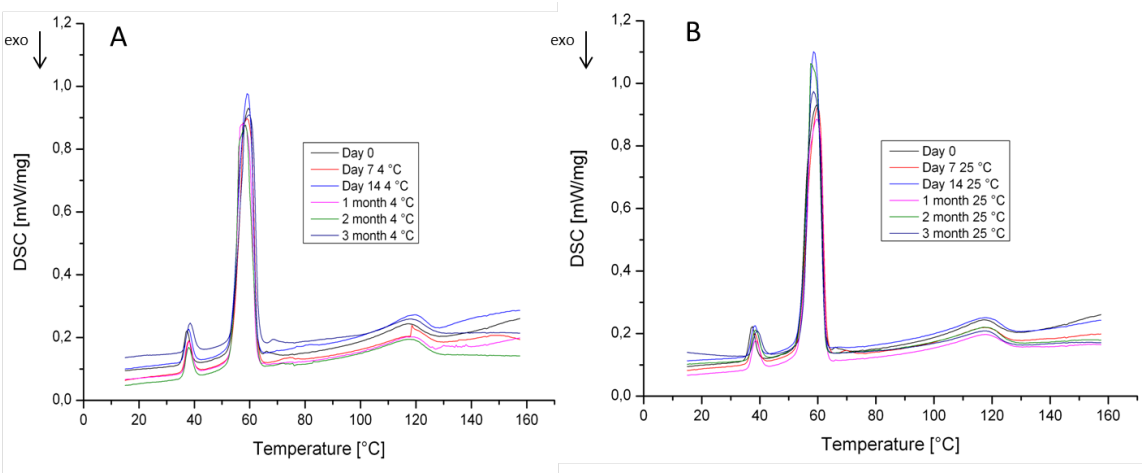

Figure 3-3: DSC thermograms of implants (50% CHOL, 30% D114. 15% soybean lecithin) (n=3) at different time points during storage. **(A)** Stored at 4°C. **(B)** Stored at 25°C.

In order to evaluate the storage stability of the extrudates over a period of 3 month, thermograms of samples stored at 25°C and 4°C were taken at different time points (Figure 3-3). No polymorphic transformations over a period of 3 month could be observed, neither for samples stored at 4°C nor for those stored at 25°C. These results support the conclusion that extrudates can be stored at either temperature. After extrusion, the initial handling of the implants showed that the systems were not pasty or brittle. The mechanical strength of the extrudates was evaluated during the storage period of 3 months. The texture analyser measurements (Figure 3-4) showed that there was no significant change in the hardness of the implants over time for either of the storage temperatures.

In addition the injectability of the implants through a trocar was investigated. Extrudates of a length of 1 cm were introduced into pig skin using a trocar. Pig skin was used as it is more similar to human skin in terms of mechanical properties [146]. Mouse skin is much thinner and therefore less force should be needed to introduce the implant in the mouse model. After insertion of the implants into pig skin, the tissue was cut open and the extrudates retrieved. Visual analysis did not show any breakage, bending or rupture of the implants caused by this procedure (Figure 3-5).

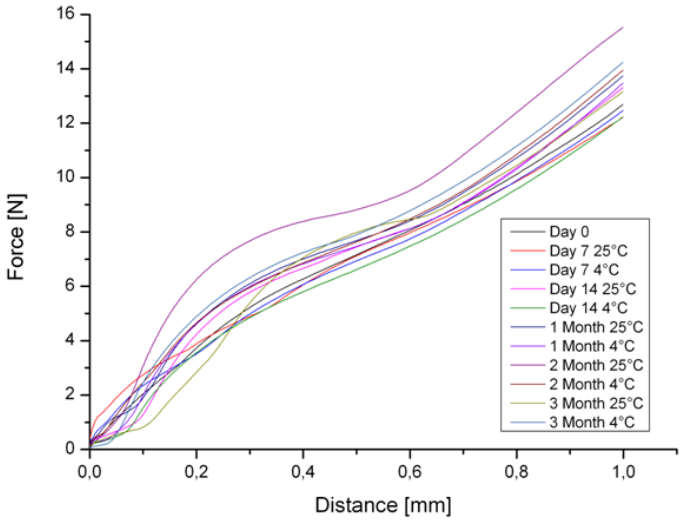

Figure 3-4: Texture analyser measurements of implants (n=3) analysed on days 0, 7, 14, 1 months, 2 months and 3 months at 4°C or 25°C, respectively.

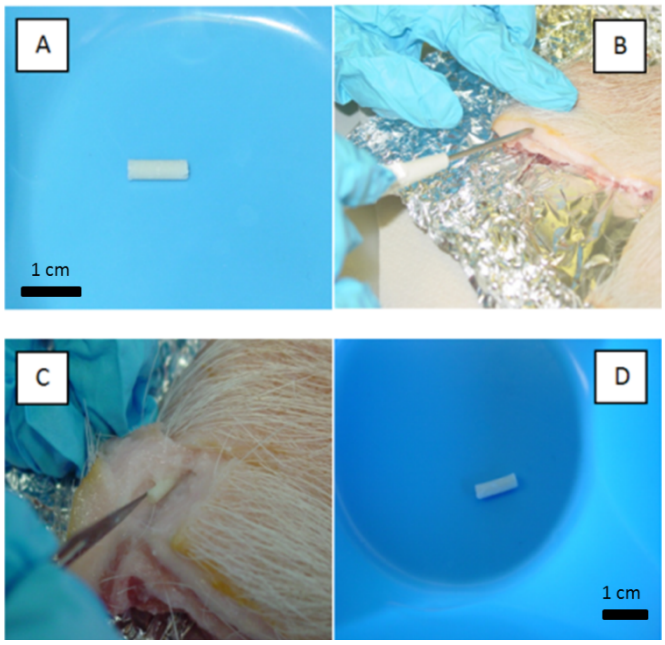

Figure 3-5: Application of an implant into pig skin using a trocar. **(A)** Implant before application. **(B)** Insertion of loaded trocar into pig skin. **(C)** Inserted implant. **(D)** Implant after application.

3.3.3 Antigen release in vitro and in vivo

Antigen release was examined directly *in vitro* and indirectly *in vivo* by assessing T cell proliferation at predetermined time points after immunisation. Implants for the *in vitro* release were composed of 55% CHOL, 30% D114, 15% soybean lecithin had a diameter

of 2 mm and were 2.5 cm long, containing 100 μg OVA per implant +/- 500 μg QA. For the *in vivo* release studies, the implants had the same composition but were only 0.5 cm long and contained therefore only 20 μg OVA per implant +/- 100 μg QA. The control implant consisted of soybean lecithin, D114 and cholesterol.

The *in vitro* investigation of the release of FITC-OVA indicated that after already 7 hours 10 to 15% of the OVA is released (Figure 1-6). However release was incomplete during the time course investigated. Release from the implants will likely occur by diffusion. As the implants do not degrade *in vitro* OVA could possibly have remained inside the implant not reaching pores to diffuse out. Therefore, it is unsurprising that not all OVA was released. However, in the *in vivo* situation we observed that after 28 days partial breakdown of the implants had occurred making it likely that release might be more complete in an *in vivo* system than *in vitro*.

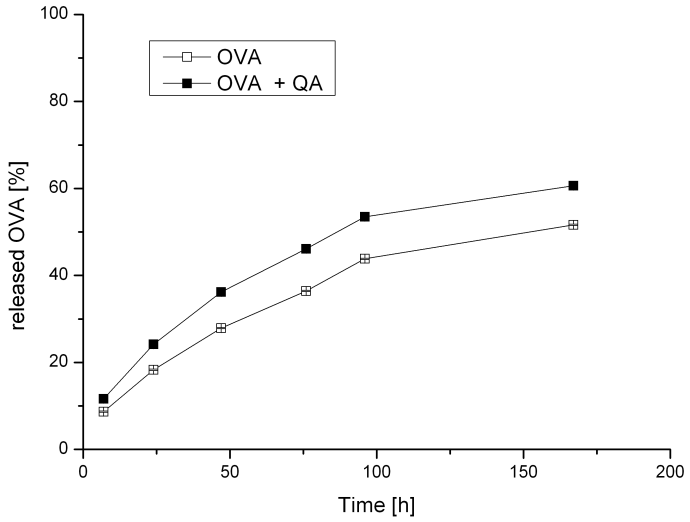

Figure 3-6: Cumulative release of FITC-OVA from implants with and without QA (55% CHOL, 30% D114 and 15% Soybean Lecithin). Data is the mean of 3 replicates and the standard deviation of n = 3.

To analyse how long OVA was released in the animals after implantation, mice were injected with CFSE stained OVA-specific T cells at predetermined times after implantation. These cells can be used to measure antigen release as they will divide as long as antigen is present in the system. CFSE spontaneously and irreversibly couples

to both cell-surface and intracellular proteins by reaction with lysine side-chains and other available amine groups [147]. When a cell divides the daughter cells will contain half the amount of CFSE which can be measured by flow cytometry, thus allowing for the investigation of release *in vivo*.

One day following administration of the vaccines, CD8 T cells from all groups of mice given OVA-containing vaccines showed similar high levels of cell division (Figure 3-7) indicating that antigen was immediately available for presentation by antigen presenting cells to the CD8 T cells. This shows good agreement with the in vitro release data which shows a release of more than 20% of the loaded OVA after 24 hours. Interestingly T cell proliferation did not appear to be dependent on the presence of an adjuvant in the vaccine. Proliferation by OT-I cells in the absence of a costimulatory signal has been previously reported and it was found that while in the absence of a costimulatory signal CD8 cells could undergo proliferation they did not gain full effector function [148]. Alternatively it could be that the physical act of vaccine administration created sufficient inflammatory signals to drive T cell proliferation at this time point.

At day 7, mice immunised with implants containing OVA and QA or with OVA in alum exhibited significantly more CD8+ proliferation than did mice immunised with implants containing OVA alone ($p<0.05$). The inclusion of liposomes in the implants appeared to have no effect on the level of CD8 proliferation. As alum is known to provide a depot effect, sustained release from this formulation was expected [139] and it was encouraging to see similar results with the implants. A possible explanation for the higher CD8+ T cell proliferation observed for the OVA/QA implants are that due to the presence of the water-soluble component QA, the release of OVA may be enhanced. Indeed the *in vitro* release (Figure 3-6) showed that implants containing QA release more OVA than implants without the adjuvant. It is also possible that the adjuvant QA is required for cell division at day 7, whereas it was not required at day 1. A greater reliance on costimulation when antigen is limited, as could be the case by day 7, has been reported [149].

The results from day 14 revealed no CD8+ T cell division for any of the groups supporting the conclusion that after 14 days there was no longer any OVA present in the system. This is in accordance with the *in vitro* release data (Figure 3-6) showing a release up to 7 days.

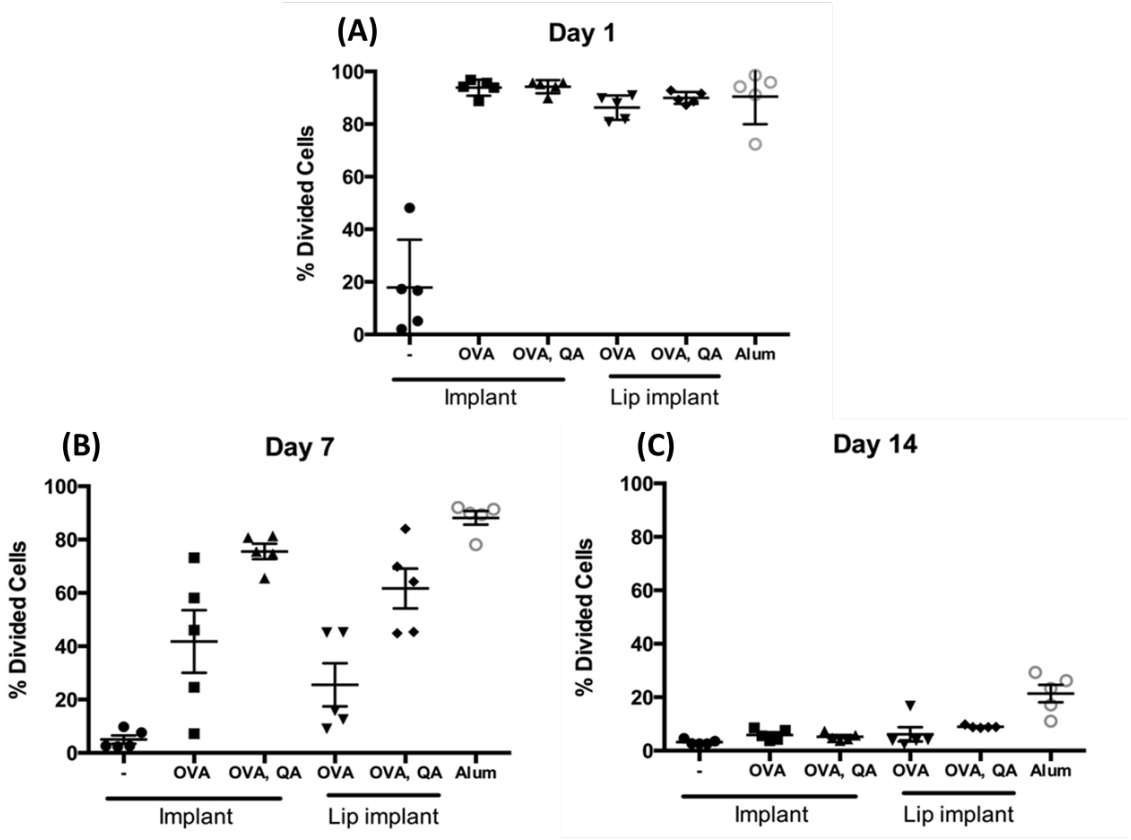

Figure 3-7: Transgenic CD8+ T cell proliferation, as a percentage of divided CD8+ T cells. Mice were immunized with blank implants (- Implant), implants containing OVA or OVA/QA either incorporated directly into the lipid mix (OVA Implant, OVA/QA Implant) or formulated into liposomes which were then freeze-dried and incorporated into the lipid mix (Lip Implant) or with OVA in alum (Alum). The adoptive transfer of CFSE stained cells was performed at **(A)** day 1, **(B)** day 7, or **(C)** day 14. Data shown are the individual results from five mice per group plus the mean and SEM.

Immune responses generated by the sustained-release lipid implants were then examined 28 days post immunisation. Both CD4 and CD8 OVA-specific T cell responses were examined (Figure 3-8). The inclusion of QA in implants resulted in a higher percentage of CD4+ and CD8+ Tg T cells in the lymph nodes of immunised mice. Similar results confirming the importance of inclusion of an adjuvant in the formulation were observed by Myschik *et al* [29].

The inclusion of liposomes into the implants did not make a significant difference on antigen specific CD4+ or CD8+ T cell expansion. Importantly measurable immune responses were detectable 28 days after immunization with implants and these responses were superior to those generated by the alum adjuvanted vaccine which was boosted at day 14.

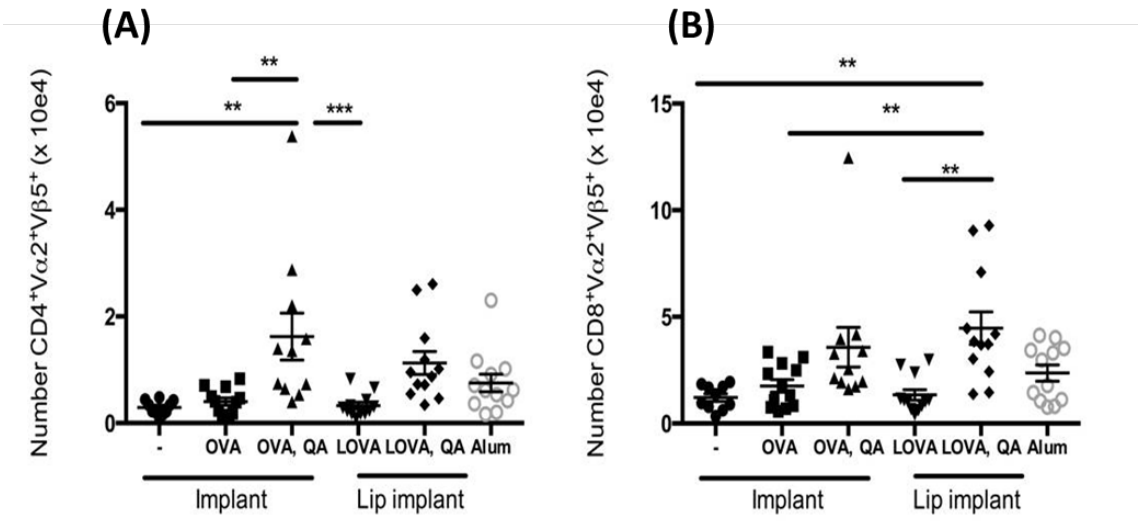

Figure 3-8: OVA-specific expansion of transgenic CD4+ **(A)** and CD8+ **(B)** T cells isolated from lymph nodes of C57Bl/6 mice on day 28. Mice were immunized with blank implants (- Implant), implants containing OVA or OVA/QA either incorporated directly into the lipid mix (OVA Implant, OVA/QA Implant) or formulated into liposomes which were then freeze-dried and incorporated into the lipid mix (Lip Implant) or with OVA in alum (Alum). Data shown are the individual results from 4 mice per group from 3 independent experiments, plus the mean and SEM. Statistical analysis of results was carried out by one-way ANOVA followed by pairwise Tukey's multiple comparisons.

Following an *in vitro* restimulation with OVA, cytokine secretion was investigated to examine antigen-specific effector function. The secretion of IL-4 (a Th2 cytokine), and IFN-γ (a Th1 cytokine) were examined (Figure 3-9). Higher IFN-γ concentrations in cell supernatants were detected for those groups that received implants containing QA compared to groups that received antigen and no adjuvant. The IFN-γ responses detected upon restimulation of cells from mice immunised with a single OVA/QA implant were significantly higher than those from mice immunised two times with OVA in alum. No IL-4 was detected in any samples, regardless of the presence of antigen and/or adjuvant (data not shown). Therefore, these results indicate that a Th1 response was observed for the groups receiving QA containing implants.

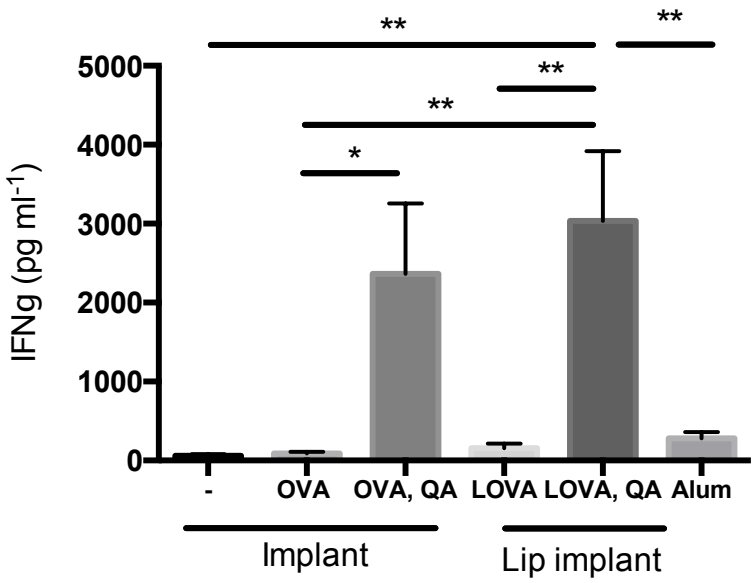

Figure 3-9: Interferon-γ concentrations for lymph node samples restimulated *in vitro* with OVA. Mice were immunized with blank implants (- Implant), implants containing OVA or OVA/QA either incorporated directly into the lipid mix (OVA Implant, OVA/QA Implant) or formulated into liposomes which were then freeze-dried and incorporated into the lipid mix (Lip Implant) or with OVA in alum (Alum). Data shown are the mean and SEM from 4 mice per group from 3 independent experiments.

Furthermore, the serum from the immunised C57Bl/6 mice was collected at the end of the experiments to measure OVA specific IgG antibodies in order to investigate the ability of the implants to stimulate a humoral response. As shown in Figure 3-10, the QA/OVA implants resulted in significantly higher antibody titres than the OVA only implants, indicating once more the importance of the adjuvant in the system. This also correlates with the results obtained by Myschik *et al.* [29] comparing the production of OVA IgG antibodies from OVA and OVA/QA implants produced by direct compression. No distinction could be made in the antibody production between QA implants and the alum + OVA injection group. This is an interesting result as the implants were administered at day 0 while the alum + OVA mice received a booster injection at day 14 of the experiment. Moreover no difference between the implants with and without preformed liposomes could be identified.

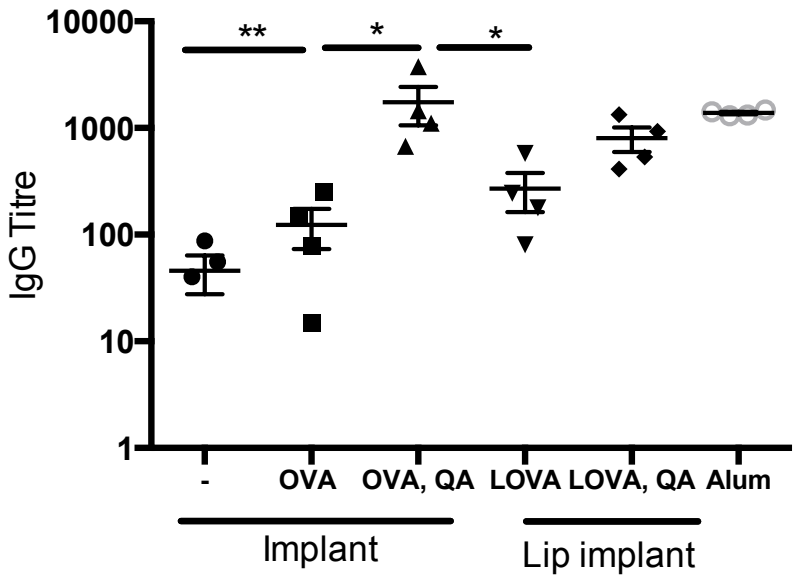

Figure 3-10: OVA-specifc IgG antibody titres determined by ELISA on day 28. Mice were immunized with blank implants (- Implant), implants containing OVA or OVA/QA either incorporated directly into the lipid mix (OVA Implant, OVA/QA Implant) or formulated into liposomes which were then freeze-dried and incorporated into the lipid mix (Lip Implant) or with OVA in alum (Alum). Data shown are the individual results from 4 mice per group and the mean and SEM from one representative experiment of three.

The reason for including liposomes in the formulation was so that antigen and adjuvant would be released in a particulate form, with the particles containing both antigen and adjuvant and therefore being able to induce optimal APC and T cell activation. A concern with antigen and adjuvant being released individually and asynchronously from a sustained release formulation is that a tolerogenic, as opposed to an effector, response could be stimulated. Indeed Kamath et al recently reported that exposure of APC to antigen before adjuvant induces an antigen-positive non-activated population of APC [128]. While the implants used here appeared to be able to stimulate an effector immune responses (as demonstrated by increased numbers of antigen specific CD4 and CD8 cells, IFN-γ production and IgG) this response could perhaps be further improved through the loading of antigen and adjuvant into a suitable particulate delivery system which could then be incorporated into the implant. Such a particulate delivery system would need to be able to efficiently load high levels of antigen and adjuvant and be compatible with a lipid based implant and tsc-extrusion. The liposomes which were used here were compatible with the lipid based delivery

system and tsc-extrusion but likely had only very low levels of antigen and adjuvant associated with the particles released [29].

In our study we used tsc-extruded implants in an attempt to mimic a natural infection as regards antigen release; we will not be mimicking the natural route of infection which can be important in situations where mucosal immunity is required. Therefore, the suitability of these kinds of implants and the required release period needs to be assessed for each application.

3.4 Conclusion

This study showed that lipid based tsc-implants can be utilised for in vivo immunisation studies. The implants have suitable physical characteristics and release the vaccine over a period of between 7 and 10 days, which would correspond to the duration of a natural infection. The results confirmed that the investigated tsc-implants can replace a prime-boost immunisation regime. Tsc-implants are therefore considered to be a promising sustained release delivery system, which can be produced by a scalable process. Further optimisation of the extrudates is possible, tuning release and decreasing the diameter of the implants, increasing their potential applications. The applicability of the system to different antigens and peptides will be investigated in future studies.

3.5 Data additional to the publication

3.5.1 Additional *in vivo* data

Appendix C shows additional *in vivo* data not shown in the publication such as the IgG titres from the two replicates of the experiment, the interferon-γ concentrations measured in the spleen and transgenic CD8+ T cell proliferation.

3.5.2 Implant changes after extraction

Figure 3-11 shows implants before administration to the mice (A) and after 28 days inside the mouse (B). During the time under the skin of the mouse, the implant lost shape and turned from a cylindrical into a ball formed shape. This is probably due to the fact that the implant becomes softer at body temperature and due to the mechanical

stress the implant was exposed to under the skin of the mouse. But the implant was still located in the neck of the mouse.

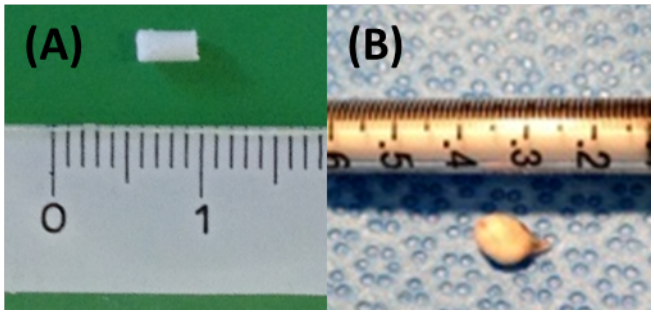

Figure 3-11: (A) Implant before administration to the mouse. **(B)** Implant after 28 days inside a mouse.

3.5.3 Antigen release *in vitro* after storage

The mechanical stability of the implants stored at 25°C and 4°C over a period of three month was investigated and showed to be given (Figure 3-4). Also the polymorph state of the lipids did not changer over a storing period of three month (Figure 3-3). To further investigate the stability of the implants during storage, implants were stored at 25°C over a period of six months. DSC measurement was performed and also the OVA release in vitro from the implants was investigated. Figure 3-12 shows the release of OVA from implants directly after extrusion, after one, three and six month for implant formulation with and without QA.

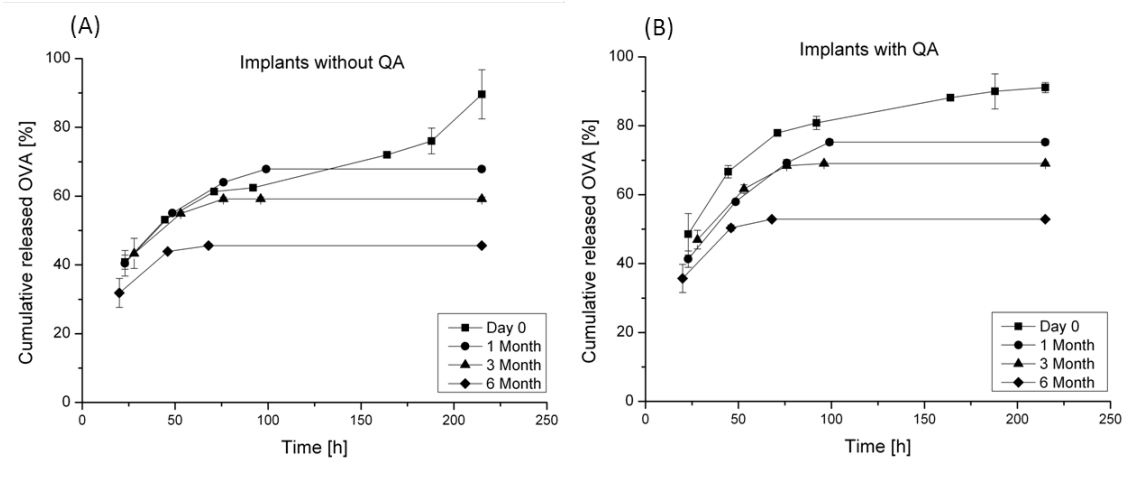

Figure 3-12: Cumulative release of OVA from implants with and without QA (55% CHOL, 30% D114 and 15% Soybean Lecithin). Implants were stored for different time period's at 25°C. **(A)**

Implants without QA. **(B)** Implants with QA. Data is the mean of 3 replicates and the standard deviation of n = 3

Implants consisted of 55% CHOL, 30% D114, and 15% soybean lecithin. Implants contained 2% OVA with and without QA (0.3%). Implants containing QA (Figure 3-12 B) showed a more complete release than implants without QA. The release gets less complete the longer implants are stored, for both implants with and without QA. Furthermore the release duration also decreases with storage time. Implants stored for 6 months release OVA over 68 hours (2.8 days), whereas the release duration measured from implants directly after extrusion is 215 hours (8.9 days). These results indicate a change is occurring during storage. The results from the DSC measurements are illustrated in Table 3-2.

Table 3-2: Shows the melting temperature and the melting energy of implants stored at 25°C over different time periods.

Implants	**Day 0**		**1 month**		**3 months**		**6 months**	
	[°C]	**[J/g]**	**[°C]**	**[J/g]**	**[°C]**	**[J/g]**	**[°C]**	**[J/g]**
Blank	59.70	49.85	60.10	54.44	59.76	48.79	59.37	54.43
OVA	59.47	52.06	59.53	53.33	59.00	43.03	59.13	53.43
OVA+QA	59.00	49.90	59.50	51.91	59.47	48.13	59.57	52.20

No shift in melting temperature or melting energy is observed over time. Furthermore, no unstable polymorphs occurred during storage. Since the DSC measurements did not offer an explanation for what occurred during storage, the assumption that the lipid matrix is changing or that interactions between the model antigen and the lipids are taking place emerged.

Chapter Four

Twin-screw extruded lipid implants containing TRP2 peptide for tumour therapy

Parts of this chapter will be submitted for peer-review and publication:

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4 Twin-screw extruded lipid implants containing TRP2 peptide for tumour therapy

In the previous chapter it was shown that implants can be stored safely up to three months without changing their mechanical strength or the polymorph form. More importantly the performed *in vivo* study proofed that our implant systems were well tolerated by mice. Furthermore, it was shown that comparable immune responses could be achieved when comparing the implant systems with two booster injections. The importance of the adjuvant in such sustained delivery systems also emerged from the study. Considering these promising results, in this chapter the use of lipid implants in tumour treatment is investigated. For this purpose, a non-mutated melanoma-associated antigen, namely the TRP2 peptide was incorporated into the implants. This peptide is a very valuable excipient. Therefore the extrusion process had to be transferred to a small size extruder, which allowed working with very small batch sizes. Before starting with the production of the TRP2 loaded implants, the process transfer from one extruder to another was analysed. Optical changes of implants produced by the two extruders were investigated by SEM. Also mechanical and polymorph differences of the implants were examined. After adapting the lipid formulation to the small scale extruder, implants containing QA and TRP2 were produced. The *in vitro* release of TRP2 and QA from the implants was investigated. Also the use of vesicular phospholipid gels (VPGs) for tumour therapy was considered, analysing the *in vitro* release of TRP2 from the VPGs. An *in vivo* tumour growth study was performed comparing lipid implants, VPGs and conventional injection in a therapeutic way. Tumour cells were injected first and 6 days later the different formulations were given.

4.1 Introduction

The discovery that CD8+ cytotoxic T lymphocytes (CTL) can recognize tumour-associated antigens has opened a new approach to the development of specific anti-tumour immunotherapies. During the last few years, much effort has been put into the identification of tumour-associated antigens [150-152]. However, most of these tumour-associated antigens are non-mutated self-antigens and are poorly immunogenic [150,153,154]. Therefore, the induction of a potent and specific T cell response still represents a major challenge [155]. The use of lipid implants as sustained delivery systems might help to overcome this challenge. A class of non-mutated melanoma-associated antigens has been identified that are recognized in an MHC class I-restricted fashion by melanoma-reactive CTLs from different patients. These antigens include tyrosinase-related protein TRP1 and TRP2, melanoma antigen reactive with T cells (MART)-1, and gp100 [156,157]. TRP1 has 40-50% amino acid sequence identity to tyrosinase and TRP2 [158]. Human TRP2 shares 84% of the sequence of its mouse counterpart [159]. TRP2 peptide (amino acids 180-188 of the TRP2 protein, SVYDFFVWL) is recognized by tumour reactive CTLs from both, mice and humans. The peptide sequence is identical between mice and humans, and is therefore a very interesting candidate for tumour studies [156,160]. However, short peptides for vaccinations show some disadvantages. Vaccination with short peptides can lead to immunological tolerance and short peptide antigens often fail to induce CD8+ T memory cells [161]. On the other hand, vaccination with long peptides (22-45 amino acids) increases vaccine efficacy with long peptides being able to induce robust CD8+ T cell responses [161]. Therefore, a long TRP2 peptide was used in this study. Peptide antigens are easy to manufacture, however, they have a very low immunogenicity and often need to be administered together with immunostimulatory adjuvants [5]. QA was used in this study. To attempt to get simultaneous delivery, both adjuvant and peptide were incorporated into a lipid matrix. In the study presented here, lipid implants composed of cholesterol, Dynasan 114, soybean lecithin, TRP2 and QA were produced by twin-screw extrusion using the small-scale ZE 5 extruder.

In early stages of formulation development, often, extensive formulation screening is necessary to achieve optimal implant characterisations. Only after the optimal formulation composition has been achieved using model compounds, the move to expensive drugs or bioactives is made. At this stage, a scale-down of the production process may be necessary. Consequently, it is important to investigate the influence a change of the production device might have. To address this issue, we compared two different extruders in this study using OVA as a model antigen. One extruder allows the preparation of lab scale batches (minimum 5 g), whereas the other extruder allows the extrusion of extremely small quantities (0.5 g), inevitable when working with the expensive drugs such as the TRP2 peptide used in this study. Our aim was to investigate the influence of the processing equipment on the extrudability of the lipid mixture, on the properties of the implants, as well as on the *in vitro* release of the model antigen. In a next step, the formulation was adapted for the release of the TRP2 peptide and the extruded implants were tested in an *in vivo* tumour study. A second lipid system was tested as a TRP2 carrier in the *in vivo* study, namely vesicular phospholipid gels (VPGs). Different studies describe the use of liposomal systems as TRP2 carriers [160,162,163], therefore VPGs were considered an interesting alternative for our experiment. They only consist of soybean lecithin and buffer and have been described in literature as potential delivery systems for pharmaceutical proteins [164-166].

4.2 Materials and methods

4.2.1 Materials

The TRP2 long peptide (SVYDFFVWLKFFHRTCKCTGNFA-OH) was purchased from peptides and elephants (Potsdam, Germany). Cholesterol (CHOL), purity 95%, was purchased from AlfaAesar (Karlsruhe, Germany). Soybean Lecithin (approx. 90% phosphatidylcholine) was purchased from APPLICHEM LIFESCIENCE (Darmstadt, Germany). Phosphate buffered saline (PBS) tablets from Oxoid Limited (Basingstoke, England). Purified Quil-A (QA) was sourced from Brenntag Biosector (Frederikssund, Denmark) as lyophilised powder and was used as supplied. trimyristin (Dynasan 114 (D114)) was kindly provided by SASOL Germany GmbH (Witten, Germany). Chloroform (HPLC grade) was purchased from Fisher Scientific. Ultrapure deionised

water having a conductivity of less than 0.055 μS/cm (Milli-Q Water systems, Millipore, MA, USA) was used throughout the study. All other chemicals were of analytical grade.

4.2.2 Preparation of lipid implants by twin screw extrusion

Implants were prepared from mixtures of soybean lecithin, CHOL, D114, with and without OVA/TRP2 and/or QA as described previously (**Chapter Three**) [127]. The final mixture was subsequently fed into a twin-screw extruder (small size extruder ZE 5, Three-Tec, Seon, Switzerland or Haake MiniLab® Micro Rheology Compounder, Thermo Haake, Germany). The ZE 5 extruder is equipped with three different individual heating zones. Each individual heating zone has a length of 1.2 cm separated by a gap of 0.3 cm. Heating zones were operated at different temperature as described in Table 1, a rotation speed of 55 rpm was applied. Implants extruded with the Haake extruder were produced at 45°C and 40 rpm. The resulting implants had a diameter of 2 mm and were subsequently cut into lengths of 2.5 resp. 0.5 cm, resulting in an implant mass of about 80 mg resp. 16 mg. Implants were produced under the laminar flow (Hera Safe, Kendro Laboratory Products GmbH, Germany) and equipment was heat sterilized at 160°C for 2 hours before use.

4.2.3 Preparation of vesicular phospholipid gels (VPGs)

Vesicular phospholipid gels were formulated by dual asymmetric centrifugation (SpeedMixer, Hauschild & co KG, Hamm, Germany). For homogenization a process speed of 3500 rpm was used for 30 minutes. QA and TRP2 peptide were incorporated into the VPGs by direct loading. QA (0.5 mg/ml) and TRP2 (0.28 mg/ml) solutions in sterile filtered PBS buffer were added to the accurately weighed soybean lecithin and homogenized by means of the dual asymmetric centrifuge in a 25 ml sterile cylindrical plastic container (25 ml, aponorm, Germany). VPGs contained 300 mg/g lipids. The PBS buffer was sterile filtrated and the containers were autoclaved. All other utensils were autoclaved or head sterilized if possible. The VPG´s were prepared under the laminar flow. TRP2 and QA were not sterilized.

4.2.4 Density measurements of implants

To determine the true density of the produced implants, defined as the density of a material excluding pores and inter-particle spaces, an AccuPyc 1330 helium pycnometer (Micromeritics, Aachen, Germany) was used at a sample holder volume of 0.718507 cm^3. Prior analysis the instrument was calibrated using a metal sphere of known volume. 10 cleaning cycles were performed using analytical grade helium. Implants were cut into small pieces, approximately 150-200 mg of the sample was analysed and the true density was calculated as an average of six measurements.

4.2.5 Scanning electron microscopy (SEM)

A Jeol JSM-6500 F (Jeol JSM-6500F, Tokyo, Japan) was used at an acceleration voltage of 2 kV and a magnification of 150. Implants were cut and attached to aluminium blocks with double adhesive tape and were analysed without further treatment.

4.2.6 *In vitro* release of OVA from implants

The release of the model antigen OVA was investigated over a period of up to 7 days. Lipid implants of a length of 2.5 cm (n=3) were incubated at 37 °C in a Heidolph 1000 shaking incubator (10 rpm/min) in vials containing 1.8 ml phosphate buffered saline (PBS) (pH 7.4, 0.01 M, 0.05% NaN_3). Lipid implants contained 55% cholesterol, 15% soybean lecithin, 30% D114, 1.6 mg OVA and 0.24 mg QA. At defined time points samples were taken and the release medium was exchanged completely. All samples were centrifuged at 14000 rpm (Mikroliterzentrifuge Z 160 M, Hermle Labortechnik, Wehingen, Germany) for 5 minutes to remove lipid particulates. OVA in the supernatant was measured by UV (Agilent Technologies 8453) at a wavelength of 280 nm. For each tested mixture an implant containing neither OVA nor QA was used as a blank for the UV measurements. Each implant was weighted before incubation and the total amount of protein present in each implant was calculated individually using a standard curve of OVA in PBS. All measured samples lay within the linear part of the standard curve (3 mg/mL – 5 μg/mL).

4.2.7 *In vitro* release of Quil A from implants

Implants were cut in 3 cm lengths and weighed. All implants contained 65% cholesterol, 600 μg of QA and 336 μg TRP2. Implants were placed into 2 mL Eppendorf tubes filled with 1 mL PBS buffer (pH 7.3) and incubated at 37°C (Clayson shaking incubator, New Zealand) at 10 rpm/min. At defined time points 300 μl of release medium was taken out and replaced with fresh PBS buffer. Samples were centrifuged for 10 minutes at 14000 rpm in a bench top centrifuge (Prism R, Labnet International Inc., Edison, USA) to pellet the lipid fractions. The supernatant was transferred into an Eppendorf tube and stored at -20°C until high performance liquid chromatography with evaporating light scattering detector (HPLC-ELSD) analysis was carried out.

4.2.8 High performance liquid chromatography with evaporating light scattering detector (HPLC-ELSD)

The HPLC system consisted of 1200 Series evaporating light scattering detector (ELSD) system from Agilent Technologies (Santa Clara, USA) equipped with a ZORBAX Eclipse XDB-C8 Column (2.1 x 50 mm; 3.5 μm, Agilent Technologies). The column was maintained at a temperature of 25°C. A guard column (2.1x12.5mm, Agilent Technologies) was used to prevent contamination of the column and was also maintained at 25°C. The injection volume was 20 μL. The mobile phase consisted of water/acetonitrile (75:25% v/v) containing 0.01% v/v formic acid. A flow rate of 0.25 mL per minute was used. The ELSD settings were as follows: nebulizing temperature of 30°C, nitrogen gas pressure at 3.5 bar, gain at 10.

4.2.9 *In vitro* release of TRP2 from implants and VPGs

Release from implants was performed in 2ml Eppendorf vials containing 1.8 mL PBS (pH 7.4, 0.01 M, 0.05% NaN_3, 0.25% SDS). Release from VPGs was analysed in 2 mL Eppendorf vials containing 1 mL PBS (pH 7.4, 0.01 M) supplemented with 0.05% NaN_3, 0.25% SDS. Release experiments were performed by complete buffer exchange in a Heidolph 1000 shaking incubator at 37 °C and 10 rpm/min. All samples were centrifuged at 14000 rpm (Mikroliterzentrifuge Z 160 M, Hermle Labortechnik, Wehingen, Germany) for 10 minutes to remove lipid particulates. TRP2 concentration was then quantified by reverse-phase HPLC (RP-HPLC).

4.2.10 Reverse-phase HPLC (RP-HPLC) for TRP2 quantification

TRP2 peptide release was quantified by RP-HPLC using a Dionex Ultimate 3000 HPLC system (Dionex, Softron GmbH, Germering, Germany). A Phenomenex Jupiter 5u C4 300 Å column (250 mm x 4.60 mm, Phenomenex, Achaffenburg, Germany) and an injection volume of 150 µL of each sample were used. The running buffer consisted of acetonitrile (0.1% TFA) and H_2O (10% acetonitrile and 0.1% Trifluoroacetic acid (TFA)), with a flow rate set to 0.75 mL/min.

4.2.11 Differential scanning calorimetry (DSC)

A Phoenix 204 (Netsch, Selb, Germany) DSC was used to record thermograms of implants directly after the extrusion. Samples of about 4 mg each were weighed into aluminium crucibles. 5 K/min were used as heating and cooling rates and were set between 20°C and 160°C. An empty crucible served as reference.

4.2.12 Texture analysis of implants

A TA.XT Plus Texture Analyser (Stable Micro Systems) was used to determine the mechanical stability of the implants. Implants were compressed using a stainless steel cylinder having a diameter of 5 mm (compression speed 0.5 mm/s, compression force 30 N). For each measurement, three replicates were analysed.

4.2.13 Tumour cells

B16-F10-luc2 melanoma cells (Thermofisher Scientific New Zealand, Auckland, NZ) were cultured in cRPMI in BD Falcon tissue flasks (BD, Biosciences, MA, USA). Cells were passaged when confluent. On the day of the tumour injection cells were washed with 10 mL of PBS, the adherent cells were dissociated by incubation with 2 mL TrypLE Exress (Life Technologies, Auckland, NZ) for 10 minutes at room temperature. To ensure that the cells are dissociated the flasks were tapped by hand from all sides. Cells were then washed with 8 mL media and centrifuged at 11000 rpm for 8 minutes (Heraeus Multifuge 3 S-R, Thermo Scientific). The supernatant was discarded, cells were resuspended and washed with 10 mL PBS. Subsequently, cells were counted and resuspended at a concentration of 1×10^6 cells/mL.

4.2.14 Animals

Male C57Bl/6 mice (n=72) were bred and maintained under specific pathogen-free (SPF) conditions at the HTRU, Dunedin, New Zealand. Mice had access to food and water *ad libitum*. All experiments were approved by the University of Otago Animal Ethics Committee, AEC-Code: D64/12.

4.2.15 Immunisation protocol

6 days prior to immunisation each mouse was injected s.c. into the left flank with 1 x 10^5 B16-F10-luc2 melanoma cells in 100 µl PBS. The formulations (implants, VPGs or aqueous control formulation) were given subcutaneously into the neck of the mice. Aqueous formulations as well as VPGs were injected in a volume of 200 µl. For the insertion of the implants mice were injected subcutaneously with carprofen (5 mg/kg) for post-operative analgesia and were anaesthetised with inhaled isoflurane. Once a surgical level of anaesthesia was reached (measured by lack of pedal withdrawal) a small incision was made at the base of the back and a trocar was used to insert the implant to the dorsal skinfold under aseptic conditions. The incision was closed with a Michel clip. The cages were placed on heating pads until all mice fully recovered from anaesthesia. Mice were weighed and physically checked for tumour growth every 2 days. Once tumours were palpable they were monitored daily (including tumour size measurements). Tumour size was monitored with a digital calliper. Mice were sacrificed if the tumour reached a size of 150 mm^2.

4.2.16 Statistical analysis

Statistical analysis was carried out using Prism (GraphPad Software Inc. La Jolla, USA).

4.3 Results and discussion

4.3.1 Differences between implants prepared using larger and small scale extruders

We were able to show in a previous study that implants for antigen delivery can be successfully produced by twin-screw extrusion (**Chapter Three**)[127]. The lipid mixtures as well as the extrusion temperature are important parameters in the production process. During extrusion, lipids are exposed to mechanical and thermal

stress, but we were able to show that the extrusion process did not change the polymorphic state of the lipids. Furthermore, we showed that storing did not compromise the physical stability or the polymorphic state of the implants (**Chapter Three**) [127]. Implants described in those studies were produced using a Haake MiniLab extruder which requires a minimum of 5 g of material. As the aim of this study was to perform a tumour experiment using implants containing TRP2 peptide, an expensive active ingredient, it was necessary to transfer the production process to a different device. In order to determine if changing the extruder impacted on implant production and on the implants produced, implants containing 55% cholesterol, 15% soybean lecithin and 30% D114 were extruded using two different extruders; all formulations contained 2% OVA as model antigen and/or 0.3% QA. The Haake extruder has screws of a length of 11 cm with a conical form going from a diameter of 10 mm to 4 mm. The ZE extruder, which enables batch sizes of 0.5 g of lipid to be formulated, was used for comparison. The ZE 5 extruder has a screw length of only 7.5 cm and a diameter of 5 mm (Figure 4-1). This difference in size consequently results in a change of the extrusion parameters such as the through-put time and heat transfer. An additional advantage of the ZE 5 extruder is that it allows the use of three different heating zones during manufacturing. Each individual heating zone has a length of 1.2 cm separated by a gap of 0.3 cm and can be tempered between room temperature and 230°C.

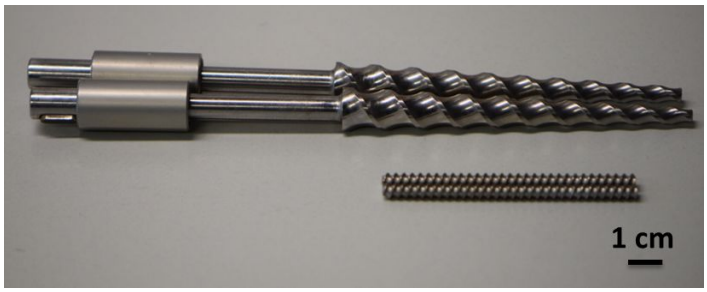

Figure 4-1: Image of the screws from the Haake MiniLab extruder (top) and the ZE 5 extruder (bottom)

In the first set of experiments, the extrusion temperature of the three heating zones was kept at 45°C (Table 4-1, run 1), as this was the extrusion temperature used in previous work with the Haake extruder (**Chapter Two and Three**).

Table 4-1: Temperature chosen for the three individual heating zones of the ZE 5 extruder for the different runs, all carried out at 55 rpm.

Run	Heating Zone 1 [°C]	Heating Zone 2 [°C]	Heating Zone 3 [°C]
1	45	45	45
2	48	48	48
3	45	48	50

OVA release *in vitro* from implants produced by the two different extruders was compared (Figure 4-2). It became clear that it is not possible to transfer the process from one extruder device to another while maintaining the same extrusion temperature. OVA release from implants produced by the ZE 5 extruder was less complete compared to OVA release observed from implants produced using the Haake extruder. The release from implants produced by the ZE 5 extruder stopped after 75 hours, whereas the release from the implants produced by the Haake extruder continued for up to 200 hours. Implants containing QA produced by either extruder released OVA faster than implants without QA.

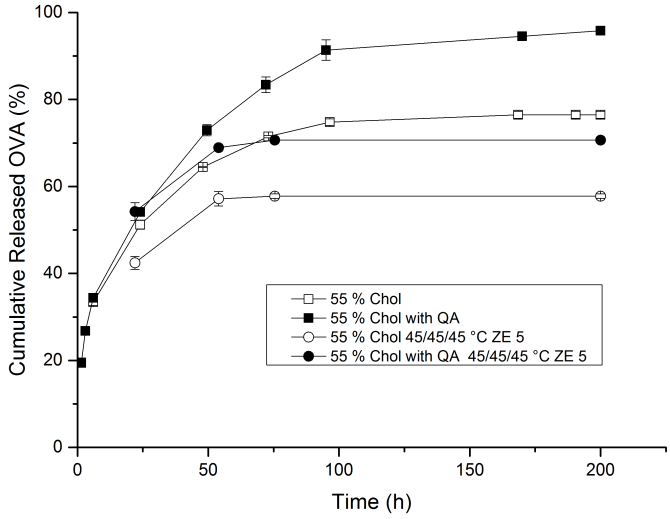

Figure 4-2: Cumulative release of OVA from implants produced at 45°C containing 55% CHOL with and without QA produced by the Haake (squares) and the ZE 5 extruder (circles). Data are the mean and SD of 3 independent replicates.

SEM micrographs of the surface and the cross section of implants produced at 45°C using the Haake and the ZE5 extruder were compared in order to investigate if differences in the structure correlated with different release behaviour from the implants. The surface of implants extruded by the ZE 5 extruder (Figure 4-3 D) appeared to be smoother compared to implants produced by the Haake extruder (Figure 4-3 A). The cross-section cut of the implants produced by the Haake extruder (Figure 4-3 B and C) revealed that these implants have pores and cracks inside the implants. The implants produced by the ZE 5 showed no cracks and only very few pores (Figure 4-3 E and F). On the cross-section of implants from the ZE 5 (Figure 4-3 E) the right upper half of the section appeared very smooth, but this is due to the cutting of the implant. The higher magnification (Figure 4-3 F) was taken from the left part of the cross-section. The network of pores and cracks in the implants produced by the Haake extruder could explain why the release of OVA from these implants is more complete compared to implants produced by the ZE 5. This is in accordance with the literature, indicating that more channels lead to a more complete drug release [134].

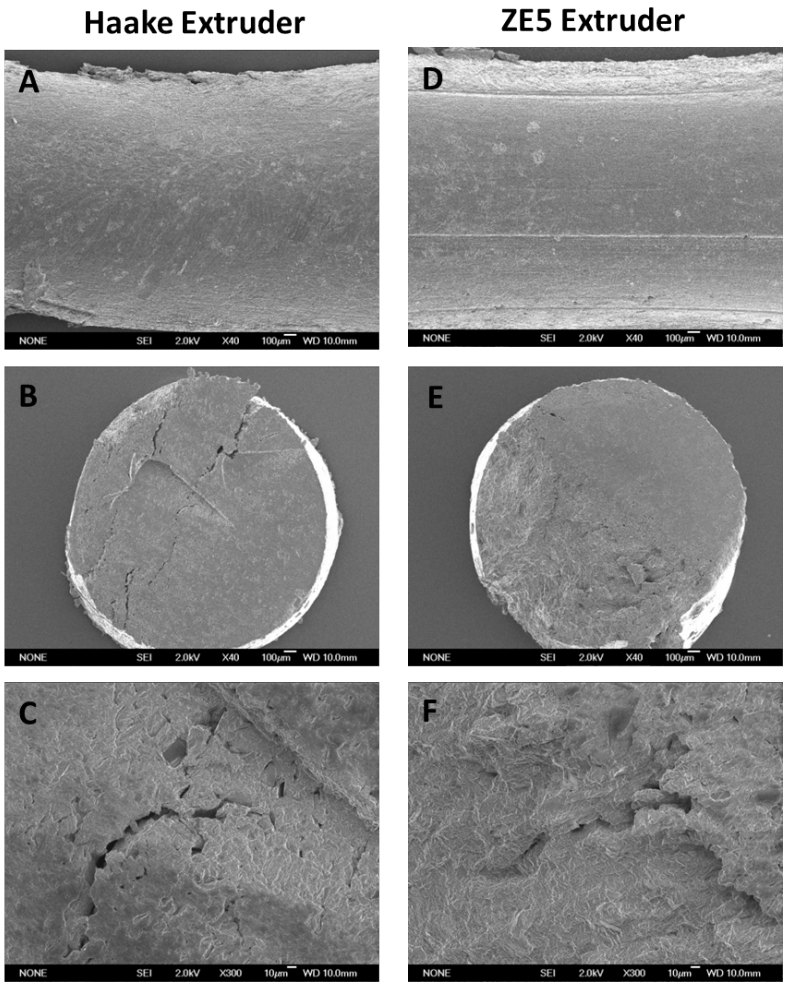

Figure 4-3: Scanning electron micrographs obtained from lipid implants after extrusion produced with the Haake and the ZE extruder (Run 1 Table 4-1). Formulations contained 55% CHOL, 15% soybean lecithin and 30% D114. Haake extruder: **(A)** surface of the implants, **(B)** cross section magnification 40 X, **(C)** cross-section magnification 300 X. ZE5 extruder: **(D)** surface of the implants, **(E)** cross section magnification 40 X, **(F)** cross-section magnification 300 X.

Reitz and Kleinebudde (2007) [167,168] showed that the extrusion temperature affects the inner morphology of the obtained system and also has a great influence on the porosity of the extrudates. In an attempt to prolong the antigen release from the implants, the temperatures of the different heating zones of the ZE 5 extruder were increased (Table 4-1 run 2 and 3). Changing the temperature in the different heating zones had a considerable influence on the resulting antigen release (Figure 4-4). Increasing the temperature in all heating zones to 48°C instead of 45°C reduced the burst release at the beginning, but did not prolong the OVA release, which still stopped after 75 h with only 40% OVA released (Figure 4-4). Gradually increasing the

temperature from 45 to 50°C (Table 1, run 3) greatly prolonged the release up to 200 hours (Figure 4-4) with sustained release of 75% OVA for implants containing QA. These results are in accordance with literature, indicating that the extrusion temperature plays an important role [167,168]. Using these parameters, a release duration of 200 hours (corresponding to 8 days) comparable to the one from the implants produced by the Haake extruder could be achieved. However, compared to the implants from the Haake extruder, the release is still less complete from implants produced by the ZE 5 extruder (Figure 4-4).

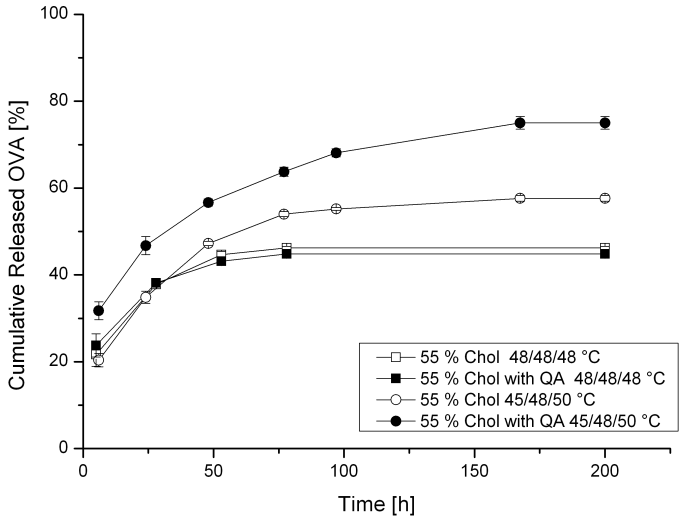

Figure 4-4: Cumulative release of OVA from implants containing 55% CHOL, with and without QA, produced at different temperatures by the ZE 5 extruder. Squares: implants extruded at 48°C (Table 4-1 run 2); Circles: implants extruded using different temperature for the heat zones (45/48/50°C) (Table 4-1 run 3). Data are the mean and SD of 3 independent replicates.

To further investigate where these differences arose from, analysis of the mechanical properties of the implants, density measurements as well as DSC measurements were performed. The thermograms obtained from implants produced using the ZE extruder indicate no changes to unstable polymorphs, even at extrusion temperatures of 50°C (Figure 4-5 A). Texture analyser measurements (Figure 4-5 B) confirmed that implants produced by the ZE 5 extruder are slightly more brittle than implants produced by the Haake extruder, which was also observed when handling the implants. The density of implants produced by the ZE 5 extruder was 1037.2 +/- 4.0E-10 mg/cm^3 (n= 6), slightly higher than that measured for implants produced by the Haake extruder

(1013.5 +/- 4.0E-10 mg/cm^3, n= 6). These physical differences between the two implants further emphasize the distinction between the implants produced by the two extruders. The higher density of implants produced by the ZE 5 extruder correlates with the SEM micrographs, showing fewer pores than for implants produced by the Haake extruder. We assume that the lipids in the ZE 5 extruder are melted more completely, due to the higher contact surface between the lipids inside the extruder and the heating walls of the extruder. Whereas in the Haake extruder, lipids presumably undergo higher mechanical stress than in the ZE 5 extruder. These differences would explain the difference in release.

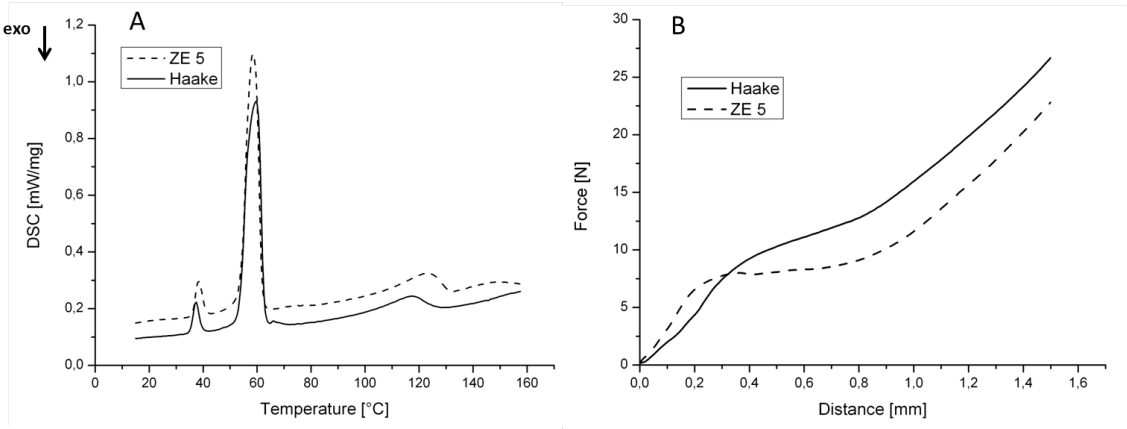

Figure 4-5: (A) Thermogram of implants extruded with the Haake extruder (45°C) compared to implants produced by the ZE 5 extruder (45/48/50°C). **(B)** Texture Analyser measurements implants from the Haake extruder (45°C) compared to implants produced by the ZE 5 extruder (45/48/50°C).

4.3.2 *In vitro* release of TRP2 peptide and QA from implants

Implants produced by the ZE 5 extruder using parameters shown in Table 1 run 3 show a promising release profile for the model antigen OVA. Also DSC measurements showed that no unstable polymorph modifications appeared after the extrusion process. Therefore, these production parameters were applied for the implants used in tumour growth study. A mixture containing 65% CHOL, 15% soybean lecithin and 20% D114 was used, the implants contained 0.53 mg QA, 0.28 mg TRP2 and 12 mg trehalose. This corresponds for an implant of a length of 0.5 cm to 100 µg QA and 56 µg TRP2, which was the dose required for the *in vivo* tumour study. The extrusion was performed as described in the materials and methods section, except for one additional

production stage. TRP2 was first admixed with 12 mg of trehalose, before blending it with the lipids. This step was necessary in order to obtain an appropriate TRP2 release from the implants. When admixing TRP2 directly with the lipid mixture, without trehalose, a very slow release was observed stopping at 3.5% released TRP2 after 10 days (Appendix D). Figure 4-6 shows the *in vitro* release of TRP2 from the produced implants. The release of TRP2 from the implants is very slow, with up to 40% TRP2 released after 58 days.

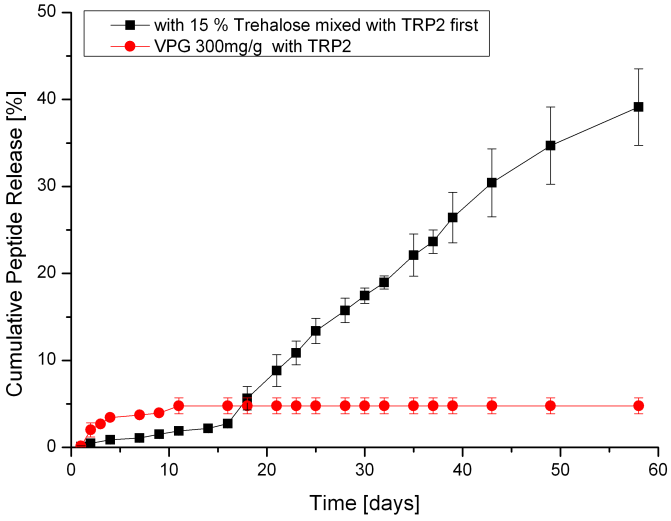

Figure 4-6: Cumulative release of TRP2 from implants produced by the ZE 5 extruder and from VPGs. Data are the mean and SD of 3 independent replicates.

In addition to the peptide release from the implants, the release of the adjuvant QA was investigated. Implants were of the same composition as described previously, except that also a batch of implants without TRP2 was produced. In contrast to what was observed for the peptide, a QA release duration of 14 days was achieved for implants containing the TRP2 peptide (Figure 4-7). The presence of TRP2 in the implants seemed to increase the release rate of QA compared to implants without TRP2. After 7 days, implants containing TRP2 have released already 87% of QA compared to 49% when no TRP2 was in the implant. These results correspond to the QA release behaviour observed from implants with and without OVA (**Chapter Two**), where it was shown that the presence of OVA in the implant formulations resulted in faster release of QA. The ample difference in the release behaviour observed between

the TRP2 peptide and the adjuvant leads to the conclusion that not only the composition of the implant, as often indicated in literature [28,120], has an influence on the release behaviour but also the molecule itself. Interactions between the peptide and the lipid matrix play a key role and have to be further investigated.

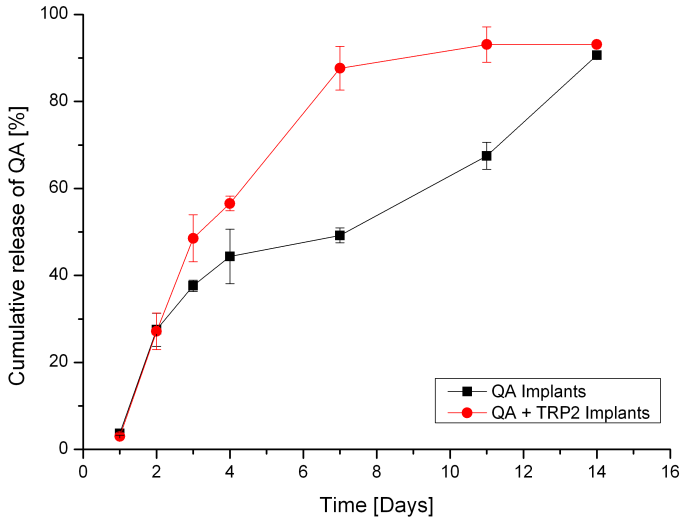

Figure 4-7: Cumulative release of QA from implants produced by the ZE 5 extruder with and without TRP2 in the formulation. Data are the mean and SD of three independent replicates.

4.3.3 *In vitro* release of TRP2 peptide from VPGs

In addition to lipid implants, TRP2 loaded VPGs were produced to investigate their efficiency in the *in vivo* tumour experiment. VPGs represent a semi-solid phospholipid dispersion and are suited to carry both lipophilic, amphiphilic and lipophilic drugs [166]. VPGs used in this study were composed of soybean lecithin and PBS (300 mg lipid/ g PBS). TRP2 and QA were added such that 200 µl of VPG contained 100 µg QA and 56 µg TRP2. Figure 4-6 shows the TRP2 release from this system. Only 5% of TRP2 were released before the release comes to an end and the remaining peptide stays inside the VPG. As mentioned already, also interactions between the peptide and the lipids can be the reason for this slow and incomplete release.

4.3.4 *In vivo* tumour study

The previously described TRP2 implants and VPGs were then used in an *in vivo* tumour study. Table 4-2 shows the immunisation groups and the number of mice used in each study. In the first study, we observed some adverse reaction in the VPG groups three days after the formulations were given to the mice, therefore VPGs were excluded from further experiments. It is likely that the adverse reactions are due to the QA, as QA is known to be have undesirable side effects [169]. Rönnberg et al (1995) stated that QA exhibits lytic activities, by reacting with the cholesterol in the cell membrane that can cause adverse reactions [170]. Interestingly they found that a dramatic decrease of the hemoltytic activity of QA when incorporated into ISCOMs. They assumed that the CHOL present in the ISCOMs interacts with QA thereby blocking QA to interact with CHOL in the cell membranes. This also is in agreement with a study by Walduck et al (1998) immunizing sheep with CHOL-lecithin implants [82]. The study showed that QA was better tolerated in implants than in the injections, leading to minor skin irritation in the sheep. In our study the VPG contained no cholesterol while the implants, which contain cholesterol, were well tolerated with no swelling, redness or skin irritation observed. However, no endotoxin values of the implants nor the VPGs were measured, which would be necessary for a substantiated conclusion on the adverse reactions provoked by the VPGS.

Table 4-2: Immunisation groups of the *in vivo* study for the two performed experiments.

Group	Description	No. of mice Study 1	No. of mice Study 2
1	QA Implant	4	6
2	TRP2 + QA Implant	8	10
3	QA VPG	4	-
4	TRP2 + QA VPG	8	-
5	TRP2 in PBS	8	8
6	No treatment	8	8

We found no statistically significant difference between the results of the no treatment groups of the two studies, therefore data from the two experiments were combined. Six days after tumour cell injection, the different formulations described in Table 4-2 were administered. Tumour size was monitored with a digital calliper and mice were sacrificed when tumour size exceeded 150 mm^2. Table 4-3 indicates the median survival time for each group corresponding to the time at which half the animals had tumours greater than 150 mm^2 in size. Survival of treated mice was not significantly different from untreated mice, however there was a trend that mice in the TRP2+QA implant group survived the longest. The numbers show that for mice in the TRP2+QA implant group the median survival time is four days longer than in the no-treatment group. According to these numbers, mice in the TRP2+QA implant group have the longest median survival time.

Table 4-3: Median survival times for each group in days.

Combined Data	No Treatment	QA Implant	TRP2 + QA Implant	TRP2 + PBS	TRP2 + QA VPG
Median survival [days]	20	22.5	24	22.5	23

Figure 4-8 shows the survival curves for each group of mice after tumour inoculation (day 0) and Figure 4-9 shows tumour growth in individual mice. Statistical analysis of the survival curves showed that there is a significant difference between the group without treatment and the group having received TRP2+QA implants ($p=0.009$) as well as between the group without treatment and the group having received TRP2+PBS injections ($p=0.035$). On the other hand, there was no significant difference between the group having received a TRP2+PBS injection and administered with TRP2+QA implants ($p=0.73$).

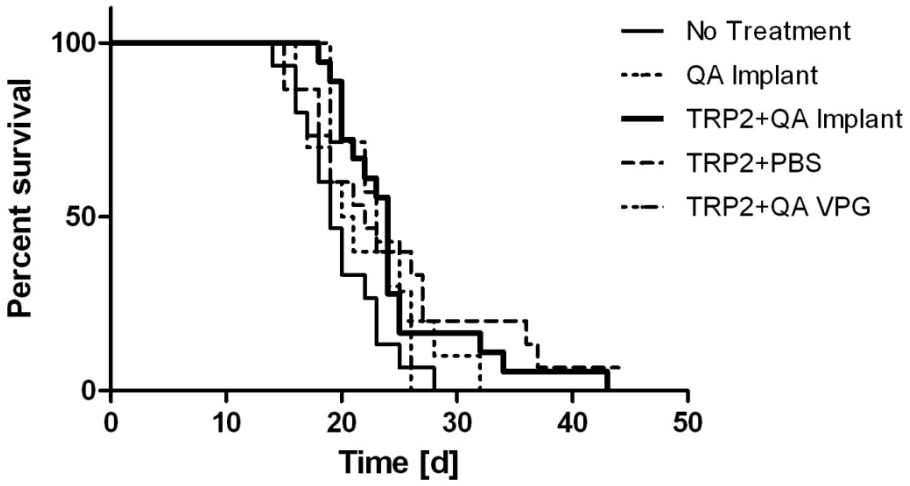

Figure 4-8: Tumour development in mice immunized with the TRP2-long peptide. Groups were inoculated on day 0 with B16-F10-luc2 tumour cells. On day six, groups were administered with formulations listed in Table 2. Tumour size was monitored with a digital calliper and mice were sacrificed when tumour size exceeded 150 mm^2.

The *in vitro* release of TRP2 from the implants showed that after 10 days, less than 2 % of TRP2 were released (Figure 4-6). In the *in vivo* experimental setup, animals were first injected with the B16F10luc2 melanoma cells s.c. into the flank. Six days later the formulations (implants, injections, VPGs) were given. Each animal, administered with a TRP2 formulation, received in total a dose of 56 μg TRP2. It appears that the TRP2+QA implants as well as the PBS+TRP2 injection group delayed the appearance of the tumour (Figure 4-9). Whether the mice received 56 μg TRP2 at once in the injection group or much lower amounts of TRP2 were released from the implants in combination with QA, seemed to have the same effect on the delay of tumour growth. In the TRP2+QA implant group tumours started to grow 17 days (mean value of all mice in this group) after tumour cells were injected, this is 11 days after the implants were given to the mice for experiment 1. For experiment 2, tumours started to grow 21 days (mean value of all mice in this group) after tumour cell injection in the TRP2+QA group, respectively 15 after the implants were given. The *in vitro* release of TRP2 out of the implants showed that after 11 days only 1.89% of the peptide was released (approximately 1.12 μg TRP2) (Figure 4-6).

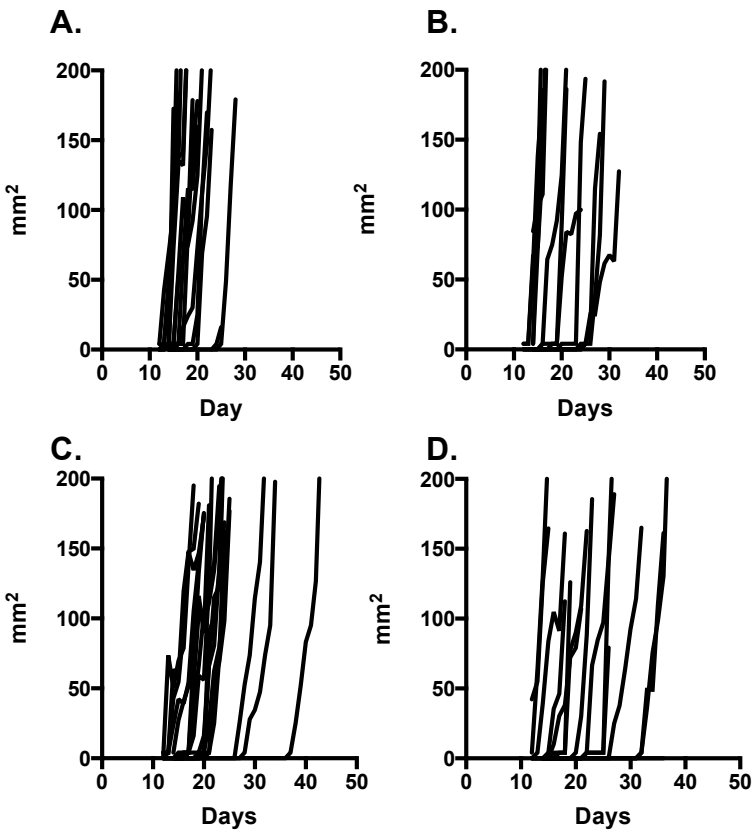

Figure 4-9: Size of tumours in mice given: **(A)** no treatment, **(B)** QA implants, **(C)** TRP2+QA implant or **(D)**TRP in PBS. Data are from individual mice.

This raises the question of the usefulness of the implants as a therapeutic vaccine or if a more appropriate use would be a prophylactic vaccine e.g. to prevent from metastases. As the *in vitro* data showed that the peptide release from the lipid implant is very slow, a prophylactic infectious disease model might be more appropriate. In the cancer setting TRP2+QA implants might be able to inhibit the growth of a tumour when a low tumour burden is present as an adjuvant therapy. For example patients having been treated with radiation or chemotherapy might be treated with TRP2+QA implants after this treatment to prevent the recurrence of tumours.

4.4 Conclusion

The process transfer from one extruder to another changed the physical properties as well as the release behaviour of the implants. A scale-down of an extrusion process by using a different device can be realized but only with engineering efforts. The lipid mixtures as well as the extrusion parameters have to be adapted to each extruder. The *in vivo* study showed that lipid implants containing TRP2 and QA were able to delay tumour growth for a short period of time, but the same retardation of tumour growth was observed for the TRP2+PBS injection group. However, the slow *in vitro* release of TRP2 gives room for a vision that such an implant vaccine could be effective in a prophylactic setting.

Chapter Five

Lipid-Peptide Interactions

5 Lipid-Peptide Interactions

The aim of **Chapter Four** was the preparation and the execution of an *in vivo* tumour study. Therefore, the TRP2 peptide, consisting of 23 amino acids, was incorporated into the implants followed by an *in vitro* investigation of the release from the implants. Against expectation, the TRP2 peptide showed a very slow release from the implants. Whereas in **Chapter Two**, the much bigger molecule OVA, showed a release duration of no more than nine days. The results of these two release studies let to the research performed in this Chapter. The assumption was that larger molecules should have more difficulties to diffuse out of the lipid matrix than small molecules, consequently showing a slower release than small molecules. But data collected in **Chapter Two** and **Chapter Four** indicate the opposite. Therefore **Chapter Five** aims to find an explanation for this, investigating possible lipid-peptide interactions, to be able to better predict drug release from lipid matrixes in the future.

5.1 Introduction

The previous chapter showed that the release of the TRP2 peptide from lipid implants is very slow. Compared to the model antigen OVA used for preliminary studies, the TRP2 peptide is a much smaller molecule (5 kDa compared to 44 kDa). As it should be easier for small molecules to diffuse out of the lipid implant through the pores and channels inside the lipid matrix, a faster TRP2 release compared to OVA was expected. Kreye *et al* (2011) showed that diffusion plays a major role in the release of molecules from lipid implants [171]. This chapter investigates the influence molecular weight of a molecule has on the release behaviour from lipid implants. Furthermore, the influence of the hydrophobicity of the molecule on the release behaviour was studied. These investigations were conducted in order to determine if peptide-lipid interactions were taking place and have an influence on the release behaviour from the lipid matrix. Reithmeier *et al* (2001) stated that the adsorption of peptides or proteins to the matrix material can be a critical factor in controlling the release kinetics [77]. Another factor influencing the release behaviour is peptide or protein aggregation inside of the controlled release device [172], caused by the interaction of the dissolving protein or peptide with the hydrophobic matrix, that might lead to unfolding of the protein or peptide [173]. Therefore, Reithmeier *et al* (2001) measured the adsorption of the peptides on the lipid matrix of their microparticles and connected an incomplete release to an absorption of the peptide on the matrix [77]. The interaction between proteins or peptides and solid lipid matrixes are poorly investigated so far, however many investigations on the interaction of peptides and lipid membranes have been conducted [174-176]. Almeida and Souto (2007) [15] stated that the interactions of the lipid matrix with lysozyme might have induced conformational changes of the lysozyme. In our study peptides of different molecular weight and hydrophobicity were incorporated into lipid implants. Their release was measured *in vitro* and a correlation between the release behaviour, their molecular weight and their hydrophobicity was tried to identify. Furthermore, the interaction between the lipid matrix and the peptides was investigated to conclude if they could be related to the release behaviour of the peptides.

5.2 Materials and methods

5.2.1 Materials

The TRP2 long peptide (SVYDFFVWLKFFHRTCKCTGNFA-OH) was purchased from peptides and elephants (Potsdam, Germany). Cholesterol (CHOL), purity 95%, was purchased from AlfaAesar (Karlsruhe, Germany). Soybean Lecithin (approx. 90% phosphatidylcholin) was purchased from APPLICHEM LIFESCIENCE (Darmstadt, Germany). Trp-Lys, Trp-Lys-Lys, Trp-Lys-Lys-Lys, Trp-Phe and Trp-Phe-Phe were purchased from Biomatik (Cambridge, Canada). Insulin was purchased from Sigma-Aldrich (Germany). Peptide 1 (P1) (SVYDFFVWLKFFHITCLCTGNFA–OH) and peptide 2 (P2) (SRYDKKRWLKKKHRTCKCTGNRA-OH) were purchased from GenScript USA Inc. (Piscataway, USA). Purified Quil-A (QA) was sourced from Brenntag Biosector (Frederikssund, Denmark), a lyophilised powder, was used as supplied. Dynasan 114 (D114) was kindly provided by SASOL Germany GmbH (Witten, Germany). Acetonitrile was purchased from VWR (France). Ultrapure deionised water having a conductivity of less than 0.055 μS/cm (Milli-Q Water systems, Millipore, MA, USA) was used throughout the study. All other chemicals were of analytical grade.

5.2.2 Preparation of lipid implants by twin screw extrusion

Implants were prepared from mixtures of soybean lecithin, CHOL, D114, with and without active ingredient and/or QA as described in **Chapter Four**. Shortly, soybean lecithin and D114 were transferred into a high-grade stainless steel beaker for milling in a swing mill Retsch CryoMill (Retsch Technology, Haan, Germany). After precooling the system with liquid nitrogen for 10 min at 5 Hz, soybean lecithin and D114 were ground for 1 min at 25 Hz. The obtained powder was mixed by hand, using a plastic mortar and pestle, with the remaining ingredients. The final mixture was then gradually blended with a mix of Active ingredient and QA and subsequently fed into a twin-screw extruder (small size extruder ZE 5, Three-Tec, Seon, Switzerland). The resulting implants had a diameter of 2 mm and were subsequently cut into lengths of 2.5 cm.

5.2.3 *In vitro* release from implants

Implants were cut into lengths of 2.5 cm and incubated at 37°C. Release from implants was performed in 2 mL Eppendorf vials containing 1.8 mL PBS (pH 7.4 or pH 4, 0.01 M, 0.05% NaN_3, 0.25% SDS) and was performed by complete buffer exchange in a Heidolph 1000 shaking incubator at 37 °C and 10 rpm/min. All samples were centrifuged at 14000 rpm (Mikroliterzentrifuge Z 160 M, Hermle Labortechnik, Wehingen, Germany) for 10 minutes to remove lipid particulates. Peptide concentrations were then quantified by Reversed-Phase HPLC (RP-HPLC) or UV metrically.

5.2.4 Adsorption Test

Peptides were incubated in 1.8 ml PBS (pH 7.4, 0.01 M, 0.05% NaN_3, 0.25% SDS) at 37°C with and without blank implants (implants containing only lipids). Four pieces of 0.5 cm long implants in each vial were used in this study. The peptide concentration was measured at pre-set time points by RP-HPLC.

5.2.5 Reversed-Phase HPLC (RP-HPLC) for peptide quantification

Peptides were quantified by RP-HPLC using a Dinoex Ultimate 3000 HPLC system (Dionex, Softron GmbH, Germering, Germany). A Phenomenex Jupiter 5u C4 300 Å column (250 mm x 4.60 mm, Phenomenex, Achaffenburg, Germany) and an injection volume of 25 to 150 µL of sample was used depending on the peptide. The running buffer consisted of Acetonitrile (0.1% TFA) and H_2O (10% Acetonitrile and 0.1% TFA), with a flow rate set to 0.75 mL/min.

5.2.6 UV-Metric quantification

OVA, lysozyme and insulin were detected by UV. The supernatant was measured by UV (Agilent Technologies 8453) at a wavelength of 280 nm. For each tested mixture an implant containing only lipids was used as a blank for the UV measurements. Each implant was weighted before the release and the total amount of protein present in each implant was calculated individually using a standard curve prepared by an 11-fold 1:1 dilution starting from a sample of 3 mg OVA, lysozyme respectively insulin in

1 mL PBS. All measured samples lay within the linear part of the standard curve (3 mg/mL – 5 μg/mL).

5.3 Results and discussion

5.3.1 Release of molecules with different molecular weight from lipid implants

Chapter Four showed that the TRP2 long peptide, having a molecular weight of 2.8 kDa, has a very slow and incomplete release behaviour. In this Chapter, interactions between peptide and lipid implants are investigated in order to try to better predict the release of a drug out of the lipid matrix. In **Chapter Two** and **Chapter Three**, the model antigen OVA, having a molecular weight of 44 kDa, was used for different studies. Initially, molecules of different molecular weight were chosen to study the influence molecular weight has on the release out of the lipid systems. Table 5-1 shows the molecular weight of the chosen molecules for this study, lying in between the molecular weight of OVA and the TRP2 long peptide.

Table 5-1: Molecules with their molecular weight, hydropathy, and isoelectric point.

Molecule	Molecular weight [kDa]	Isoelectric point	Hydropathy – Kyte Doolittle
Ovalbumin	44	4.54	0
Lysozyme	14.3	11.35	-0.43
Insulin	5.7	5.4	A Chain: 0.18 B Chain: 0.22

Figure 5-1 shows the release of OVA, lysozyme and insulin from lipid implants composed of 65% CHOL, 15% soybean lecithin and 20% D114. Implants contained 2% of OVA, lysozyme or insulin respectively, with and without 0.3% of the adjuvant QA. The first assumption implies that the diffusion of the molecule out of the pore and channel system insight the implant, plays a dominant role in the release kinetics as suggested by literature [171]. Therefore, it was assumed that molecules with a smaller molecular weight should diffuse faster out of the implant than molecules with a larger molecular weight. Opposite to the assumption made, insulin, having the smallest molecular weight, showed the slowest release behaviour, with a nearly linear release over a period of ten days. Lysozyme on the other hand, nearly three times as big as

insulin, showed a much faster release, of merely five days, half as long as the release of insulin (Figure 5-1). OVA was released over a period of ten days, showing a faster release than insulin during the first 150 hours. Even though insulin and OVA are both released over a duration of ten days, the release profile is different. Where for insulin we observed a nearly linear release, OVA showed a burst release which than slows than down after approximately 100 hours.

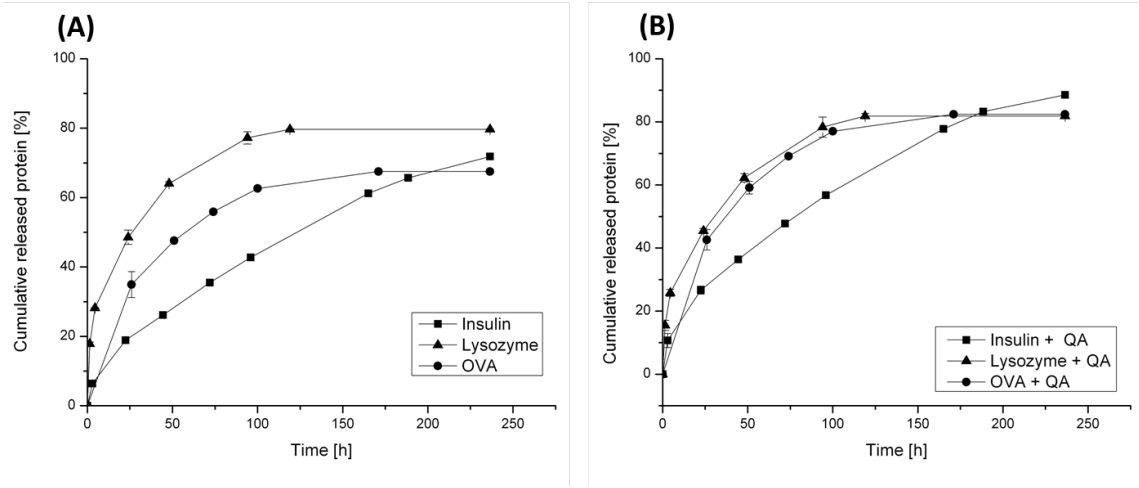

Figure 5-1: Cumulative release of molecules with different molecular weight from lipid implants (OVA, lysozyme and insulin are released). **(A)** without QA. **(B)** with QA. Data are the mean and SD of 3 independent replicates.

Chapter Two showed that the presence of QA in the implant formulation leads to a faster and more complete release of the model antigen OVA. This result was confirmed for OVA as well as for insulin. However, QA seems to have no influence on the release of lysozyme. In **Chapter Two** it was revealed that QA forms pores on the implant surface during release. This led to the assumption that the presence of these pores was responsible for a more complete OVA release when QA was in the formulation. Considering the fact that this is not true for lysozyme, the assumption arises that not only the pore formation, but also some interactions between QA and the released molecule are influencing the release behaviour of the latter. This could also be the reason why adverse reactions to QA VPGs in the *in vivo* study described in **Chapter Four** were more extensive than to QA+TRP2 VPGs. QA is known to have undesirable side effects [169]. It exhibits lytic activities, by reacting with the cholesterol in the cell membrane that can cause adverse reactions [170]. A decrease of the haemolytic activity

of QA was observed in the presence of CHOL blocking QA to interact with CHOL in the cell membranes. Possibly similar interactions took place between the TRP2 peptide and the QA, decreasing the toxicity of QA in the TRP2+QA VPGs. These results indicate that the chemical and physical characteristics of a molecule influence the diffusion out of the implants rather than their size.

Since no direct relation between the size and the release behaviour could be identified, the isoelectric point (IEP), hence the net charge, of the molecules at the release buffer pH 7.4 was taken into consideration (Table 5-1). OVA and insulin are negatively charged, lysozyme positively. At first sight, it appears that the negatively charged molecules are released over a longer period of time. However, to confirm or refute this, further molecules have to be considered.

5.3.2 Interaction of peptides of different hydropathy and molecular weight with lipid implants

Since the size gave no indication about the release behaviour out of lipid implants, peptides with different hydropathy were chosen to investigate the relation between release rate and hydropathy (Table 5-2). The Kyte-Doolittle hydropathy scale was used, taking into consideration the hydrophilic and hydrophobic properties of each of the 20 amino acid side-chains [177]. A hydropathy < 0 corresponds to hydrophilic molecules, a hydropathy > 0 to hydrophobic molecules. In a first step, the release of small peptides was analysed (Table 5-2, peptides 1 to 5) to comprehend if there is a relation between the release behaviour and the hydropathy of the peptide.

Table 5-2: Peptides, their hydropathy, molecular weight and isoelectric point.

Nbr	Peptide	Hydropathy – Kyte Doolittle	Molecular weight [Da]	Isoelectric point
1	Trp-Lys-Lys-Lys	-3.16	588.73	10.84
2	Trp –Lys-Lys	-2.9	460.56	10.1
3	Trp –Lys	-2.4	332.39	10.1
4	Trp –Phe	0.95	351.4	6.01
5	Trp –Phe-Phe	1.56	498.5	6.01
6	P2	-1.99	28882.4	11.15
7	TRP2 long peptide	0.25	2834.31	9.02
8	P1	0.9739	2777.27	7.16

Figure 5-2 shows the release of these peptides from lipid implants composed of 65% CHOL, 15% soybean lecithin and 20% D114. Implants contained 2% peptide and 0.3% of QA. The release rate from the implants did not show a correlation with the hydropathy of the peptide. However, Figure 5-2 shows that there is a difference in the shape of the release curves comparing hydrophobic and hydrophilic molecules. The release curve of Trp-Phe and Trp-Phe-Phe showed steep slopes at the beginning of the release, flattening down after two to three days. The curves of the hydrophilic peptides (Trp-Lys, Trp-Lys-Lys and Trp-Lys-Lys-Lys) on the other hand showed a steep slope at the beginning and nearly all the peptide was already released after two days. This might be an indication that the hydropathy does not influence the amount of peptide that is released but the way it comes out of the implant (i.e. with a burst, linear…). Trp-Phe and Trp-Phe-Phe present the same release speed and duration, however, after nine days a complete Trp-Phe was observed, but merely 50% of Trp-Phe-Phe was released.

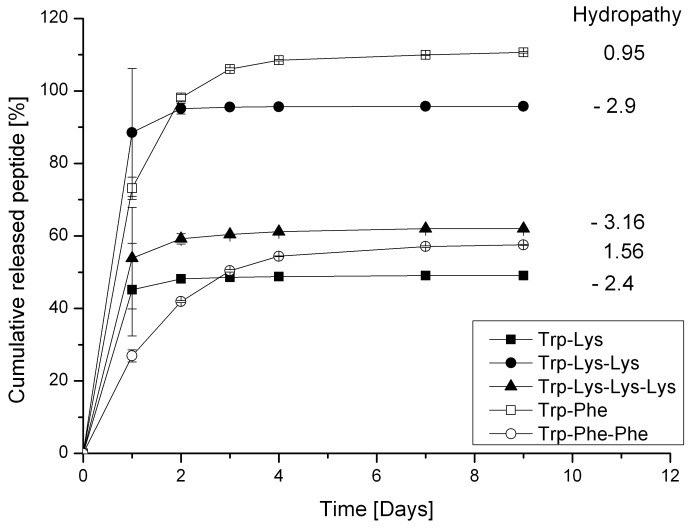

Figure 5-2: Cumulative release of peptide with different hydropathy from lipid implants (at pH 7.4). Data are the mean and SD of 3 independent replicates.

In a next step, the adsorption of the different peptides onto lipid implants was investigated. Protein aggregation inside the controlled release device as well as peptide interactions with the hydrophobic lipid matrix can have an influence on the release [77,172,173]. Peptides (Table 5-2, peptides 1 to 5) were incubated at 37°C in PBS for

different time frames, with and without blank implants (implants containing only lipids).

Figure 5-3 presents the changes in peptide concentration over time for the different samples. Figure 5-3 confirms that when blank implants were present in the sample, the decrease in peptide concentration over time was greater than in the absence of implants. By comparing Figure 5-3 A and B, it becomes clear that in the presence of implants, the concentration of peptides with a hydropathy > 0 decreased much faster than for peptides having a hydropathy < 0. As expected, hydrophobic peptides adsorbed much faster to the implants, showing the important role hydropathy plays. The concentration of Trp-Phe decreased to 73% after seven days. For Trp-Phe-Phe an even more substantial alter in concentration could be observed, after seven days merely 40% of the peptide´s initial concentration were measurable in the PBS buffer. In samples containing no lipid implants, the concentration decreased no more than 12%, even after 7 days incubation, whether the peptide was hydrophilic or hydrophobic did not make a difference.

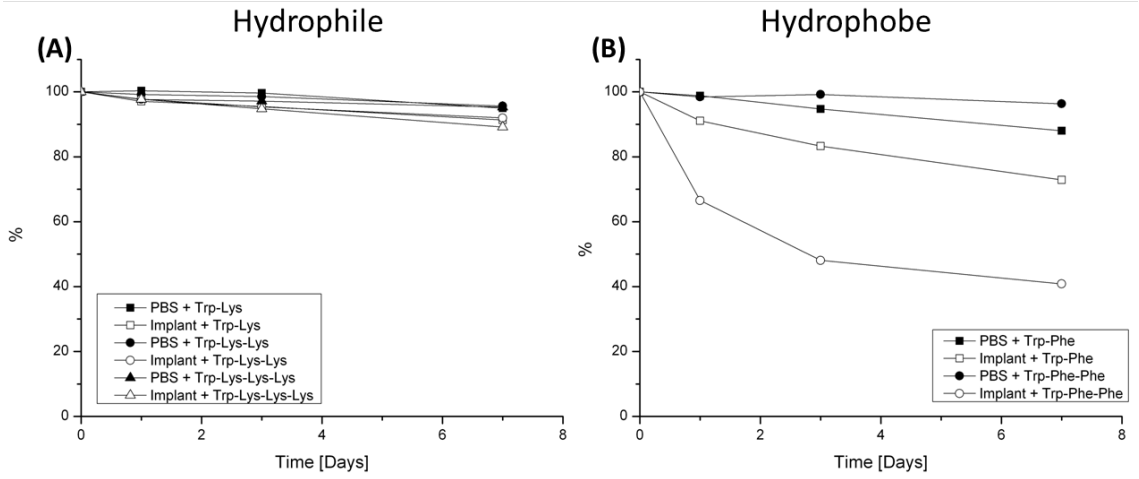

Figure 5-3: Indicates the percentage of peptide present in PBS for different peptides (Table 5-2, peptides 1 to 5) after different incubation durations with and without blank implant present in the sample. **(A)** Peptides with a hydropathy < 0. **(B)** Peptides with a hydropathy > 0. Data are the mean and standard deviation (SD) of 3 independent replicates.

All peptides used in the previous study were short peptides, but all of different molecular weight. Therefore, two peptides of the same molecular weight as the TRP2 long peptide, used in **Chapter Four**, were analysed (Table 5-2, peptide P1 and P2). P1 is

more hydrophobic and P2 more hydrophilic than the TRP2 peptide. The same experiment as described previously was performed; peptides were incubated in PBS with and without blank implants. Figure 5-4 shows the results of this study. After only one day incubation, there was no more TRP2 measurable in the sample containing the blank implant. The same holds true for the P2 peptide. A different behaviour was observed for the P1 peptide, the concentration decreased gradually, showing a decrease of 30% after one day, going further down to a remaining concentration of 14% P1 after seven days incubation with lipid implant in the sample. This was the opposite of what was expected considering the results from the previous study (Figure 5-3), as P2 is more hydrophilic than P1. The concentrations in the samples containing only PBS on the other hand decreased only slightly (no more than 10% over seven days) for the three studied peptides. This result is consistent with the results from the experiment performed with the short peptides.

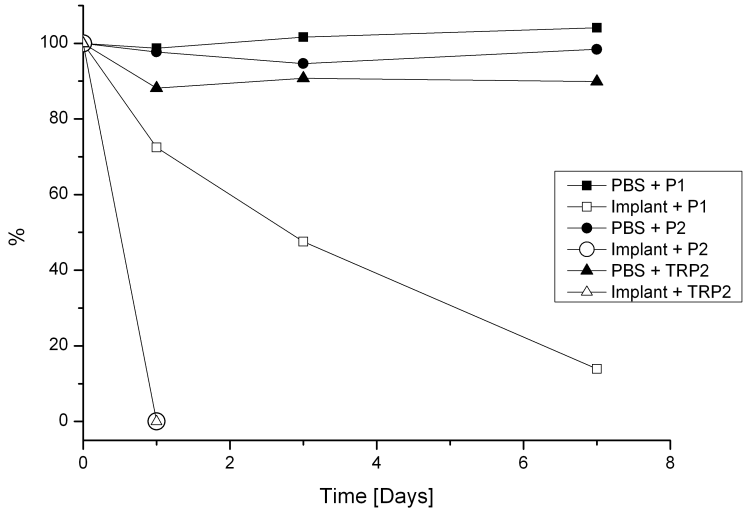

Figure 5-4: Percentage of peptide present in PBS for different peptides (Table 5-2, peptide P1, P2 and TRP2) after incubation up to seven days with and without blank implant present in the sample. Data are the mean and SD of 3 independent replicates.

Since the two studies led to conflicting results concerning the absorption behaviour onto the implants in relation with the hydropathy, peptides of the two studies were compared. Considering the hydropathy of peptide P1 and P2, it can be noticed that the hydropathy value of P1 is close to the value for Trp-Phe, the same can be observed for

P2 and Trp-Lys. The main difference between the two peptides in each pair is the molecular weight. Figure 5-5 shows the data for those four peptides. Even though the hydropathy is the same, the behaviour of the peptides was different. These results indicate that neither the hydropathy nor the size alone influenced the behaviour of the peptides when incubated together with lipid implants. The fact of how fast a peptide adsorbs on the lipid implants correlated well with the hydropathy value for short peptides (two to four amino acids). This correlation could not be observed for longer peptides. The folding of the peptide might also play a role, and should be investigated. Conformational changes of lysozyme due to interactions with a lipid matrix have been stated by Almeida and Souto (2007) [15]. Therefore, the conformation of the peptides should be further analysed. The correlation for shorter peptides was maybe very good because there are not many possible ways for them to fold, which is not the case for lager peptides.

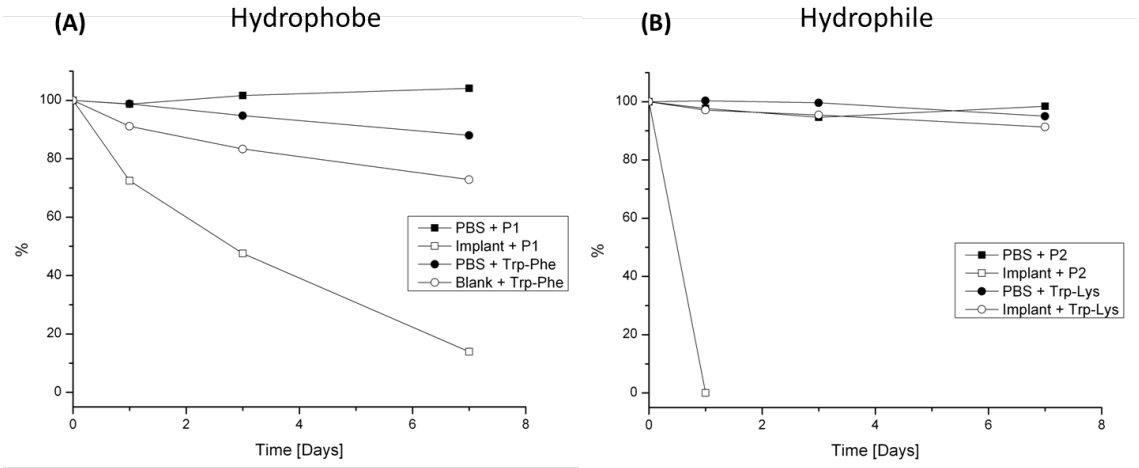

Figure 5-5: Indicates the percentage of peptide present in PBS for different peptides (Table 5-2, peptides 3,4,6 and 8) after different incubation durations with and without blank implant present in the sample. **(A)** Peptides with a hydropathy > 0. **(B)** Peptides with a hydropathy < 0. Data are the mean and SD of 3 independent replicates.

5.3.3 Interaction of peptides with pure lipids

As the implants consist of a mixture of CHOL, D114 and soybean lecithin, peptides TRP2, P1 and P2 were incubated with each of the lipids individually, in order to see the effect each lipid has on peptide concentration. Figure 5-6 shows the change in concentration of each peptide over time upon incubation with the different lipids. The

data clearly showed that all three peptides strongly interacted with cholesterol. After one day, an important concentration decrease was noticed for all three peptides.

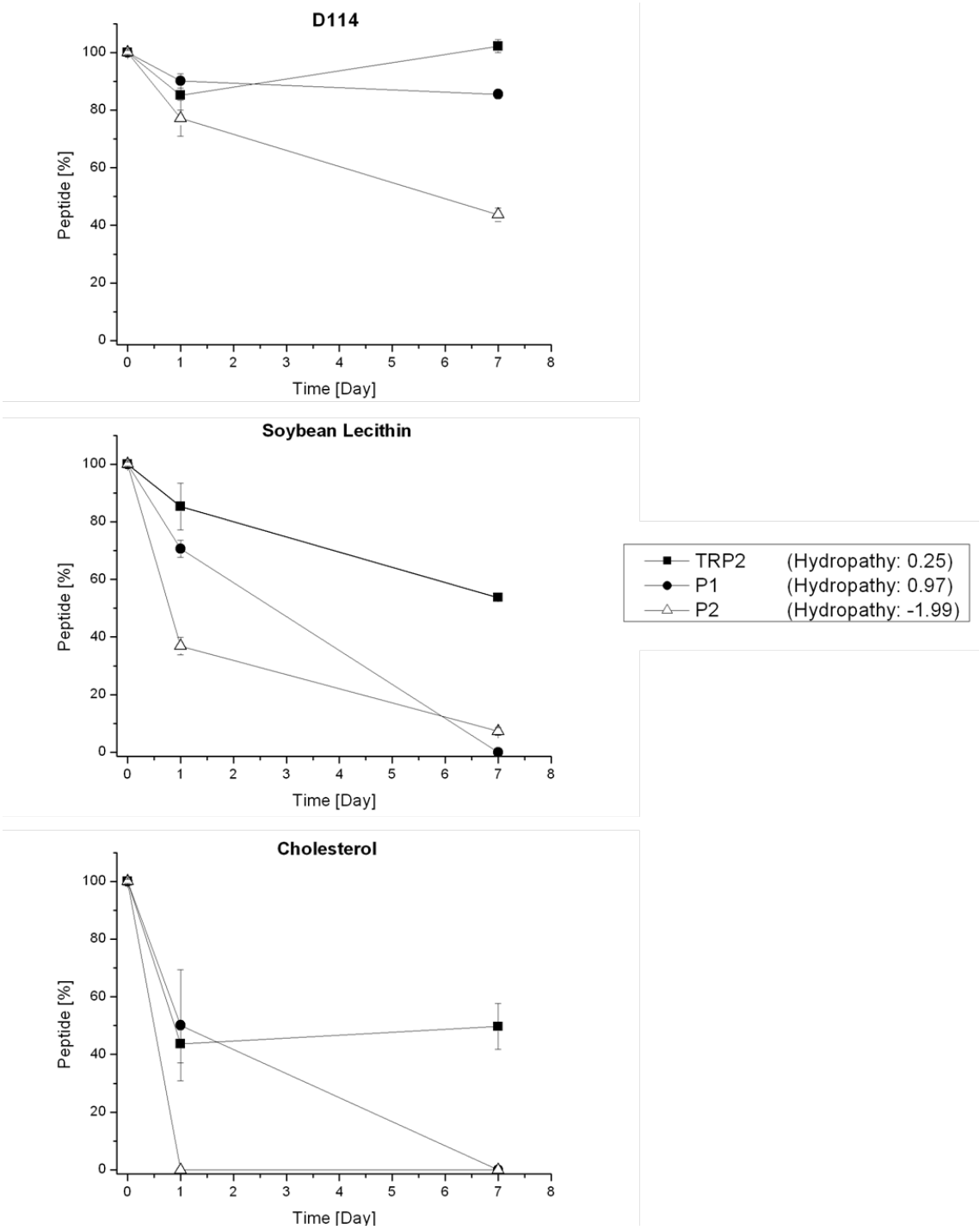

Figure 5-6: Indicates the percentage of peptide incubated in PBS at 37°C together with either, D114, soybean lecithin or CHOL, for different peptides (TRP2, P1 and P2) at day 0, after 1 day and after 7 days. Data are the mean and SD of 3 independent replicates.

Incubated together with cholesterol, already after one day, there was no P2 measurable in the sample anymore. The same was observed for P1 after seven days. TRP2 reacted least with cholesterol, after seven days there were still 50% of the initial TRP2 concentration measurable.

All three peptides interacted also with soybean lecithin, already after day one, a decrease in concentration could be determined. According to the results of this study, peptides interacted least with the D114. One assumption is that as cholesterol and lecithin are amphiphilic molecules, there is more room for interaction than with the D114. Furthermore, lecithin is known to form lamellar structures, and some of the peptide could be enclosed into micelles or liposomal structures when incorporated together with lecithin. The interaction with all three lipids was strongest for the peptide P2, even though P2 is the most hydrophilic of the three analysed peptides. These results confirmed the data obtained from the previous study, showing that the interaction between the lipids and the larger peptides is not in direct correlation with the hydropathy of the considered peptide.

5.4 Conclusion

This chapter aimed to investigate the potential influence size and hydropathy have on the release out of lipid implants. It was demonstrated that not size alone plays a role in the release rate of a molecule from the lipid matrix. Besides the size, the hydropathy of a peptide in relation with its release was also analysed. No direct relation between the hydropathy and the release rate was identified for peptides. Merely the release profile showed similarities for peptides of the same hydropathy whether they were hydrophilic or hydrophobic. Furthermore, the role that the adjuvant plays in the release has been reweighed. It appeared that not only the pore former quality of QA has to be considered, but also the possibility of interactions between the peptide and the adjuvant should be considered. When examining the adsorption of peptides onto the lipid implants, there was a nice correlation, indicating that hydrophobic peptides adsorbed faster than hydrophilic peptides. However, this was only true for short peptides. When analysing peptides of higher molecular weight (P1, P2 and TRP2) the opposite was observed. These results led to the conclusion that neither the size nor the

hydropathy of the peptides allow to predict the release behaviour or the interactions with the implant. It is assumed that the folding of the molecules might play an important role and should be investigate. Furthermore, a broader range of molecules has to be investigated to identify which role the charge of the molecule plays in the release behaviour.

Chapter Six

Summary and future work

6 Summary and future work

As discussed in **Chapter One**, the delivery of subunit vaccines in a sustained manner results in most cases in a better immune response than given by one or multiple booster injections. Furthermore, sustained delivery systems allow to reduce the number of immunisations and thereby make the need for multiple administrations redundant. Different delivery systems, consisting of different materials, have been discussed, each presenting its own advantages and disadvantages. However, an optimal system has not been found jet, showing the need to carry on the research in this area, as new, optimized delivery systems are essential for the successful use of subunit vaccines in the future.

The work presented in this thesis investigated the use of lipid implants as systems for the sustained delivery of subunit vaccines. They appear to be well tolerated in a mouse model and were able stimulate antibody production as well as T cell expansion. The production of these lipid systems by twin-screw extrusion makes the production of large batch sizes possible within a small amount of time. These lipid systems are promising candidates for the future of sustained vaccine delivery.

The successful preparation by direct compression and the immune response stimulated by such lipid implants was described in literature [28,29]. However, the preparation of lipid implants for vaccine delivery by twin-screw extrusion has not been investigated before. In **Chapter Two** the search for an extrudable mixture was described as well as the important parameters for the extrusion process. Different extrudable mixture were identified and the release behaviour of the model antigen OVA was investigated. It was shown that the composition of the lipid formulation influenced the release of OVA. Particularly the amount of cholesterol in the mixture affected the release of OVA, showing that higher amounts of cholesterol in the formulation led to a slower OVA release. In the investigated implant systems, the adjuvant QA was used, as previous studies indicated that it proves to be the most effective adjuvant for these systems [29,178]. In **Chapter Two** it was shown that the presence of the QA in the formulation influenced the release of OVA from the implants. When QA was present, OVA release was faster and more complete. SEM micrograms showed that during the release, QA

forms pores on the implant surface, explaining why the OVA release is more complete when there is QA in the system. Moreover, the treatment of implants by heat after extrusion made it possible to slow down the OVA release from the implants up to 14 days, compared to a release duration of seven days without the curing step. Furthermore, the release of QA from the implants was investigated, as literatures showed that an optimal immune response could be achieved when the antigen and adjuvant are released together [128]. Results indicated that QA is released over a period of 12 days from our implants, thereby longer than OVA was released. This implies that as long as OVA is released, there is also adjuvant released from the implants, increasing the chance that a strong immune response can be trigged by the investigated implants. What was observed for the release of OVA in presence of QA holds also true for the release of the adjuvant. The presence of OVA in the formulation enhances the release of QA from the implants. Thus, **Chapter Two** proved that lipid implants can be produced by twin-screw extrusion and that a reasonable antigen and adjuvant release from the implants could be obtained. As the lipids undergo thermal and mechanical stress during the extrusion process, the polymorph state of the lipids was investigated in **Chapter Three.** Neither the extrusion process, nor storing the implants did change the polymorphism of the lipids. Also the mechanical strength of the implants stayed stable during storage. However, when investigating the release of implants stored over several months, a change in the release of OVA was observed. The longer the implants were stored, the shorter and less complete OVA was released. This observation could not be correlated with changes in polymorphism or melting energy in the implants. The assumption that interactions between OVA and the lipid matrix takes place occurred. This should be further investigated by trying to investigate the state OVA is in when released from the implants. Another explanation could be the rearrangement of the lipid matrix during storage.

To investigate the compatibility of the produced implants, an *in vivo* study was conducted in a mouse model. The *in vivo* release of OVA was studied and the advantage of depot versus double shot was investigated. **Chapter Three** shows the data of these studies. The *in vivo* release correlated nicely with the *in vitro* data, and was about seven days long. Antibody response as well as specific T cell proliferation

was comparable for implants containing QA and the injection group. These results indicate the importance of the adjuvant in such vaccine delivery systems. Implants showing an OVA release of seven days resulted in the same immune response than two injections given at day one and day 14. An interesting future approach would be to test the cured implants *in vivo* and compare them to double OVA shots, as the OVA release *in vitro* from the cured implants is double as long as from the implants investigated in the *in vivo* study presented in **Chapter Three**. Furthermore, cytokine secretion was analysed to examine antigen-specific effector function. The results indicated that a Th1 response was observed for the groups receiving QA containing implants.

Considering the promising results obtained from the *in vivo* studies while using the model antigen OVA, **Chapter Four** investigated the use of these implant systems in tumour therapy in a mouse model. For this purpose a non-mutated melanoma-associated antigen, namely the TRP2 peptide was incorporated into the implants. First the production device had to be changed to a small size extruder, enabling the production of small batch sizes. The characteristics of implants produced by the two different extruders were compared. Results clearly showed that a transfer from one extruder device to another couldn't be performed without adjusting the extrusion parameters and lipid formulation, if the same implant characteristics are to be maintained. Implants and VPGs containing QA and TRP2 were produced to perform an *in vivo* tumour growth study. VPGs were considered an interesting alternative for our experiment as numerous studies describe the use of liposomal systems as TRP2 carriers [160,162,163]. *In vitro* release of TRP2 turned out to be slow and incomplete from both systems, VPGs and implants. *In vivo*, the two systems were compared to a no-treatment group and one group of mice receiving TRP2 in PBS injections. There was a statistical difference between the TRP2+QA implants and the no treatment group. On the other hand, the TRP2 in PBS injection achieved the same results as the TRP2+QA implants. VPGs were not well tolerated by the mice and were therefore considered as a non-suitable system. Most likely the adverse reactions are due to the QA, as QA is known to be have undesirable side effects [169]. In our study, the VPG contained no cholesterol while the implants, which contain cholesterol, were well tolerated with no

swelling, redness or skin irritation observed. The CHOL present in the implants probably interacts with the QA, thereby withdrawing the toxicity of QA. To confirm this assumption, VPGs containing no QA would have to be administered to the mice. Furthermore, adverse reactions were less severe for VPGs containing TRP2+QA than for VPGs containing only QA. We suppose that some interactions between the peptide and the QA might take place, thereby decreasing the toxicity of QA. Therefore, the interaction between the peptide and QA should also be investigated. Also testing the VPGs for their endotoxin level should be considered before deciding if VPGs are adequate delivery systems or not. Another interesting question arising from this *in vivo* study is related to the results showing that TRP2+QA implants have the same effect as the TRP2+PBS injections. Both formulations were able to delay tumour growth. A total dose of 56 μg TRP2 was administered to each animal. In the injection group mice received 56 μg TRP2 at once whereas much lower amounts of TRP2 were released from the TRP2+QA implants. The *in vitro* release of TRP2 out of the implants showed that at the moment tumours were starting to grow; only 1.89% of the peptide were released (approximately 1.12 μg TRP2). This brings us to reconsider the implants as a therapeutic vaccine system. A prophylactic use of the implants as tumour vaccine might be more appropriate. In the cancer setting TRP2+QA implants might be able to inhibit the growth of a tumour when a low tumour burden is present. To investigate the future use of these implants, first the release of TRP2 *in vivo* should be analysed. In a next step, a prophylactic *in vivo* study would have to be performed, administering the implants first and afterwards the tumour cells.

In **Chapter Five** the interactions of peptides and lipid implants were investigated in order to better predict the release of a drug from the lipid matrix. The slow and incomplete release observed for TRP2 raised questions about what drug characteristics influence the release out of the lipid matrix. Up to present, the common knowledge was that diffusion plays a major role in the release of molecules from lipid implants [171]. Consequently, larger molecules should be released slower than smaller ones. Though our results showed that OVA was released much faster than TRP2, which is a much smaller molecule. This observation led to the assumption that maybe some interactions between the molecule and the lipid matrix take place, influencing the

release of a molecule, rather that the size of the molecule. Literature suggests that another factor influencing the release behaviour is peptide or protein aggregation inside of the controlled release device [172], caused by the interaction of the dissolving protein or peptide with the hydrophobic matrix that might lead to unfolding of the protein or peptide [173]. This is a really important point, which should be investigated, in order to try and predict the release of a molecule from a lipid implant system. To learn more about the interactions between lipid implants and peptides, we investigated the adsorption behaviour of peptides, characterized by different hydropathies, on the lipid matrix. No direct correlation between size, hydropathy and the release behaviour could be identified. Only when considering short peptides, a correlation between the hydropathy and the adsorption behaviour on lipid implants could be identified. But this was not true for larger peptides. We assume that the interactions between peptides and the lipid implants are dependent on the folding of the peptides. This should be investigated by analysing the folding of peptides of different hydropathie and different size before and during incubation with and without lipid implants present. Including peptide folding could offer valuable clues on the release behaviour of peptides from lipid implants.

Another controversial subject is the size of the implants. The implants used in our studies had a cylindrical shape with a diameter of 2 mm. The length of the implants was varying, using for example a size of 5 mm for the *in vivo* studies. The question arises if a smaller size should be used for further investigations. Table 6.1 shows some of the implants approved for human use as well as their size. Implanon® and Zoladex®, implants applied s.c., they are both longer than the implants used in our *in vivo* study,except for Zoladex® having a smaller diameter than implants in our study. Intravitreal implants seem to be smaller in size than our systems, but Retisert® for example is also 5 mm long and has a width of 2 mm. Compared to what is on the market, the implants analysed in this work seem to have a reasonable size for s.c. application. However, smaller sized implants, resulting in the use of a smaller trocar for application, would of course increase patience compliance and make implants an even more attractive delivery system. Therefore, the investigation of smaller sized implants should be considered when carrying on studies with implants as sustained

delivery systems. In case that the implants would be used at some point in veterinary medicine, these size considerations would be of less importance.

Table 6-1: Examples of implants approved for human use and their size

Name	Active component	Application	Size
Implanon®(Nourypharma GmbH)	etonogestrel	s.c.	d: 2 mm l: 4 cm
Zoladex® (Zeneca)	goserelinacetate	s.c.	d: 1 mm l: 1.5 cm
Retisert®(Bausch and Lomb)	fluocinolone acetonide	intravitreal	l: 5 mm w: 2 mm h: 1.5 mm
Ozurdex® (Allergan)	dexamethasone	intravitreal	d: 0.45 mm l: 6.5 mm
Iluvien® (Alimera)	fluocinolone acetonide	intravitreal	d: 0.37 mm l: 3.5 mm

* d: diameter, l: length, w: width, h: height

The work conducted in this thesis showed that lipid implants are an interesting candidate for sustained vaccine delivery. But the results also indicated that at the moment there are no reliable criteria to predict the release behaviour from such lipid systems. Lipid-peptide interactions seem to play a major roll. It would be of great interest to further investigate these interactions to gain better understanding of the role they play in the peptide release behaviour from lipid implants.

In conclusion, biodegradable lipid implants produced by twin-screw extrusion show great promise as sustained vaccine delivery systems. They show good biocompatibility, good biodegradability and producing them by twin-screw extrusion easily enables the production of large batch sizes, therefore their development should be further perused.

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Appendix

8 Appendix

8.1 Appendix A

Solutions used in this thesis

Solutions for in vitro release studies

PBS pH 7.4

$Na_2HPO_4 * H_2O$	1.44 g
KH_2PO_4	0.2 g
NaCl	8 g
KCl	0.2 g
NaN_3	0.5 g
Distilled water to	1000 mL

Solutions used for Enzyme-linked Immunosorbent Assays (ELISAs)

0.1 M Carbonate-Bicarbonate buffer pH 9.6

Sodium carbonate (Na_2CO_3)	0.318 g
Sodium hydrogen carbonate ($NaHCO_3$)	0.586 g
Milli-Q water qs to	200 mL
Adjust pH to 9.6	

(This buffer was also used for coating of plates with anti-CD3)

Wash Buffer

Phosphate buffered saline	1000 mL
Tween 20	500 μL
Adjust pH to 7.4	

Blocking Buffer

Phosphate buffered saline	200 mL
Bovine serum albumin (BSA)	4 g
Adjust pH to 7.4	

Assay Buffer

Wash Buffer	200 mL
Bovine serum albumin (BSA)	1 g
Adjust pH to 7.4	

Cell culture work

Lysis Buffer

(A) 0.16 M Ammonium chloride (NH_4Cl) solution

Ammonium chloride	8.29 g
Milli-Q water to	1000 mL
Adjust pH to 7.4	

(B) 0.17 M Tris hydrochloride (Tris-HCl) solution

Tris-HCl	20.6 g
Milli-Q water to	1000 mL
Adjust pH to 7.65	

Mix 9 parts of solution A with 1 part of solution B, filter sterilise through a 0.22 μm filter prior to use.

Fluorescence-activated cell sorting (FACS) buffer

Sodium azide (NaN_3)	0.1 g
Bovine serum albumin (BSA)	10.0 g
PBS (pH 7.5) to	1000 mL

Complete Iscove's Modified Dulbecco's Medium (cIMDM)

Penicillin/Streptomycin solution	10.0 mL
2-mercaptoethanol	1.0 mL

Foetal calf serum (FCS)	50.0 mL
Glutamax	10.0 mL
IMDM to	1000 mL

Complete RPMI Medium

Sodium carbonate (Na_2CO_3)	2 g
D-glucose	4.5 g
Penicillin/Streptomycin solution	10.0 mL
Foetal calf serum (FCS)	100.0 mL
Glutamax	10.0 mL
Sodium Pyruvate	10 mL
RPMI to	1000 mL

8.2 Appendix B

Scanning electron micrographs of the surface of cured implants.

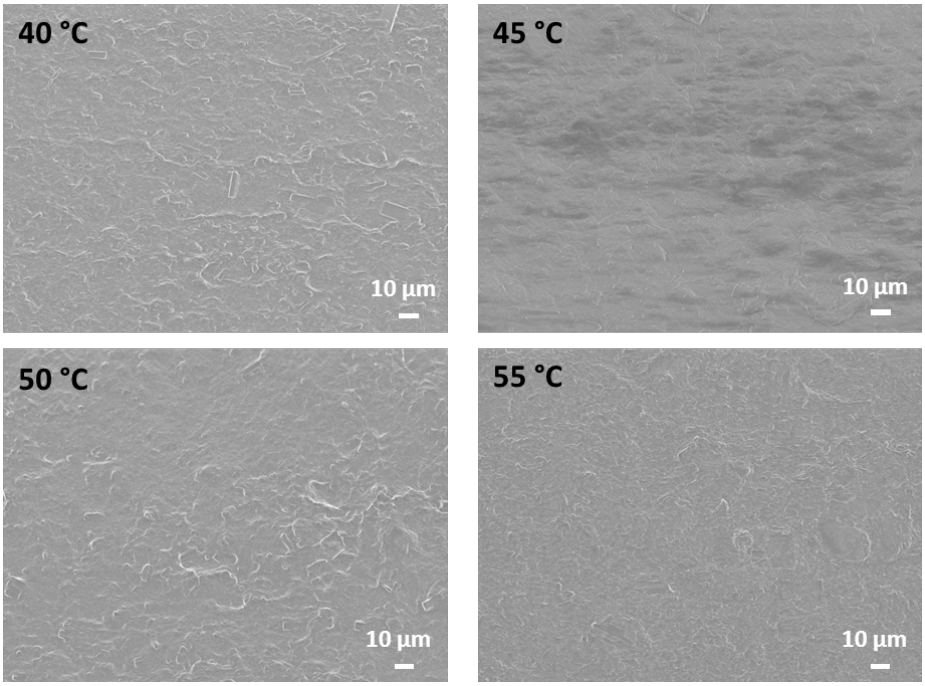

Figure B - 1: Scanning electron micrographs obtained from lipid implants after curing. at 40°C, 45°C, 50°C respectively 55°C. Scale bar = 10 µm. Magnification 500 x.

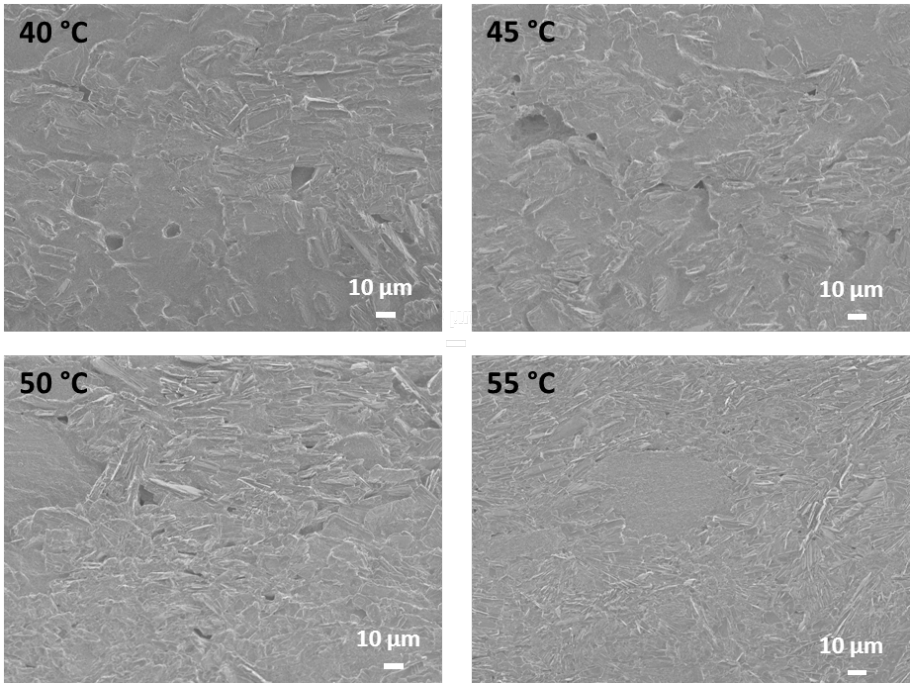

Figure B - 2: Scanning electron micrographs obtained from blank lipid implants after 14 days in PBS buffer (pH 7.4) at 37°C. Implants were cured at 40°C, 45°C, 50°C respectively 55°C. Scale bar = 10 µm. Magnification 500 x.

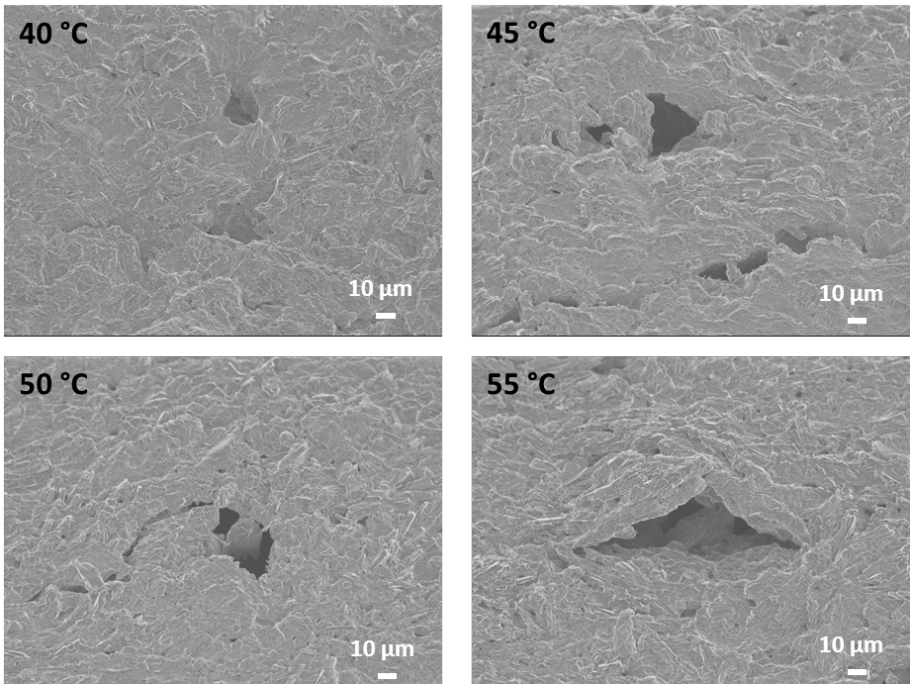

Figure B - 3: Scanning electron micrographs obtained from OVA+QA lipid implants after 14 days in PBS buffer (pH 7.4) at 37°C. Implants were cured at 40°C, 45°C, 50°C respectively 55°C. Scale bar = 10 µm. Magnification 500 x.

8.3 Appendix C

Additional data from the *in vivo* study described in Chapter Three

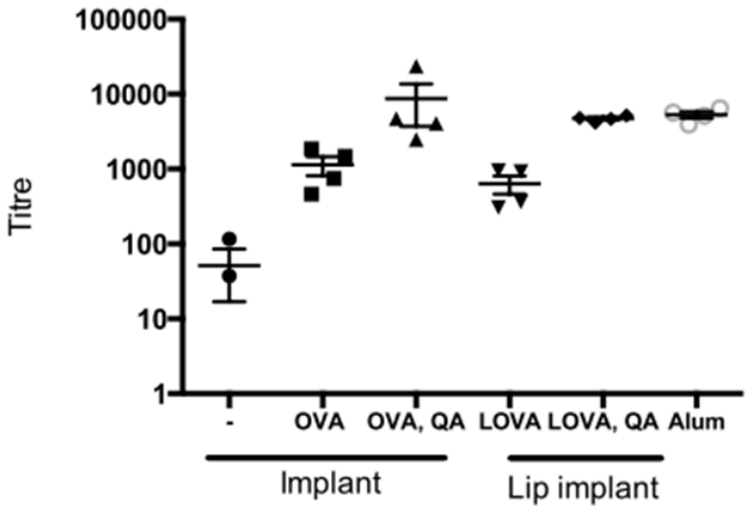

Figure C - 1: OVA-specifc IgG antibody titres determined by ELISA on day 28. Mice were immunized with blank implants (- Implant), implants containing OVA or OVA/QA either incorporated directly into the lipid mix (OVA Implant, OVA/QA Implant) or formulated into liposomes which were then freeze-dried and incorporated into the lipid mix (Lip Implant) or with OVA in alum (Alum). Data shown are the individual results from 4 mice per group and the mean and SEM.

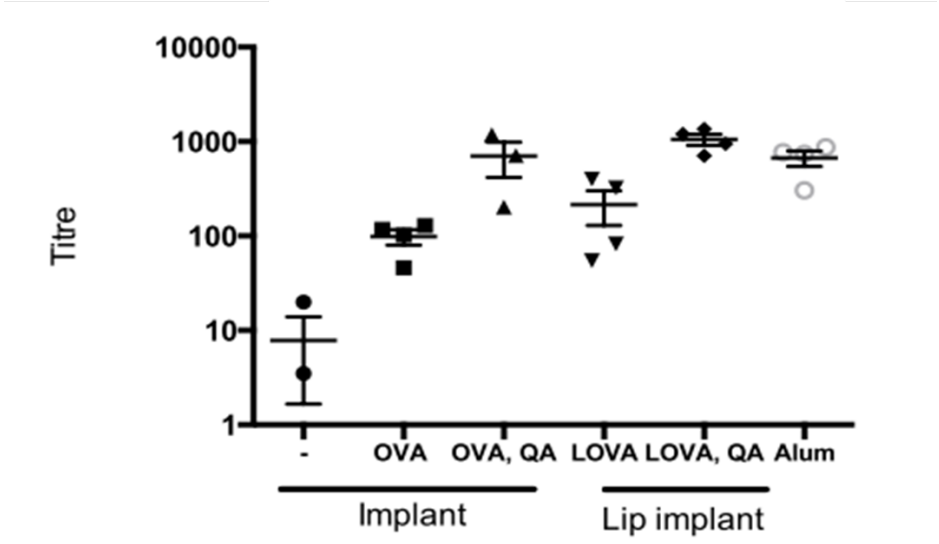

Figure C - 2: OVA-specifc IgG antibody titres determined by ELISA on day 28. Mice were immunized with blank implants (- Implant), implants containing OVA or OVA/QA either incorporated directly into the lipid mix (OVA Implant, OVA/QA Implant) or formulated into liposomes which were then freeze-dried and incorporated into the lipid mix (Lip Implant) or with OVA in alum (Alum). Data shown are the individual results from 4 mice per group and the mean and SEM.

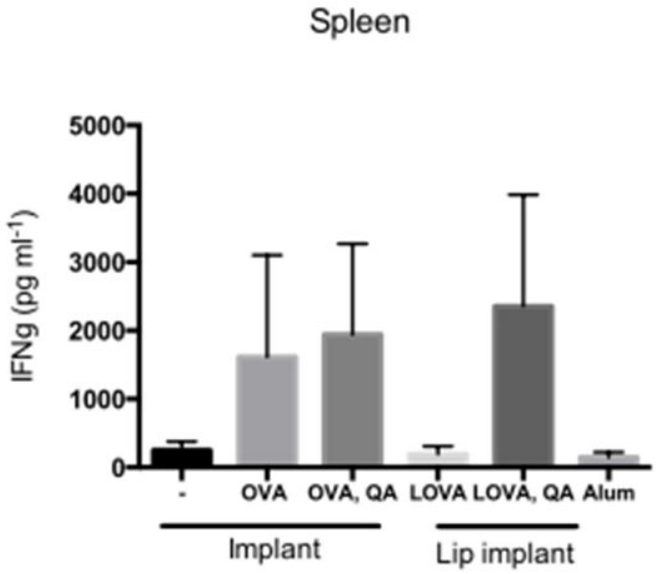

Figure C - 3: Interferon-γ concentrations for spleen samples restimulated in vitro with OVA. Mice were immunized with blank implants (- Implant), implants containing OVA or OVA/QA either incorporated directly into the lipid mix (OVA Implant, OVA/QA Implant) or formulated into liposomes which were then freeze-dried and incorporated into the lipid mix (Lip Implant) or with OVA in alum (Alum). Data shown are the mean and SEM from 4 mice per group from 3 independent experiments.

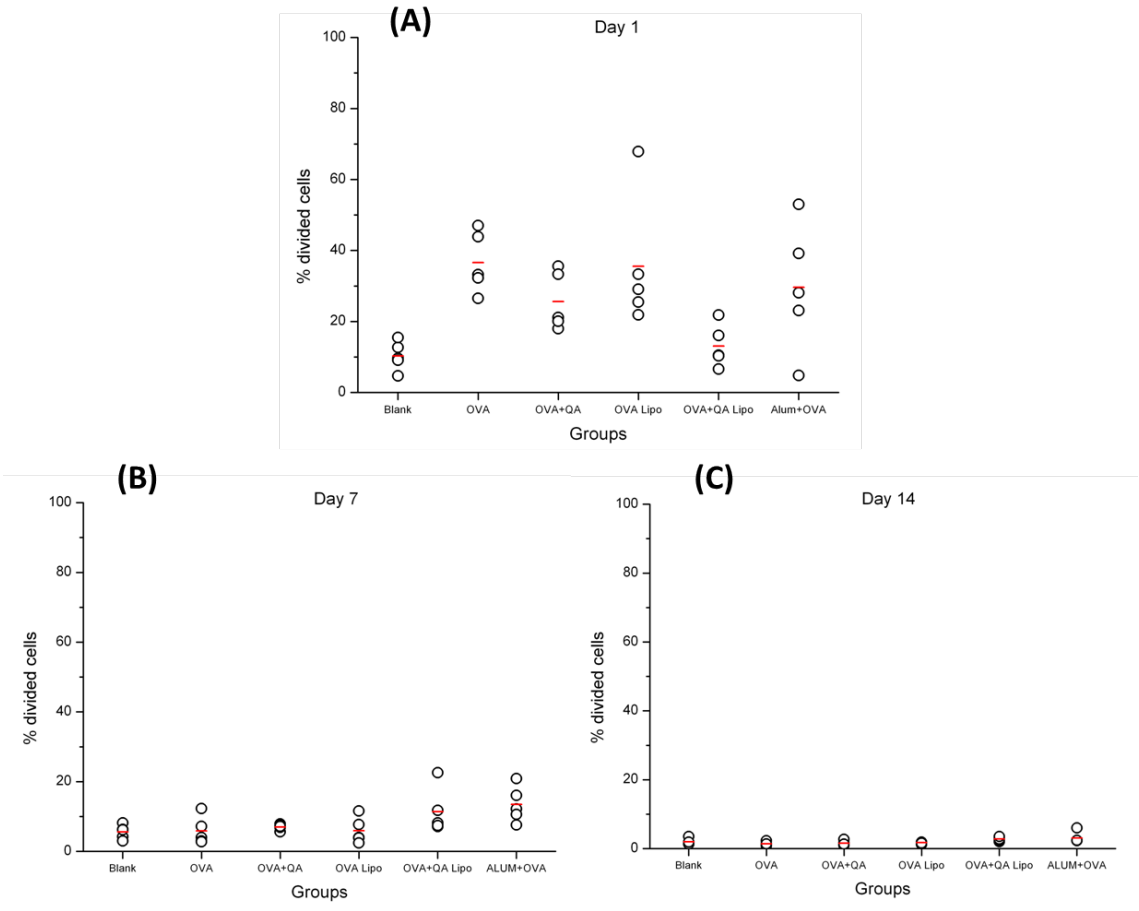

Figure C - 4: Transgenic CD4+ T cell proliferation, as a percentage of divided CD4+ T cells. Mice were immunized with blank implants (- Implant), implants containing OVA or OVA/QA either incorporated directly into the lipid mix (OVA Implant, OVA/QA Implant) or formulated into liposomes which were then freeze-dried and incorporated into the lipid mix (Lip Implant) or with OVA in alum (Alum). The adoptive transfer of CFSE stained cells was performed at **(A)** day 1, **(B)** day 7, or **(C)** day 14. Data shown are the individual results from five mice per group plus the mean and SEM

8.4 Appendix D

TRP2 release from lipid implants

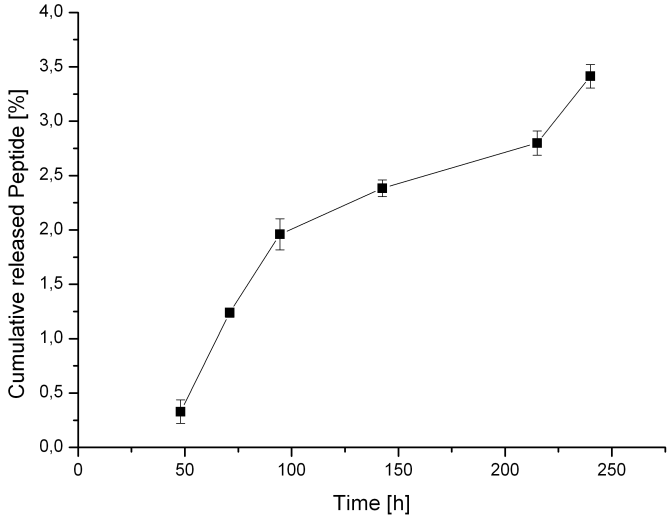

Figure D - 1: Cumulative release of TRP2 from implants produced by the ZE 5 extruder. Implants consist of 65% CHOL, 15% soybean lecithin and 20% D114, containing 0.53 mg QA, 0.28 mg TRP2 Data are the mean and SD of 3 independent replicates.