

# Biodegradable microparticles as a single dose delivery system for *Ehrlichia ruminantium* vaccines

By

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A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science (Veterinary Science) in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa

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# Declaration

I, Ndavheleseni Phanuel Tshikhudo, declare that this dissertation is my own work. It has been submitted to the University of Pretoria for the degree Master of Science. It has not been submitted before for any other degree at any other university.

.....

Ndavheleseni Phanuel Tshikhudo

Date.....



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# Abbreviations

| AE               | adsorption efficiency               |
|------------------|-------------------------------------|
| AMP              | ampicillin                          |
| APC              | antigen presenting cells            |
| ATP              | adenosine triphosphate              |
| A <sub>260</sub> | absorbance at 260 nm                |
| BCA              | bicinchoninic acid                  |
| BMP              | bone morphogenetic protein          |
| bp               | base pair                           |
| BSA              | bovine serum albumin                |
| CBP              | chitin binding protein              |
| CD               | cluster of differentiation          |
| CMI              | cell mediated immune response       |
| CTAB             | cetyltrimethylammonium bromide      |
| CTL              | cytotoxic T-lymphocytes             |
| °C               | degree Celsius                      |
| DC               | dendritic cells                     |
| DCM              | dichloromethane                     |
| DH               | dog histiocytic                     |
| DL               | DNA loading                         |
| DNA              | deoxyribonucleic acid               |
| DT               | diphtheria toxoid                   |
| EBs              | elementary bodies                   |
| EDTA             | ethylenediaminetetraacetic acid     |
| EE               | encapsulation efficiency            |
| ELISA            | enzyme linked immunosorbent assay   |
| ELISPOT          | enzyme linked immunospot            |
| ESAT6            | early secreted antigen target 6 kDa |
| FCS              | fetal calf serum                    |
| FITC             | fluorescein isothiocynate           |



| g      | grams   |
|--------|---|
| GAPDH  | glyceraldehyde-3-phosphate dehydrogenase      |
| GFP    | green fluorescent protein                     |
| GMO    | glycerol monooleate                           |
| GST    | glutathione-S-transferase                     |
| h      | hour  |
| HBSS   | Hank's balanced salt solution                 |
| HBsAg  | hepatitis B surface antigens                  |
| HIV    | human immunodeficiency virus                  |
| HS     | hot saline                                    |
| HSp65  | heat shock protein-65 kDa                     |
| i      | insoluble                                     |
| i.d.   | intra dermal                                  |
| IFA    | indirect immunofluorescence assay             |
| IFN-γ  | interferon gamma                              |
| IgA    | immunoglobulin A                              |
| IgM    | immunoglobulin M                              |
| IL     | interleukin                                   |
| IL-2Rα | interleukin-2 receptor alpha                  |
| IPTG   | isopropyl-β-D-thiogalactopyranoside           |
| i.m.   | intra muscular                                |
| IMAC   | immobilized metal ion affinity chromatography |
| kDa    | kilodalton                                    |
| kg     | kilogram                                      |
| kV     | kilovolts                                     |
| LB     | Luria Bertani                                 |
| LHRH   | luteinizing hormone-releasing hormone         |
| LSDV   | lumpy skin disease virus                      |
| MAP    | major antigenic protein                       |
| MBP    | maltose binding protein                       |
| mg     | milligram                                     |



| MHC   | major histocompatibility complex                               |
|-------|--|
| min   | minutes  |
| ml    | millilitres  |
| mM    | millimolar   |
| MP    | microparticles   |
| mRNA  | messenger RNA  |
| mVA   | millivolts ampere  |
| μg    | microgram  |
| μΜ    | micromolar   |
| M.W.  | molecular weight   |
| MWCO  | molecular weight cut-off point                                 |
| NO    | nitric oxide   |
| OC    | open circular  |
| ORFs  | open reading frames  |
| OVI   | Onderstepoort Veterinary Institute                             |
| PBMC  | peripheral blood mononuclear cells                             |
| PBS   | phosphate buffered saline                                      |
| PBS-T | phosphate buffered saline supplemented with 0.05% (v/v) Tween- |
|       | 20   |
| PCR   | polymerase chain reaction                                      |
| pDNA  | plasmid DNA  |
| PEC   | poly-e-caprolactone  |
| PEI   | poly(ethylene-imines)  |
| PL    | protein loading  |
| PLA   | poly(lactic acid)  |
| PLGA  | poly(lactide-co-glycolide) acid                                |
| PLL   | polylysine   |
| PVA   | polyvinyl alcohol  |
| PVDF  | polyvinyl difluoride   |
| r     | recombinant  |
| RBs   | reticulate bodies  |



| ribonucleic acid  |
|---|
| revolution per minute                                     |
| reverse transcription-PCR                                 |
| soluble   |
| subcutaneous  |
| super-coiled  |
| sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| scanning electron microscopy                              |
| stimulation index   |
| tuberculosis  |
| tris-borate EDTA  |
| tris ethylenediaminetetraacetic acid                      |
| T helper  |
| tumor necrosis factor                                     |
| thioredoxin   |
| tetanus toxoid  |
| water-in-oil-in-water                                     |
| times   |
| relative centrifugal force                                |
|   |



# Abstract

# Biodegradable microparticles as a single dose delivery system for *Ehrlichia ruminantium* vaccines

# By

# Ndavheleseni Phanuel Tshikhudo

| Promoter    | : | Dr Mirinda van Kleef  |
|-------------|---|---|
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|             |   | And New Generation Vaccines Programme, Onderstepoort                |
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Four 1H12 *E. ruminantium* open reading frames cloned into the pCMViUBs mammalian expression vector and used as a recombinant DNA vaccine against heartwater repeatedly provided complete protection in sheep (using a cocktail or the individual ORFs) against a laboratory needle challenge while 1/5 of sheep were protected after a natural tick challenge. The lack of protection under natural field conditions could be attributed to the delivery strategy used and therefore there is a need to investigate other delivery methods. Polymeric microparticles based on PLGA polymers have been used extensively to target the delivery of vaccine to antigen presenting cells, play a role in the induction of cellular immunity and can be used as a single dose vaccine mimicking prime/boost vaccination. In this study, the four 1H12 pCMViUBs\_ORFs and their respective recombinant proteins were either encapsulated into or adsorbed onto microparticles using a modified double emulsion solvent evaporation technique. The particles were formulated to release DNA on day zero and day 21 and recombinant proteins on day 42 thus mimicking a two times DNA prime/recombinant protein-boost immunization strategy. Encapsulation did not have any detrimental effects on the stability of the recombinant proteins as determined by



gel electrophoresis and western blotting. The *in vitro* incubation of microparticles in either a Float-A-Lyzer<sup>®</sup> dialyzer or an eppendorf tube showed the potential of microparticles to be used as a vaccine because of their release profiles that mimics a heterologous prime/boost immunization strategy. Microparticles formulated using polymers with low glycolide ratios released 80% of the encapsulated proteins within the first week of *in vitro* incubation with most of the proteins released on day 1. Microparticles formulated using polymers with 50:50 monomer ratios released the recombinant proteins during week 1 and 3 of *in vitro* incubation. These microparticles did not release any protein in week 2 (day 7-14). Microparticles with 0.5% cetyltrimethylammonium bromide (CTAB) on their surfaces adsorbed DNA and released more than 40% of DNA on day 1 with 100% release by day 14. RG502H microparticles formed with PVA as the internal phase viscosity enhancer released intact DNA only from day 12 to day 21. A cocktail of these microparticles could therefore be used as an autobooster vaccine thus reducing the need for repeated immunizations needed to obtain protective immunity.

#### Potential scientific publication

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#### Publication of results in conference proceedings / abstracts

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# Chapter 1:

# Literature review

# 1.1. Introduction

Since the eighteenth century when Louis Trichardt recorded the first observation of heartwater in sheep in the now Limpopo province of South Africa (reviewed by Provost, Bezuidenhout 1987), heartwater remained one of the major drawbacks for livestock production in areas where the tick vector occurs. Heartwater is caused by a tick-borne rickettsial parasite known as Ehrlichia ruminantium. E. ruminantium is a small pleomorphic, coccoid or ellipsoidal, occasionally rod shaped organism occurring intracytoplasmically but not intranuclearly (reviewed by Yunker 1996). In southern Africa, heartwater is controlled by the use of acaricides, antibiotic prophylaxis, and the infection and treatment method of vaccination (reviewed by Uilenberg et al. 1984). Tetracycline antibiotics are commonly used to treat E. ruminantium infection (reviewed by Yunker 1996). These procedures are expensive and therefore cannot be used effectively in heartwater endemic areas, which are mainly rural with limited infrastructures. Vaccination with attenuated and inactivated heartwater organisms is also inadequate due to lack of cross-protection of isolates from different origins. All these factors underline a pressing need for a recombinant (r) vaccine that will reduce the need for cold storage and virulence as with the infection and treatment method.

A heartwater DNA vaccine consisting of a cocktail of four 1H12 open reading frames (ORFs) cloned into the pCMViUBs expression vector protected all sheep against a homologous needle challenge while only 1/5 sheep were protected in the field (Pretorius et al. 2007). It was also shown that the individual gene products of the four 1H12 ORFs are capable of inducing a protective immune response. Furthermore, a heterologous prime-boost immunization strategy that protected sheep against virulent *E. ruminatium* Welgevonden needle challenge also confirmed these observations (Pretorius et al. 2008). In that study all the sheep immunized with a DNA prime-rprotein boost vaccine survived a homologous needle challenge while immunization with DNA prime-lumpy skin disease virus (LSDV) boost strategy protected 9/10 sheep. Animals challenged in the field



(natural tick challenge) again did not survive despite an observed increase in IFN-γ (interferon gamma) production. These findings suggest that for a natural tick challenge to succeed, an even stronger immune response directed to these ORF products must be obtained. This could be achieved by simplifying and improving the delivery system used such as a microparticle-based controlled release delivery system. Microparticles are capable of reducing the number of vaccine administrations needed to obtain protective immunity. Moreover, microparticles can target the delivery of antigens to antigen presenting cells (APCs). Thus in this study, poly(D,L-lactide-co-glycolide) (PLGA) microparticles were formulated to adsorb and encapsulate each of four plasmid (p)DNA as well as to encapsulate each of the four rErum1H12 proteins by using a double emulsion solvent evaporation technique.

# 1.2. Vectors of E. ruminantium

Ticks of the genus *Amblyomma* are the main vector for heartwater transmission (reviewed by Purnell 1984). There are ten *Amblyomma* species that could be involved in the transmission of heartwater organisms in Africa (reviewes by Uilenberg et al. 1984; and by Bezuidenhout 1987), namely *A. hebraeum, A. gemma, A. lepidum, A. pomposum, A. sparsum, A. variegatum, A. astrion, A. cohaerens, A. marmoreum* and *A. tholloni*. In sub-Saharan Africa, the most important of these vectors are *A. hebraeum, A. pomposum* and *A. variegatum*. The latter is the most widely spread vector in Africa. *A. pomposum* is the major vector in Angola and Mozambique, while in Madagascar and Zambia *A. variegatum* is the major vector. In Zimbabwe, minor vectors such as the *A. sparsum* and *A. tholloni* were observed together with the major vectors (reviewed by Purnell 1984). The major vector known to transmit heartwater in South Africa is *A. hebraeum*.

# 1.3. Heartwater reservoirs

A study done in the Kruger National Park, South Africa, confirmed the existence of *E. ruminantium* infection in wild life as reported earlier (reviewed by Burridge 1985). In this study pheromone traps inside the park collected *A. hebraeum* ticks. *E. ruminantium* was isolated from these ticks and used to infect a susceptible goat that exhibited signs of an acute heartwater attack (Peter, Mahan & Burridge 2001, reviewed by Burridge, Mahan



2002). This report indicated that wild animals could act as *E. ruminantium* reservoirs and thus play a role in the transmission of heartwater to ticks that in turn can also transmit it to domestic animals. Blesbok, black wildebeest, blue wildebeest, African buffalo, eland, giraffe; greater kudu and sable antelope are all wild animals susceptible to *E. ruminantium* infection thus further complicating the control of heartwater infection (Peter, Burridge & Mahan 2002).

#### 1.4. The lifecycle of E. ruminantium

Ticks are infected with E. ruminantium parasites after feeding on a heartwater-infected animal (reviewed by Bezuidenhout 1987; Kocan et al. 1987; Prozesky 1987a). Infection of the vector can occur during its nymph, larva or adult stages. This life journey begins in the tick gut where the microorganisms invade the gut epithelial cells and proceeds to acini cells and salivary glands on day 2 after infection (Prozesky 1987b). The tick bite transfers organisms from saliva to livestock or wild ruminant host where the organisms multiply in the vascular endothelial cells, neutrophils, macrophages and reticulo-endothelial cells (Du Plessis 1970). The incubation period of heartwater varies between 9-29 days in cattle (Alexander 1931) and 5-35 days in goats and sheep (Uilenberg 1983) which is followed by an increase in temperature. Once parasites are transmitted to the ruminant host, they will drain to the lymph nodes and infect reticulo endothelial cells (Du Plessis 1970). The infection mechanism involves the virulent form of organism called the elementary bodies (EBs) (Jongejan 1991). The EBs reorganise their DNA inside the host to become reticulate bodies (RBs). The RBs are regarded as the multiplication stage of the organism. The RB stage reverts to EBs when the organism is ready to infect new host cells. Once replication in the lymph nodes is complete the EBs are released into the blood of the host. The parasites are then available to be transmitted back to the tick vector (during tick feeding on the infected animal) and to infect host artery epithelial cells and epithelial cells of major organs including the heart, lungs and brain (reviewed by Yunker 1996).

#### 1.5. Symptoms of heartwater

The microorganisms survive in the endothelial cells of various organs thereby causing vascular permeability (Owen et al. 1973). An excessive amount of fluid accumulates in



the sac surrounding the heart (hydropericardium) hence the name heartwater. Fluid is also observed in the lungs (oedema) and in the chest (hydrothorax) (Prozesky 1987a). Animals that are resistant or exposed to a mild form of heartwater only exhibit transient fever, apathy and slight tachypnoea (Van de Pypekamp & Prozesky 1987). Other signs of heartwater manifest in behavioural changes such as excessive chewing and overly rigid posture, hypersensitive to bright light and sudden noise (Prozesky 1987a).

# 1.6. Diagnosis of heartwater

Heartwater diagnosis has been based on brain smears and microscopy done as part of a post-mortem. These smears are stained with Giemsa that facilitates the observation of heartwater organisms (Prozesky 1987a). Serology based tests such as indirect fluorescent antibody (IFA) (Du Plessis & Malan 1987; Semu et al. 1992) and ELISA (Neitz et al. 1986) were also developed. These tests were used during *E. ruminantium* infection surveys. The IFA test uses infected peritoneal macrophages as antigen (Martinez et al. 1990) while the ELISA test measures the anti-major antigenic protein-1 (MAP1) antibody in serum. However, both these assays fail to distinguish *Erhlichia spp* from *Anaplasma spp* (Logan et al. 1986).

Recent diagnosis is based on the polymerase chain reaction (PCR)/probe method using a conserved gene region of *E. ruminantium*, known as pCS20 (Mahan et al. 1992). The PCR/probe was modified to amplify a 903 bp product from characterized *E. ruminantium* stocks combined with a 279 bp probe for detection of low levels of pCS20 in Welgevonden stock (Van Heerden et al. 2004). The most recent diagnostic improvement of this assay is the quantitative real-time PCR Taqman probe assay (Steyn et al. 2008). This assay is able to detect *E. ruminantium* in livestock blood and ticks collected from the field with higher sensitivity than the standard PCR. Even though the real-time PCR and the PCR/Probe methods are sensitive, they can still only detect parasites during the febrile stages of the disease and therefore early detection and treatment is not yet feasible.



#### **1.7. Immune responses to heartwater**

Pathogens can either induce cell mediated and/or humoral immune responses (reviewed by Mosmann, Sad 1996). CD4<sup>+</sup>T-cells play a major role in the regulation of immune responses. These cells recognize antigens after they have been presented by major histocompatibility complex II (MHC II) on APCs. After antigen specific activation, CD4<sup>+</sup>T-cells could differentiate into two functional subsets: T helper 1 (Th1) or T helper (Th2). Th1 cells respond by secreting cytokines, mainly IFN- $\gamma$  and interleukin-2 (IL-2) that contribute to macrophage activation and generating opsonins of the IgG2a isotype. In addition, Th1 responses mediate the killing of intracellular pathogens by cytotoxic Tlymphocytes (CTL). CD8<sup>+</sup>T-cells recognize antigens presented by MHC I molecules and mediate effector functions through secretion of cytokines such as of IFN- $\gamma$  and tumor necrosis factor-alpha (TNF- $\alpha$ ). CD8<sup>+</sup>T-cells release granules such as perforin and granzyme thus inducing cytotoxic cell death. In contrast, Th2 cells secrete IL-4; IL-5; IL-6 and IL-10 that induce growth and differentiation of B-cells. B-cells generate circulating antibodies of IgG1, IgA and IgE isotype.

Because *E. ruminantium* is an intracellular pathogen, cell mediated immunity (CMI) is expected to play a major role in protection against infection. Observations in mice, suggested that antibodies are not important in the protective immunity against heartwater (Du Plessis, Berche & Van Gas 1991). Mice immunized with immune serum before or after infection with the Kümm stock of *E. ruminantium* did not show any protective immune response, but peritoneal macrophages incubated in the presence of immune serum reduced the infection rate of heartwater organisms (Du Plessis, Berche & Van Gas 1991). Experiments like these therefore do not completely rule out possible protection against obligate intracellular parasites induced by antibodies (reviewed by Casadevall 2003). Intracellular pathogens also reside in extracellular spaces at some point in their infectious cycles (reviewed by Casadevall 2003). A study done on *E. chaffeensis* (an obligate intracellular organism) emphasized the existence of intracellular bacterium in extracellular spaces where it is susceptible to serum antibodies (Li & Winslow 2003). Similarly, *E. ruminantium* is found extracellularly before it re-infects a new endothelial cell.



In their review of immune responses to E. ruminantium infections, Totté and co-workers implicated the involvement of cellular immune responses and the production of IFN- $\gamma$  as the protective mechanism against E. ruminantium infection (reviewed by Totté et al. 1999). IFN- $\gamma$  is secreted by Th1-type T-cells and acts as an induction and modification factor in cell-mediated immunity (reviewed by Mosmann & Sad 1996). It has been shown that IFN- $\gamma$  inhibits the growth of *E. ruminantium* regardless of the isolates and endothelial cell lines used, but IFN- $\alpha$  failed to inhibit the growth of the organisms even at high levels (reviewed by Totté et al. 1999). In another immune response study, antigen specific proliferation of CD4<sup>+</sup>T-cells and  $\gamma\delta^{+}$ T-cells were observed in cattle immunized by the infection and treatment method (Mwangi et al. 2002). In this experiment peripheral blood mononuclear cells (PBMCs) were incubated with surface exposed major recombinant proteins (MAP1 and MAP2). The cells responded by generating CD4<sup>+</sup>T-cells and  $\gamma\delta^+$ Tcells. Reverse transcription polymerase chain reaction (RT-PCR) revealed increased expression of mRNA encoding for IFN- $\gamma$ , IFN- $\alpha$  and IL-2R $\alpha$ . The supernatant of the culture was dominated by IFN- $\gamma$  protein. Elevated levels of IFN- $\gamma$  production were also observed in goats after immunization with a killed *E. ruminantium* vaccine as compared to naïve animals (Esteves et al. 2002; Esteves et al. 2004b). Other antigens that showed potential as vaccine antigens are the low molecular weight proteins obtained after fractionation of E. ruminantium proteins, especially those of 11, 12, 14 to 17 and 19 to 23 kDa (Van Kleef et al. 2002). Immune lymphocytes cultured in the presence of fractionated *E. ruminantium* proteins proliferated and produced high levels of IFN- $\gamma$ . The dominant subset was CD4<sup>+</sup>T-cells. Similar findings were observed in goats immunized with Gardel stock of E. ruminantium (Esteves, Martinez & Totté 2004a). In order to discover the mechanism whereby IFN- $\gamma$  inhibits the growth of *E. ruminantium*, Esteves and colleagues used an inactivated E. ruminantium (Gardel) vaccine to study lymphocyte subsets responsible for production of IFN- $\gamma$  (Esteves et al. 2004a). A better understanding of immune mechanisms responsible for protection against heartwater would facilitate the development of such a vaccine. Using flow cytometry it was discovered that the source of IFN- $\gamma$  in goats immunized with inactivated *E. ruminantium* was CD4<sup>+</sup>T-cells and CD8<sup>+</sup>Tcells (Esteves et al. 2004b). Further experiments showed that CD8<sup>+</sup>T-cells depended on CD4<sup>+</sup>T-cells in order to produce this cytokine. Interestingly, all animals vaccinated with a



killed *E. ruminantium* vaccine responded by high production of *E. ruminantium* specific  $CD8^{+}T$ -cells late after challenge (reviewed by Totté et al. 1999). This report confirms the fact that immunity towards intracellular pathogens often depends upon generation of  $CD8^{+}$  memory T-cells (Huster, Stemberger & Busch 2006). Thus, the vaccine that provokes both  $CD4^{+}$  and  $CD8^{+}T$ -cells responses and IFN- $\gamma$  expression will probably render protective efficacy against heartwater.

#### 1.8. Heartwater vaccines

Vaccination remains the only feasible control measure for most diseases in Africa. A number of heartwater vaccine strategies have been tried in the past (Mahan et al. 1999). The only vaccine available on the market is a sheep blood live vaccine based on the Ball3 E. ruminantium stock (reviewed by Oberem & Bezuidenhout 1987). It can be used to vaccinate young animals that have never encountered the heartwater organisms. For older animals, the vaccine needs to be combined with antibiotic treatments (thus the infection and treatment method of vaccination) (reviewed by Purnell 1984). Unfortunately, the Ball3 stock does not protect against all isolates in the field, but due to its mildness, it can be used successfully in a live vaccine. The more virulent Welgevonden stock (that protects against all known stocks) does not work well with the infection and treatment method. Ball3, unlike Welgevonden, enables antibiotic treatment by causing a temperature rise in animals a few days before the onset of serious disease. When this rise in temperature is observed, tetracycline treatment can be administered effectively. The live vaccine suffers major drawbacks such as the need for cold storage and transportation (in cryopreserved form). This makes this vaccination method inconvenient in rural settings (Van der Merwe 1987).

It was because of these limitations that scientists were forced to look for an alternative vaccine. Such a vaccine should be able to engender cross-protective immunity against heterologous isolates and not capable of causing illness in animals. In 1991, Jongejan reported the first attenuated *E. ruminantium* vaccine (Jongejan 1991). This vaccine was based on the Senegalese stock of *E. ruminantium*. Attenuation was achieved after 229 days of *in vitro* culture. The supernatant of infected bovine umbilical endothelial culture



was able to induce protective immunity in goats challenged with the same isolate. Again, lack of cross-protective immunity was a major disadvantage of this vaccination method (Mahan et al. 1999). Recently, investigators were able to attenuate a virulent Welgevonden stock (Collins et al. 2003). This stock was attenuated in canine histiocytic cell line (DH82). This vaccine completely protected mice and sheep against homologous challenge after a single immunization. Unfortunately, the DH82 cell line is cancerous and cannot be used to grow vaccine materials. It was later shown that the attenuated Welgevonden stock re-adapted to a bovine endothelial cell-culture line was able to protect all sheep against virulent homologous and heterologous (Ball3, Gardel, Mara 87/7 and Blaauwkrans) needle challenge (Zweygarth et al. 2005).

Inactivated vaccines were also investigated, thus circumventing any risks of reversion to virulence. In 1994, Martinez and co-authors reported that the inactivated E. ruminantium Crystal Springs (stock) elementary body vaccine protected goats against homologous challenge (Martinez et al. 1994). This vaccine consisted of chemically inactivated E. ruminantium organisms derived from bovine endothelial cell culture and formulated in Freund's adjuvant and in Montanide ISA50. The results were reproduced in sheep and all the sheep were protected against homologous (Crystal Springs stock) needle and field challenge (Mahan et al. 1995). The Zimbabwean Mbizi strain of E. ruminantium was also used as an inactivated vaccine (Mahan et al. 1998). This resulted in an increase in crossprotective ability of the vaccine because it protected sheep against homologous and heterologuous (such as Welgevonden, Mbizi, Beatrice and Isiolo) needle challenge. However, only 50% or partial protection was achieved against field tick challenge in heartwater endemic areas. The inactivated vaccine prevents death but not infection because tests done on brain biopsies indicated the presence of heartwater organisms. All the above vaccine strategies suffer the major drawback of lack of cross-protection against field tick challenge.

The ability to use inactivated material of heartwater organisms to obtain protective immunity paved a way for the subunit vaccine. The identification of genes encoding for major antigenic membrane proteins (MAP1 and MAP2) followed by their expression



(Mahan et al. 1994; Van Vliet et al. 1994) and demonstration of protective capacity in mice (Nyika et al. 1998) was the major highlight for the possibility of a subunit vaccine. One of the first heartwater experimental sub-unit vaccines was based on the immunodominant major outer membrane protein MAP1 (Nyika et al. 1998). The *map1* DNA vaccine partially protected mice against challenge with a lethal dose of *E. ruminantium* (Nyika et al. 1998). Booster vaccination with a recombinant MAP1 protein conferred protection against homologous (Crystal Springs) needle challenge (Nyika et al. 2002). The protective immune response observed in the mouse model of *E. ruminantium* induced by a subset of genes encoding for recombinant antigens increased the number of antigens that could be incorporated into a recombinant vaccine (Barbet et al. 2001). These genes showed significant resemblance to those of *Rickettsia prowazekii* that encode for outer membrane proteins and those encoding for unusual tandem repeat structures.

Another nucleic acid vaccine investigated was the OVI-DNA vaccine. This vaccine consists of a cocktail of four E. ruminantium ORFs derived from the 1H12 clone from a genetic locus involved in nutrient transport (Collins et al. 1998). Erum2540 is believed to encode for an exported protein with properties similar to that of *Wolbachia* species; Erum2550 and Erum2590 are probable ATP binding ABC transporter genes while Erum2580 is also believed to encode for a solute binding ABC transporter (Pretorius et al. 2007). These ORFs were cloned into a DNA vaccine vector pCMViUBs. Sheep vaccinated with this vaccine cocktail (gene gun and i.m. administration) were completely protected against a virulent needle challenge, either with the homologous or with heterologous stocks. However, protection against a tick field challenge in a heartwater endemic area was low (Collins et al. 2003). It was also shown that the individual four 1H12 ORFs can confer protective immunity against a virulent Welgevonden needle challenge. Boosting this vaccine with four 1H12 ORF products transfected into rLSDV protected 9/10 sheep against homologous virulent needle challenge, while boosting with rproteins protected all the sheep (Pretorius et al. 2008). Lymphocytes responded by proliferation and production of IFN- $\gamma$  only from animals boosted with r1H12 proteins. This report suggests that rproteins are better at boosting the protective immune response



than the rLSDV. In light of the above, it was then decided to try to improve the delivery of this vaccine by using biodegradable microparticles. Biodegradable microparticles are capable of providing long lasting immunity by releasing their entrapped antigen at predetermined time points following a single administration (Singh et al. 2000; Feng et al. 2006; Aguilar, Rodríguez 2007). Moreover, biodegradable microparticles are capable of enhancing the potency of DNA vaccines (reviewed by O'Hagan 2006).

#### 1.9. Microencapsulation

Microencapsulation technology was developed in the 1950s and is currently widely used in drug delivery (reviewed by Morris, Steinhoff & Russell 1994). It is defined as a technology of packaging solid, liquid and gaseous materials in small capsules that releases their contents at a controlled rate over prolonged periods of time (Champagne & Fustier 2007). This technology is used to form biodegradable microparticles encapsulating drugs of pharmaceutical importance. It has also been used in studies where for example the prime-boost vaccination strategy was employed. Turkeys were vaccinated with PLGA microparticles adsorbing pDNA encoding a fusion protein gene against metapneumovirus (Liman et al. 2007). The booster vaccination contained the fusion rprotein encapsulated into PLGA microparticles. This vaccination resulted in higher viral clearance and production of CD4<sup>+</sup>T-cells than observed in the negative control groups. The negative control birds showed more clinical signs and histological lesions and less significant production of CD4<sup>+</sup>T-cells. DNA prime/rprotein boost strategy is able to utilize the benefits of DNA and protein vaccines to induce both cell mediated and antibody responses against pathogens (reviewed by Lu 2006).

The widely used methods for microparticle formulation are spray drying, double emulsion solvent evaporation technique and phase separation (reviews by Morris, Steinhoff & Russell 1994; Hanes, Cleland & Langer 1997). In spray drying, the polymer is dissolved in organic solvents such as dichloromethane (DCM) and acetyl acetone (reviewed by Gharsallaoui et al. 2007). The protein or drug of interest is added in a solid state and subjected to homogenization. This mixture is atomized in a stream of heated air. Spray drying is more reproducible than other encapsulation procedures and the particle size is



controllable. It is, however, an expensive procedure. It has been used to successfully encapsulate rhuman erythropoietin (Morlock et al. 1997). Double emulsion involves the emulsification of watery protein or drug solution with the oil phase solution of polymer dissolved in organic solvent such as dichloromethane (Jeffery, Davis & O'Hagan 1993). The solution is sonicated or homogenized to form a primary water-in-oil (w/o) solution, which is stabilized by the addition of surfactants such as polyvinyl alcohol (PVA). Further homogenization leads to the formation of a double emulsion of w/o/w. The organic solvent is evaporated or extracted leaving the hardened microparticles behind which are eventually harvested. The method does not need freeze drying of protein prior to encapsulation and can be scaled accordingly. It is cheaper than spray drying, however, it is difficult to modify the release profiles of the drug released from microparticles. Double emulsion has been used to encapsulate *influenza A* vaccine (Moldoveanu et al. 1993), bovine serum albumin (BSA) (Kohn et al. 1986) and luteinizing hormone-releasing hormone (LHRH) agonist (reviews by Morris, Steinhoff & Russell 1994; Cleland 1997; Hanes, Cleland & Langer 1997).

The main challenge of protein encapsulation is to optimize the procedure that will preserve the folded, three-dimensional structure of a protein (reviewed by Hanes, Cleland & Langer 1997; Bilati, Allémann & Doelker 2005; Morlock et al. 1997). Steps involved in the encapsulation process can lead to physical or chemical degradation of protein. This should be avoided as it can result in the induction of an undesired immune response. To counter these stability challenges, stabilizing agents are added during the encapsulation process. These additives include other proteins such as BSA, gelatin, chelating agents and sugars such as trehalose, sucrose, maltose and glucose. Surfactants such as polysorbate 20 and PVA are also preferred (Sinha & Trehan 2003).

# 1.9.1. Immune responses induced by microparticles

Microparticles can present multiple copies of antigens on their surfaces, which is requirement for successful B-cell activation. The ability of microparticles to constantly furnish APCs with antigens can also lead to protective T-cell responses (reviewed by O'Hagan 2006). In addition, biodegradable microparticles can protect antigen from acidic



and enzymatic degradation in the gastrointestinal tract and provide a stable vaccine vehicle with prolonged shelf life (reviewed by Morris, Steinhoff & Russell 1994). Microparticles can induce both IgA and IgG responses for mucosal as well as humoral immune responses when administered orally (reviewed by Morris, Steinhoff & Russell 1994). Encapsulated antigens are capable of stimulating both humoral and CMI responses (reviewed by O'Hagan, Singh & Gupta 1998). It is widely reported that microparticles with a small diameter (less than 10 µm), a smooth surface and the highest possible antigen encapsulation efficiency are internalized easily by macrophages and provoke a cellular immunity (reviews by Hanes, Cleland & Langer 1997; Gupta, Singh & O'Hagan 1998). This is because microparticles can be formulated to have sizes similar to the pathogens that the immune system has evolved to combat (reviewed by O'Hagan 2006). Macrophages play a major role in cell-mediated immunity as antigen presenting cells (reviewed by Gupta, Singh & O'Hagan 1998). These encapsulated antigens are brought into contact with the CD4<sup>+</sup>T-cells by macrophages, thus leading to the activation of Th1cells (reviewed by Shedlock & Weiner 2000). In addition, the process of phagocytosis of particulate bacteria or even polystyrene beads (2 µm) can induce IL-12 and thus Th1 cellular immunity (Fulton et al. 1996). Besides being phagocytosized by macrophages, microparticles also form a depot at the site of administration, thus provoking the immune system in a continual, prolonged manner (reviewed by Hanes, Cleland & Langer 1997). It was shown that immunization of mice with a rHIV envelope protein entrapped in biodegradable PLGA microparticles induced consistent HIV-specific CD4<sup>+</sup> and CD8<sup>+</sup>Tcell responses (Moore et al. 1995).

As mentioned earlier, the protective mechanism to heartwater involves the secretion of IFN- $\gamma$  by CD4<sup>+</sup>T-cells (Moore et al. 1995; Totté et al. 1999; Esteves et al. 2004c). The use of biodegradable microparticles may therefore enhance the production of IFN- $\gamma$  by CD4<sup>+</sup>T-cells thus leading to a more improved heartwater vaccine. It is widely reported that encapsulation of vaccine antigens or drugs in biodegradable microparticles such as PLGA resulted in the improvement of targeted delivery and vaccine protective efficacy. Furthermore, adsorption of a DNA vaccine onto cationic microparticles resulted in



significantly enhanced serum antibody responses in comparison to naked DNA and induction of potent CTL responses at low doses (Singh et al. 2000).

# 1.9.2. Controlled release of PLGA microparticles

Polymers, especially those of PLGA and poly (D, L-lactide) (PLA) have been preferred for vaccine delivery and drug discovery (reviews by O'Hagan, Singh & Gupta 1998; Bilati, Allémann & Doelker 2005). Some of the reasons cited included biocompatibility, biodegradability, availability, can encapsulate molecules of any size, capable of controlling the release of vaccine antigens slowly and continuously for up to 4 months and the ability to induce cell mediated immunity (reviews by Morris, Steinhoff & Russell 1994; Gupta, Singh & O'Hagan 1998; Jiang et al. 2005; O'Hagan 2006). PLGA is biodegradable and biocompatible because it consists of glycolic acid and lactic acid polymer. The polymer degrades into two monomers that are easily absorbed by the body without any serious side effects (O'Hagan, MacKichan & Singh 2001). Using PLGA polymers, microspheres of 1 to 300  $\mu$ m in diameter can be produced.

The rate of biodegradation of polymer, the surface area and the porosity of formed microparticles encapsulating active compounds play an important role in the release of entrapped materials. The bioerosion of polymer due to hydrolysis of ester linkages has been exploited to control the release of encapsulated materials. This erosion leads to fragmentation of polymer and the subsequent release of monomeric subunits such as lactic acid and glycolic acid in PLGA. The rate of polymer degradation is controlled by the ratio of monomer composition with PLGA with 50:50 ratios (i.e. equal amounts of lactide (L) and glycolide (G) with the fastest erosion rate of 50-90 days *in vitro* (reviewed by O'Hagan, Singh & Gupta 1998). This is followed by PLGA ratios of 65:35 > 75:25 > 85:15. PLGA 85:15 can last for 150 days while the PLA homopolymer for 12-14 months. In animals, the half-life of 100% polyglycolide polymer microspheres is 5 months and it could be decreased to 7 days with the addition of co-polymers including polylactide (50:50). Increasing the amount of lactide added to the polymer mix will again increase the resulting microparticles half-life until a maximum of 6 months with 100% PLA. A variety of release patterns for encapsulated vaccines can be pre-programmed by changing the



microparticles sizes, molecular weights and monomer ratios (reviewed by Morris, Steinhoff & Russell 1994). One can combine microparticles with different release rates as a single formulation. PLGA microparticles therefore offer the potential for the development of single dose vaccines because it is possible to control the rate of release of entrapped antigens at predetermined time points.

# 1.9.3. Single dose rprotein vaccines

The ability to encapsulate and control the release of encapsulated materials from PLGA micropaerticles led to encapsulation of most of the traditional vaccines such as neonatal tetanus, diphtheria and hepatitis (reviewed by O'Hagan 2006). The main objective was to convert multiple dose traditional vaccines into single dose vaccines, thus reducing the rate of non-compliance to vaccination schedules that results in unnecessary death in less developed communities. So far, a substantial body of work has been done on mice as a model for human diseases such as neonatal tetanus, diphtheria, hepatitis, etc. (reviewed by O'Hagan 2006). A continuous and discontinuous release profile has been shown to induce potent immune responses comparable to several administrations of alum-adjuvanted vaccines following a single administration.

Numerous attempts have been made to establish a single dose vaccine that will reduce the number of repeated vaccinations needed to prevent neonatal tetanus. PLGA microencapsulated tetanus toxoid (TT) (immunogenic antigen) was investigated as a possible single dose vaccine against tetanus (reviewed by O'Hagan 2006). Long lasting antibody responses have been observed in mice immunized with the microencapsulated TT. These responses were comparable to two injections of TT with alum as adjuvant. The major drawback with microencapsulated TT vaccine has been the instability, which resulted in aggregation of TT within the microparticles, thus causing incomplete release. Non-covalent interactions (like polymer to protein interaction) and disulfide interchange have been cited as the major problems behind these instabilities (Schwendeman et al. 1995). Recently, TT was encapsulated into PLGA and chitosan microparticles together with trehalose and  $Mg(OH)_2$  protein stabilizer and antacid respectively. Single immunization of guinea pigs with these microparticles resulted in the induction of



antibody responses equivalent to that obtained following booster injections of alum based vaccine (Jaganathan et al. 2005).

Similar results were obtained when encapsulating diphtheria toxoid (DT). The current diphtheria vaccine also requires multiple injections to establish immunity. In contrast, a single microencapsulated DT injection into mice induced IgG serum levels similar to that of three doses of alum associated DT. Higher IgG responses were obtained by blending alum adjuvanted DT with microparticles based DT in mice (Singh et al. 1997). Another disease studied to produce encapsulated antigen as a vaccine is hepatitis B. The hepatitis B virus is a major cause of liver associated problems such as liver cirrhosis and is still a cause of health concern in developing countries. The identification of an immunogenic hepatitis B surface antigen has led to the development of a hepatitis B vaccine. This vaccine requires three doses to be effective and is therefore too expensive to use as a vaccine in developing countries (Feng et al. 2006). A PLGA vaccine against hepatitis B virus was formulated based on hepatitis B surface antigens (HBsAg). Polymers with different physico-chemical properties (PLGA 75:25, PLGA 50:50 and PLGA 50:50-COOH) were used to form microparticles. A once off subcutaneous administration of a mixture of these microparticles in mice provoked antibody responses comparable to three injections of alum adjuvanted vaccine (Feng et al. 2006). In another study, the immunodominant B-cell epitope of HBsAg was encapsulated into PLGA microparticles. Mice were immunized once orally with B-cell epitope loaded microparticles and responded by induction of HBsAg-specific serum IgG and IgM antibodies (Rajkannan et al. 2006). Brucella ovis antigenic hot saline (HS) extract was also encapsulated in PLGA 75:25 and poly- $\epsilon$ -caprolactone (PEC) microparticles. Excipients like  $\beta$ -cyclodextrin and pluronic F-68 were added for increased stability of antigen. Mice were immunized with these microparticles and resulted in the elicitation of Th1 responses (Murillo et al. 2002).

# 1.9.4. Microparticles as delivery system for DNA vaccines

DNA based vaccines are essential for combating infectious diseases such as malaria in humans, foot and mouth disease, swine fever, brucellosis and rabies in animals (reviewed by Dhama et al. 2008). More than four decades ago, when naked DNA was first shown to



be able to transfect mammalian cells *in vivo*, that led to the development of DNA and RNA based vaccines. DNA immunization may provoke CMI which is often not seen when sub-unit protein or inactivated pathogen vaccines are used. One way to determine CMI is to measure the amount of IFN- $\gamma$  secreting cells present, which assists in the eradication of pathogens *in vivo* (reviewed by Dhama et al. 2008).

Advancement in the field of rDNA technology has heightened the use of DNA vaccines to elicit cell mediated and antibody-mediated immune responses. The crucial components of a pDNA vaccine is the gene encoding an immunogenic antigen specific for the targeted pathogen (reviewed by Shedlock & Weiner 2000) and the promoter as well as the origin of replication (reviewed by Dhama et al. 2008). DNA vaccines are more stable, cost effective and do not require a cold chain as do most other protein/inactivated vaccines. Moreover, immune responses elicited by DNA immunization closely resemble that of natural infection, because of the endogenous production of pathogen proteins. This phenomenon is necessary for cell based and antibody based immune responses (reviewed by Dhama et al. 2008).

The main limitations of DNA vaccines include a lack of knowledge regarding the amount of the encoded protein expressed *in vivo* and large amounts needed to be administrated in order to provoke the necessary immune responses. The amount of DNA required depends on the weight of the animal (Singh et al. 2000). Parenteral i.m. administration of 10-100  $\mu$ g of pDNA is required in small animals like mice, 100-300  $\mu$ g for larger animals including rabbits and up to 2.5 mg in humans and bigger animals (reviewed by Dhama et al. 2008). However, gene gun DNA administration is capable of significantly decreasing the amount of DNA required to induce an immune response. Once expressed, the protein encoded by gene of interest in the plasmid, should undergo the necessary posttranslational modifications and fold properly before being processed by macrophages and dendritic cells. DNA vaccination is often good in priming the immune responses in a prime-boost immunization strategy, but still large amounts are needed in bigger animals (reviewed by Shedlock & Weiner 2000). Recently pig skin and muscle electroporation


has been shown to effectively induce protective immunity against bovine herpes virus glycoprotein gene gD (Babiuk et al. 2003).

One way of enhancing the immunogenicity of DNA vaccines is the use of adjuvant such as alum based salts and cytokines as well as the polymeric based microparticles. pDNA can be encapsulated into or adsorbed onto microparticles. The crucial goals of DNA encapsulation are: preservation of integrity which is often at risk, controlled release of pDNA, transfection rate and cytotoxicity. DNA adsorption onto cationic surfaces (Oster et al. 2005) and encapsulation of DNA polyplexes have been used to address the question of stability and transfection activity (Howard et al. 2004; reviewed by Luten et al. 2008). Cationic lipids and cationic polymers are non-viral systems mainly used for pDNA vaccine delivery (Jilek, Merkle & Walter 2005). Cationic polymers can be toxic to the cell membranes and thus lead to the collapse of the potential of the affected membrane. Biodegradable polymers are often used to avoid this problem. These kinds of polymers do not accumulate in cells because of their biodegradability caused by water sensitive ester bonds dominant in their structure. This feature is used as a tool to release DNA into the cytosol. They are often stable enough to allow the immunogenic plasmid to reach the targeted site with unaltered integrity. Water-soluble polyesters such as poly (4-hydroxy-L-proline ester) (reviewed by Luten et al. 2008), Poly (ethylene-imines) PEI (Zhou et al. 2008) and polylysine (PLL) (Gebrekidan, Woo & DeLuca 2000) are well researched for DNA delivery. These poly cations are able to attract polyanions such as the DNA and degrade rapidly (Oster et al. 2005). They also have the ability to protect the DNA against scavengers such as DNAses. However, the use of low molecular weight PLL (<25 kDa) often results in DNA complexes with low transfection efficiency and with considerable degree of toxicity (Howard et al. 2004). PEI is currently the most used cationic polymer for gene delivery because of its high transfection efficiency. Unfortunately, only PEI with molecular weight cutt-off (MWCO) above 25 kDa can be used because of the zero-order transfection rate observed of lower molecular weights. pDNA adsorbed onto cetyltrimethylammonium bromide (CTAB) has been shown to elicit CTL responses using low concentration of DNA as compared to naked DNA (Singh et al. 2003).



PLGA polymers are the most widely used matrix for DNA delivery. Their continued use in surgical implants and low toxicity is the main reason for their worldwide preference. However, encapsulation of high amounts of pDNA is often difficult to achieve. The highest recorded DNA load is  $5 \mu g/mg$  of particles (Greenland & Letvin 2007). The difficulty in DNA loading and encapsulation efficiency (EE) is due to the large size of pDNA and its hydrophilic nature, which makes the hydrophobic polymers unable to encapsulate.

Erosion of PLGA can also lead to low microenvironment pH which can affect the stability of DNA negatively thus reducing the rate of transfection of that DNA. Coencapsulation of DNA into PLGA microparticles with excipients such as NaHCO<sub>3</sub>, sodium hydroxide, Ca(OH)<sub>2</sub> and antacid magnesium hydroxide have been shown to remedy the pH problem (reviewed by Luten et al. 2008). To improve the DNA loading and the EE process parameters such as PLGA solubility, molecular weight, viscosity and organic solvents are adjusted. DNA loading can be improved by pegylation of polymers or by using hydrophilic polymers such as those containing hydrophilic terminal groups. High molecular weight polymers have also been shown to encapsulate better than low molecular weight polymers. Complexing DNA with pLL that reduces the negative charge of DNA by condensing it before encapsulation also resulted in improvement of EE (reviewed by Luten et al. 2008).

A single dose of PLGA microparticles protected mice against *Mycobacterium tuberculosis* challenge at levels similar to three doses of naked DNA or with *Mycobacterium bovis* BCG (Cai et al. 2005). The PLGA microspheres encapsulated a mixture of DNA genes of Ag85B, MPT-64 and MPT-83 tuberculosis antigens with dimethyldioctyldecyl ammonium bromide (DDA). Mice splenocytes reacted by IFN- $\gamma$  secretion and production of high titers of antibodies to a single subcutaneous administration of PLGA microparticles loaded with pDNA encoding mycobacterial heat shock protein 65 (Hsp65) (Johansen et al. 2003). The use of cationic microparticles adsorbing pDNA reduces the amount of DNA needed to provoke a protective immune response by orders of magnitude below that of naked DNA. This was observed when



mice were intramuscularly immunized with PLGA-CTAB microparticles adsorbing anti-mycobacterium tuberculosis antigen Ag85A (Mollenkopf et al. 2004).

#### 1.10. Rational

This study uses biodegradable PLGA and PLA polymers of different physico-chemical characteristics to adsorb and/or encapsulate pDNA/rproteins onto/into microparticles that are capable of releasing intact antigens in a way that mimics the DNA prime/rprotein boost vaccination strategy. Biodegradable PLGA polymers were investigated to encapsulate rproteins of the four 1H12 Erum ORFs used in a DNA vaccine against heartwater in sheep. The objective was to formulate microparticles that can release rprotein at day 42 of *in vivo* administration. This is described in chapter 2. The four 1H12 ORFs were encapsulated into PLGA microparticles and adsorbed onto cationic microparticles. The aim was to formulate the microparticles in such a way that intact pDNA is released on day 0 and day 21 of *in vivo* administration. This is described in chapter 3. The major findings are summarized and analyzed from previous chapters and future research is highlighted as described in chapter 4.



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# Chapter 2:

# Recombinant protein expression and encapsulation into biodegradable microparticles

#### 2.1. Introduction

The advent of rDNA technology has generated many proteins that are used to protect against various diseases. For a protein to be used in a vaccine its corresponding genes have to be cloned and expressed by a system that is easy to manipulate and capable of producing sufficient quantities of high quality product. For this purpose, E. coli is the commonly used bacterium for routine heterologous protein expression that does not require posttranslational modification such as glycosylation (Singh & Panda 2005; Geng & Carstens 2006; Terpe 2006; Ahn et al. 2007). The bacterium is easy to manipulate, has the ability to grow rapidly to the required biomass and is inexpensive to grow and to induce bacterial expression. The E. coli BL21 (DE3) strain is mostly used for this purpose. However, expression in this bacterium induces inclusion body formation because of protein misfolding during high-level expression (Peti & Page 2007). Another problem is poor expression due to toxicity of expressed proteins and the accumulation of endotoxins such as lipopolysaccharide. This problem is solved by utilizing other bacterial hosts such as Bacillus brevis or Bacillus subtilis (Peti & Page 2007). The accumulation of inclusion bodies is decreased by lowering the expression temperature or by incorporation of additives such as sorbitol and sucrose into the growth medium (Geng & Carstens 2006). Alternatively, the inclusion bodies can be purified by use of denaturants such as urea and/or guanidine hydrochloride that will assist in solubilisation of the inclusion bodies. Refolding using suitable buffers is however required after purification for the protein to be bioactive (Tsumoto et al. 2003).

The nature of the protein of interest dictates the choice of the promoter system to be used by the expression vector. The T7 RNA Polymerase, the *lac* promoter and the *tac* and *trc* promoters are the main promoters that have been used with *E. coli* (Dumon-Seignovert,



Cariot & Vuillard 2004; Terpe 2006). Of these, the most widely used promoter is the T7 RNA Polymerase system. This promoter is stronger, faster, isopropyl-ß-D-thiogalactopyranoside (IPTG) inducible and can achieve high levels of expression using the easily manipulable BL21 (DE3) cells. Basal expression of T7 RNA Polymerase occurs that could be problematic if the rprotein is toxic to the host cell and may lead to premature cell death. This can be circumvented by using *E. coli* strains that contain the pLysS plasmid such as the BL21 (DE3) pLysS. This enables the expression of toxic proteins because low levels of T7 lysozyme is produced, an inhibitor of T7 RNA polymerase, thus allowing the expression of toxic proteins with the pET expression system (Studier 1991).

Once the protein of interest is expressed in sufficient amounts, the next step is to purify that protein from unwanted proteins. Purification is an important step in the study of proteins' biological and physiological properties (Schmidt 2004; Lichty et al. 2005). Most of the protein purification procedures involve exposure of proteins to detergents, extreme pH and temperatures as well as isolation from original location. These conditions can have drastic effects on the stability of the proteins. Affinity tags are used to purify rproteins from the crude biological source using the affinity technique. These tags such as GST (glutathione-S-transferase), CBP (chitin binding protein) and MBP (maltose binding protein) and the poly (His-tag) are attached as fusion proteins to rproteins (Wilchek & Miron 1999; Lichty et al. 2005). The commonly used method of histidine tagged protein purification involves the use of immobilized metal ion affinity chromatography (IMAC) where proteins can be purified using nickel or cobalt bound columns (Donnelly et al. 2006). The histidine tag enables the protein to bind to these immobilized ions through histidine side chains, thus making the separation of that particular protein from other unwanted proteins much easier (Mondal & Gupta 2006).

Purity of a protein is not the only prerequisite for its successful use as a therapeutic and like all sub-unit vaccines, it still requires an appropriate delivery carrier system to prolong its half-life in the body and to protect its biological activity from enzymatic degradation (Kang & Singh 2001). For this purpose, biodegradable polymeric



microparticles, especially those based on PLGA, have been used successfully to enhance the therapeutic efficacy of protein drugs by safeguarding their stability and controlling their release *in vivo* (Lavelle et al. 1999). Without an efficient delivery system, even potent immunogens such as TT failed to induce protective immunity following a single administration (reviewed by Hanes, Cleland & Langer 1997). In contrast, the use of biodegradable polymeric microparticles encapsulating TT, Diphtheria toxoid, envelope glycol protein gp120 (HIV vaccine) (reviewed by Hanes, Cleland & Langer 1997) and Hepatitis B surface antigen (Feng et al. 2006) enhanced immunogenicity of these vaccines by targeting the delivery of immunogens to induce protective immunity in a sustained and controlled manner.

As mentioned in chapter 1, an *E. ruminantium* 1H12 subunit vaccine induced protection against virulent *E. ruminantium* needle challenge when used in a DNA only as well as in a DNA prime/rprotein boost strategy. However, both these vaccine strategies failed to induce protection against natural tick challenge. These findings therefore suggest that for a natural tick challenge to succeed, an even stronger immune response directed to these ORF products may have to be obtained. This could be achieved by simplifying and improving the delivery system used such as a microparticle-based controlled release delivery system. Microparticles are capable of reducing the number of vaccine administrations needed to obtain protective immunity. Moreover, microparticles can target the delivery of antigens to APCs (Gupta, Singh & O'Hagan 1998). Thus in this study, PLGA microparticles were formulated to encapsulate each of the four rErum1H12 proteins by using a double emulsion solvent evaporation technique. Microparticles should be less than 10  $\mu$ m in diameters and have smooth, non-porous surface morphology. Each formulation was aimed to encapsulate 150 µg or more of rprotein and capable of releasing intact protein on day 42 mimicking a single administration of 2 x DNA prime 1 x rprotein boost immunisation in a controlled and sustainable manner.



#### 2.2. Materials and Methods

See appendix A for list of all materials used and buffer compositions.

#### 2.2.1. Protein expression

The rproteins of the four *E. ruminantium* 1H12 ORFs (namely, rErum2540, rErum2580, rErum2550 and rErum2590 according to the Collins et al. 2005 annotation) were expressed. These ORFs were previously cloned into the pET102/D-TOPO® expression vector by using the Invitrogen Champion <sup>TM</sup> pET Directional TOPO<sup>®</sup> Expression kit, according to the manufacturer's instructions (Pretorius et al. 2008). However, in this study, the expression of rErum2540 was modified by removing the signal peptide sequence. Briefly, the forward primer specific for the rErum2540 ORF was re-designed down stream of the signal peptide (Table 1). The new forward primer and the previously designed reverse primer (Pretorius et al. 2008) were used in a PCR catalyzed by a proof reading polymerase (Pfu, Promega) that produces blunt ended products. A total of 176 ng of genomic *E. ruminantium* Welgevonden DNA was added to a reaction mixture (25 µl) containing final concentrations of  $200 \,\mu\text{M}$  of each dNTP, 200 nM of each primer, and 0.7 U Pfu in 1 x reaction buffer. The reaction was performed in the GeneAmp<sup>®</sup> PCR system 9700 from Applied biosystems. The samples were activated at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s (denaturation); 46 °C for 30 s (annealing) and 72 °C for 2 min (extension). The strands were finally extended for 7 min at 72 °C.

The PCR products were purified and concentrated using the MSB<sup>®</sup> Spin PCRapace kit (Invitek). The pET-TOPO<sup>®</sup> vector was combined with purified PCR products in a cloning reaction mixture and incubated at room temperature for 30 min. The reaction mixture was used to transform One Shot<sup>®</sup>TOP10 competent cells using heat shock (30 s at 42 °C). The transformed TOP10 cells (50 µl) were plated on LB/agar/ampicillin (Amp) (50 µg/ml) plates and incubated overnight at 37 °C. Transformed colonies were picked, grown overnight in LB/Amp (50 µg/ml) broth and DNA extracted and checked using PCR. The clones were screened for insert with PCR where Go-taq<sup>TM</sup> (Promega) 2 x master mix was used in a PCR reaction containing 0.2 µM pET Trx Fus F and T7R primers (Table 1) according to the manufacturer's instructions. This allowed the PCR products to be loaded



directly onto 1% agarose gel without the addition of loading dye for electrophoresis and ethidium bromide (0.02 mg/ml) staining. A sample of the construct with correct insert size was sequenced using the T7R and TrX Fus F to confirm that the gene of interest was in frame. The staden spin software confirmed the attachment of the His<sub>6</sub>-tag (Staden, Beal & Bonfield 1998).

| Primer     | Primer sequence $(5' \rightarrow 3')$ | $Ta (^{o}C)^{a}$ |
|------------|---------------------------------------|------------------|
| Erum2540 R | CCAAAAATTTATTAATCCTAAATTTTC           | 45               |
| Erum2540 F | CACCACATACCAGAATGGGTATTCTG            | 47               |
| T7R        | TAGTTATTGCTCAGCGGTGG                  | 55               |
| TrX Fus F  | TTCCTCGACGCTAACCTG                    | 59               |
| 3          |                                       |                  |

 Table 2.1. Primer sequences

<sup>a</sup>Ta = annealing temperature

A pilot expression was performed by transforming plasmid into electrocompetent BL21 Star<sup>TM</sup> (DE3) cells at 1.4 kV using the BIORAD Gene Pulser <sup>R</sup> II. Transformed cells were first incubated at 37 °C for 30 min followed by an overnight incubation at 37 °C in 10 ml of LB/Amp (50 µg/ml) medium. The overnight culture (500 µl) was used to inoculate 10 ml of fresh LB/Amp (50 µg/ml). The culture was grown at 37 °C until an OD<sub>600</sub> of 0.5-0.8 and was divided in two. One culture was subjected to 1 mM IPTG induction. The cultures were sampled each hour (up to 4 h) to check the expression of the His<sub>6</sub>-tagged rproteins by SDS-PAGE gel electrophoresis.

Large scale production of rproteins of all Erums were obtained by auto-induction of BL21 Star<sup>TM</sup> (DE3) bacterial cells in 1 litre of Overnight Express<sup>TM</sup> Instant TB Medium containing Amp (50  $\mu$ g/ml) following electroporation in the BIORAD gene Pulser<sup>®</sup> II at 1.4 kV. The bacterial cells were incubated overnight at 37 °C and rproteins were extracted from the cell pellet (10,000 x g, 10 min) using the BugBuster<sup>®</sup> protein extraction reagent (Novagen<sup>®</sup>). The extracted proteins were purified using the Protino<sup>®</sup> Ni-TED 1000 packed columns according to the manufacturer's instructions. The purified proteins were concentrated by centrifugation at 4,000 x g for 45 min using the Amicon<sup>®</sup> Ultra-4 Centrifugal Filter Device (5,000 MWCO, Millipore) and concentration determined using the bicinchoninic acid protein (BCA) protein assay (BCA1-1KT,



Sigma-Aldrich). The protein sizes were confirmed using SDS-PAGE gel (Criterion<sup>™</sup> XT Precast gel, 4-12% Bis-Tris from BIORAD) and anti-His<sub>6</sub> (Roche) western blot done according to the instructions of the manufacturer.

# 2.2.2. Microencapsulation

Bovine serum albumin (BSA), (Fraction V, Roche) was used as an encapsulation test protein. Biodegradable PLGA polymers with different physio-chemical characteristics ( such as molecular weight, monomer composition, viscosity and degradation) from Boehringer Ingelheim were used: Poly(D,L-lactide-co-glycolide) 50:50 (RG502H 12 kDa, RG503H 34 kDa and RG504 65 kDa); Poly(D,L-lactide-co-glycolide) 75:25 RG 752S; 80:20 and 85:15; Poly(D,L-lactide) R203S, Appendix A). Polymers with these characteristics were chosen to encapsulate BSA and rproteins, because they form microparticles with different protein encapsulation efficiencies and release profiles.

The final protocol below was developed after several adjustments of encapsulation process variables such as sonification, homogenisation, volume of internal water phase, viscosity of the internal water phase, concentration of protein and the amount of PLGA as suggested by (Jeffery, Davis & O'Hagan 1993). Microparticles were formulated using a modified double emulsion water-in-oil-in-water (w/o/w) solvent evaporation technique (Jeffery, Davis & O'Hagan 1993). Equal volumes containing 2.5% PVA (MW15,000, MP Biomedicals, Inc), in deionised water and between 0.5-3 mg/ml rprotein solution were added to an organic phase solution of 100 mg PLGA/PLA in 5 ml DCM. The mixture was sonicated (Branson 250 micro tip sonifier) for 1 min on ice to obtain a w/o primary emulsion. The primary emulsion was added drop-wise into 15-20 ml aqueous solution of 0.03% PVA before being homogenized for 1 min using the Ultra-Turrax® homogenizer (Janke and Kunkel, IKA-Labortechnik). The formed microparticles were hardened by stirring overnight at room temperature in 100 ml solution of 0.03% PVA. Microparticles were collected by centrifugation at 10,000 x g for 10 min and washed three times in distilled water. The washed microparticles were freeze-dried overnight using Edwards freeze dryer. The freeze-dried microparticles were stored in desiccators at room temperature until further use.



## 2.2.2.1. Particle size and surface morphology

A thin layer of Japan Gold Size (Winsor & Newton) artists resin was applied to an aluminium scanning electron microscope viewing stub and allowed to dry (air dry or oven dry) to a sticky, but not liquid consistency. Dried microparticles were then applied to the sticky surface of the stub using a fine (No. 1 or 2) artist's paint brush. The stub was then tapped (side on) to dislodge any loose particles. The sample was sputter coated with gold and viewed at 8 kV using a Hitachi S-2500 scanning electron microscope. Micrographs were taken at between 300-3000 X magnifications.

#### 2.2.2.2. Determination of protein loading (PL) and encapsulation efficiency (EE)

The amount of rErum protein entrapped into the microparticles was determined by digesting between 4-30 mg of microparticles overnight with end-to-end rotation at room temperature in 1.5 ml solution of 5% SDS in 0.1 M NaOH. After centrifugation at 13,000 x g for 5 min, supernatants were collected and analyzed for protein content using the BCA assay. Alternatively, between 4-30 mg of microparticles were dissolved in 1.5 ml DCM at 37 °C for 10 min. The protein was extracted twice by addition of 400  $\mu$ l distilled water followed by 5 min centrifugation at 13,000 x g. The aqueous top layers were pooled together and used in the BCA protein assay.

#### 2.2.2.3. Determination of in vitro protein release kinetics

A Float-A-Lyzer<sup>®</sup> (100,000 MWCO 3 ml capacity, Spectra/Por<sup>®</sup>) was used for *in vitro* release kinetic studies (D'Souza & DeLuca 2005). Pre-weighed amounts (between 10-60 mg) of protein-loaded freeze-dried microparticles were suspended in the release media (12 mM NaH<sub>2</sub>PO<sub>4</sub>; 75 mM NaCl; 2 mM urea and 62 mM imidazole pH 8) supplemented with 5 mM SDS as solubilising agent and 0.02% sodium azide as bacteriostatic agent. The samples were loaded into the Float-A-Lyzer<sup>®</sup> in duplicate. The Float-A-Lyzer<sup>®</sup> was incubated at 37 °C within a measuring cylinder filled with 25 ml release media for six weeks. The release media was continually stirred with a small magnetic stir bar (2 rpm). At predetermined time points (once a week) 1 ml of the release media was collected and kept at -20 °C. The collected supernatants were replaced with fresh medium. Between 15-



30 µl of the protein sample was loaded on SDS-PAGE gel (Criterion<sup>TM</sup> XT Precast gel, 4-12% Bis-Tris from BIORAD) and (800 µl) kept for BCA protein assay (Feng et al. 2006). The dialysis method was compared with the sample and separate method (Float-A-Lyzer<sup>®</sup>). Briefly, a pre-weighed (between 10-60 mg) amount of microparticles were placed in an eppendorf tube containing 1.5 ml of release buffer and shaken with a Stuart<sup>®</sup> SB3 rotary shaker set at 10 rpm and kept at 37 °C (D'Souza & DeLuca 2005; D'Souza & DeLuca 2006). At predetermined time intervals (once a week) the supernatant release media were removed after centrifugation at 13,000 x g for 5 min and replaced with an equivalent amount of fresh media. The collected supernatants were also kept at -20 °C until further use. Between 15-30 µl of the protein sample was loaded on a SDS-PAGE gel (Criterion<sup>TM</sup> XT Precast gel, 4-12% Bis-Tris from BIORAD). All the release supernatants were used in BCA protein assay. The percentages of released proteins were plotted against time to determine the *in vitro* release profile of each r1H12 protein from the microparticles.

#### 2.2.2.4. Stability of encapsulated and released proteins

The stability of proteins after undergoing the microencapsulation process was analyzed by SDS-PAGE gel electrophoresis. The alkaline hydrolysis supernatants and the *in vitro* release samples collected in the above sections were analyzed by SDS-PAGE gel electrophoresis (Criterion<sup>TM</sup> XT Precast gel, 4-12% Bis-Tris from BIORAD). About 28 µl of native or alkaline hydrolysis supernatants were mixed with 1 x XT reducing agent (BIORAD) and with sample buffer (1 x XT sample buffer, BIORAD). The mixture was heated at 100 °C for 10 min. The sample (20 µl) was loaded onto a Criterion<sup>TM</sup> XT Precast gel, 4-12% Bis-Tris from BIORAD. The gel was subjected to electrophoresis at 100 V for 90 min and proteins visualized by staining for 1 h with Bio-Safe <sup>TM</sup> Coomassie Blue G250 Stain from BIORAD or the PageSilver<sup>TM</sup> staining kit (Fermentas Life Sciences). The gel picture was captured using the AutoChemi System (UVP<sup>®</sup> BioImaging). Alternatively less than 3 mg of protein loaded freeze-dried microparticles were soaked with 50 µl of sample buffer and 20 µl loaded directly into the wells and subjected to electrophoresis as above.



# 2.2.2.5. Integrity of encapsulated and released proteins

The integrity of encapsulated and released protein was confirmed by western blotting. The mouse monoclonal antibody for the detection of histidine-tagged rprotein (Ant-His<sub>6</sub>) from Roche was used. The protein in the supernatants of the release tests and alkaline digestion supernatants were run on a SDS-PAGE gel (Criterion<sup>™</sup> XT Precast gel, 4-12%) Bis-Tris from BIORAD) and blotted (using SEMI-PHOR<sup>™</sup> TE70, Hoefer Scientific) onto a wetted polyvinylidene difluoride (PVDF) membrane (Immobilon<sup>TM</sup>-P transfer membrane 0.45 µm, Millipore) at 110 mA for 90 min. The membrane was incubated overnight with shaking at 15 rpm (GyroTwister, LABNET) in a solution of 75 ng/100 ml anti-His<sub>6</sub> antibody (Roche) in blocking buffer (1% BSA in PBS). After washing three times by rotating for 15 min at 45 rpm with wash buffer (PBS; 0.1% Tween-20) the membrane was transferred into a solution of 1/20,000 diluted anti-mouse-peroxidaseconjugate in blocking buffer and shaken for 90 min at 15 rpm at room temperature. The membrane was washed again for 15 min three times by shaking at 45 rpm in 100 ml of the wash buffer. The blots were visualized using the SuperSignal<sup>®</sup> West Pico chemiluminescence substrate from Pierce following developing and fixing of film for 1 min in the dark room.



#### 2.3. Results

## 2.3.1. Expression of rErum proteins in E.coli

At least 2 mg per protein was required for encapsulation of protein into the microparticles. The previously constructed plasmids containing the ORFs of Erum2550, - 2580 and -2590 (Pretorius et al. 2008) were used for large-scale protein expression. However, a signal peptide sequence that was not removed previously hampered the expression of rErum2540. Therefore, Erum2540 was re-cloned into the pET vector system after the exclusion of the signal peptide sequence. This was done by redesigning the forward primer sequence (Table 2.1). The thermostable proofreading polymerase was able to amplify the Erum2540 ORF and this is evident from the discrete band in lane 1 (Figure 2.1) that shows the quality and size of the PCR product. This amplified PCR product was cloned into the pET vector and clones with correct insert size were selected for sequence analyses (Figure 2.2). Sequence analysis of these clones (represented in lanes 3, 7, 9, 10 and 11 in Figure 2.2) revealed that the inserts were cloned in frame with the polyHis tail intact.



**Figure 2.1.** Agarose gel showing: Lane 1, a single band of blunt end PCR product produced using new primers specifically designed for Erum2540 gene to exclude the signal sequence. Lane 2, negative control (empty vector). Lanes M, Hae 111 Phix 174 markers.



**Figure 2.2.** Agarose gel electrophoresis of Erum2540 PCR amplified plasmids purified from colonies: Lanes 3; 7; 9; 10 and 11, plasmids with the correct size and therefore selected for sequencing.

The pDNA containing the correct insert size and sequence was transformed into BL21 (DE3) cells and induced with IPTG to produce histidine-tagged rproteins. The amount of protein expressed increased over time as can be seen in Figure 2.3 with an optimum time of harvesting between 4-5 h. There was also some background expression observed in the non-induced fractions.

For large scale production of rErum proteins the Auto-inducing Overnight Express<sup>TM</sup> TB Instant medium (Novagen<sup>®</sup>) was used and all the proteins with the correct predicted sizes were produced (Figure 2.4). The rproteins were all purified from the insoluble fraction, but soluble protein was also recovered for rErum2540. The amount of protein expressed differed for each protein. Protein expression of rErum2580 and rErum2590 was too low ( $20 \mu g/ml$ ) for EE and *in vitro* release kinetic determinations as final concentrations would be below the detection limit. The use of other protein expression *E. coli* strains such as BL21 or Rosetta2 (DE3) pLysS or expression at 18 °C did not improve protein yields. In order to obtain enough protein, expression in BL21 (DE3) had to be repeated several times.





**Figure 2.3.** *SDS-PAGE gel showing a pilot expression of rErum2540 in BL21 (DE3)* E. coli *cells following IPTG induction. The non-induced samples taken hourly were loaded in lanes 2-6 while samples taken hourly after induction were loaded in the next 5 lanes as indicated. Lane 1, molecular weight markers. The arrow indicates the rErum2540 protein band of interest.* 



**Figure 2.4.** *SDS-PAGE gel electrophoresis of four rErum proteins expressed overnight in 1 l of Overnight Express*<sup>TM</sup> *TB instant medium containing ampicillin. The arrows indicate the respective expressed proteins.* 



# 2.3.2. Optimisation of encapsulation protocol using BSA

In this study, BSA was used as a model encapsulation protein due to its hydro-solubility and commercial availability. The well-published double emulsion solvent evaporation technique was used to encapsulate BSA into Poly (D,L-lactide-co-glycolide) 50:50, trade name Resomer<sup>®</sup> (RG502H) as a test polymer. Ideally, the microparticles should have a non-porous, smooth surface and be smaller than 10  $\mu$ m.

Some of the main factors that were taken into consideration before fabrication of the microparticles were the stability of BSA and the boiling point of the organic solvent. In order to keep the fabrication condition (temperature) constant the Branson sonifier 250 and MSE ultrasonic disintegrator were chosen as the instruments for the formation of both the primary and secondary emulsions respectively. This is because the temperature can be maintained at 4 °C by keeping the emulsions in an ice bath during processing. These conditions also help keep the emulsion temperature far below 40 °C that is the boiling point of DCM. A 40 mg/ml BSA solution in water was emulsified with an 8 ml polymer in DCM solution using the Branson sonifier for 10 s at high speed to form w/o emulsion. This emulsion droplets. The microdroplets were processed as detailed in the materials and methods section. Following examination using the SEM it was clear that this procedure did not result in the formation of spherical microparticles (Figure 2.5a). Increasing the sonication time of the primary emulsion to 20 s resulted in the formation of spherical microparticles but with rough and porous surfaces (Figure 2.5b).

The next goal was to improve the conditions in such a way that microparticles with smooth surface morphology were formed. To achieve this Ultra-Turrax<sup>®</sup> homogeniser was used to form the secondary emulsion. The first attempt using the Ultra-Turrax<sup>®</sup> was undertaken using the following parameters: RG502H (100 mg) dissolved in 8 ml of DCM sonicated for 10 s with 40 mg/ml BSA dissolved in 500  $\mu$ l distilled water. This primary w/o emulsion was added into 15-20 ml of 0.03% PVA solution. After homogenising for 10 s, the formed double emulsion was hardened overnight by stirring in 100 ml solution of 0.03% PVA. Microparticles were washed three times by centrifugation for 10 min at



1000 g, freeze-dried overnight and stored in desiccators at room temperature. This resulted in the formation of irregularly shaped microparticles (Figure 2.5c) not suitable for vaccination but with smooth surface morphology. The protocol was further modified by keeping all the parameters constant except for the sonication and homogenisation times that were increased to 30 s and 60 s respectively. Although these microparticles were between 5-10  $\mu$ m, and spherical in shape, these modifications were unsuccessful, because their surfaces were porous (Figure 2.5d).



**Figure 2.5.** SEM micrographs of RG502H microparticles loaded with BSA. Microparticles formed using the MSE ultrasonic disintegrator to prepare the double-emulsion (A and B). Microparticles fabricated using the Ultra Turrax<sup>®</sup> to form the double emulsion (C and D). Microparticles prepared with 10 s sonication and 10 s homogenization times (C). Microparticles formed by increasing sonication time to 30 s and the homogenisation time to 60 s (D). Micrographs were taken at 3000 X (A and B) and at 6000 X (C and D) magnifications.

The formation of microparticles with less than  $5 \,\mu m$  diameters and with a smooth spherical shape (Figure 2.6) was finally achieved by decreasing the BSA concentration



from 40 mg/ml to 22 mg/ml in the internal water phase and using the longer sonication and homogenisation times. This encapsulation protocol resulted in two different microparticle sizes: One was highly individualized with small (~2  $\mu$ m) microparticles (Figure 2.6a) characterised by smooth surface morphology, while the other population had bigger (~5  $\mu$ m) microparticles (Figure 2.6b) with small minor dimples on their surfaces. Thus optimum conditions for encapsulation using BSA were: 100 mg RG502H polymer, 30 s sonication, 60 s homogenisation and a protein concentration of 22 mg/ml. These conditions were also tested on RG504 polymer (high molecular weight and hydrophobic) and microparticles with similarly good morphological features were formed (not shown).



**Figure 2.6.** SEM micrographs showing highly spherical RG502H-BSA microparticles with non-porous surfaces and diameter of less than  $2 \mu m$  (A) and highly spherical RG502H-BSA microparticles with non-porous surfaces and diameter of less than is  $5 \mu m$  (B). Both microparticles were formed by using the protocol with longer homogenisation (60 s) and lower concentration of BSA. Micrographs were taken at 6000 X magnification.

# 2.3.3. Encapsulation of rErum proteins using different polymers

The conditions for encapsulating proteins were used as optimised with BSA. Expression of the rErum proteins resulted in inclusion body formation of all rproteins except for rErum2540 that was isolated from both the soluble protein and inclusion body (insoluble) fractions as mentioned previously. Isolation of protein from the insoluble fraction



required the addition of chaotropic agents such as urea that might influence particle formation. Therefore, further optimisation of encapsulation was required for the expressed rErum proteins. The insoluble rErum proteins were first concentrated using the Amicon<sup>®</sup> Ultra Centrifugal filters to obtain 3 mg/ml solution before encapsulation into RG504 polymer using the optimised RG502H-BSA encapsulation conditions (30 s sonication and 60 s homogenisation) with the exception of protein concentration made difficult by low protein yield. The microparticles encapsulating rErum2590, rErum2550 and rErum2580 were not individualised (aggregated) and the surface morphology was rough and porous with foam-like appearance (Figure 2.7a). In contrast, the non-concentrated rErum2540 insoluble protein (500 µg/ml) was successfully encapsulated (Figure 2.7b) with smooth ~2 µm size particles but the EE and the PL were below the detection limits of microBCA protein assay and that of SDS-PAGE and silver staining.



**Figure 2.7.** *SEM micrographs of rErum protein loaded-microparticles showing effect of protein concentration: (A), RG504 microparticles (representative of all insoluble rErum proteins) encapsulating concentrated insoluble rErum2590 (3 mg/ml). (B), RG504 microparticles encapsulating diluted rErum2540 insoluble protein (500 µg/ml). Micrographs were taken at 3000 X magnification.* 

Increasing the volume of the internal water phase from  $500 \,\mu$ l to 2 ml in order to try to dilute the rprotein in the suspension did not improve the morphology of RG504 microparticles (Figure 2.8a). These microparticles were irregularly shaped with sponge-like appearance (Figure 2.8a). Encapsulation of 2 ml of insoluble, concentrated



rErum2540 into copolymer PLGA 80:20 resulted in spherically shaped microparticles with small diameters but hollow with obvious cracks on their surfaces, which may lead to increase in burst release (Figure 2.8b).



**Figure 2.8.** *SEM micrographs of rErum protein loaded-microparticles showing the effect of increasing the volume of internal water phase:* RG504 *microparticles prepared by increasing the volume of internal water phase containing insoluble rErum2540 from 0.5 ml to 2 ml (A), and PLGA 80:20 microparticles encapsulating 2 ml of rErum2540 insoluble protein (B). Micrographs were taken at 3000 X (A) and at 6000 X (B) magnifications.* 

Lactide homopolymer R203S and co-polymer PLGA 85:15 were successfully used to encapsulate a lower concentration of insoluble rErum2540 ( $500 \mu$ l solution) and rErum2590 respectively (Figures 2.9). However, the EE and PL were also very low (data not shown). These results show that the volumes of internal water phase also play a role in morphology of microparticles.





**Figure 2.9.** SEM micrographs of diluted (less than 1 mg/ml) insoluble rErum2540 protein loaded into R203S microparticles (A) and rErum2590 loaded into PLGA 85:15 microparticles (B). Micrographs were taken at 3000 X magnification.

Co-encapsulation of 2.5% PVA with both the soluble and insoluble rproteins (500  $\mu$ l solution) resulted in the improvement of the EE irrespective of the polymer and protein concentration used (Table 2.2). The addition of PVA surfactant in the internal water phase did not only increase the EE and PL efficiency of microparticles but also resulted in microparticles with satisfactory physical characteristics such as 5  $\mu$ m and lower diameters, spherical shape as well as smooth and non-porous surface morphology (Figure 2.10-2.13). Thus all subsequent protein encapsulations were carried out with PVA as internal viscosity enhancer.

The four rErum proteins were expressed, concentrated (if very dilute i.e. less than 500  $\mu$ g/ml) and encapsulated with addition of PVA using different polymers and the PL and EE determined as indicated in Table 2.2. The lowest encapsulation efficiency was 48% for microparticles formulated using RG504 polymer and insoluble rErum2540 protein. The protein loading ranged from 3.4 to 59.3  $\mu$ g/mg. Copolymer PLGA 80:20 encapsulated with rErum2540 soluble protein gave the highest protein loading. Initial low protein concentrations, especially for rErum2580 and rErum2590, influenced the protein



loading values: the higher the isolated protein concentration the better PL values were obtained. The protein loading of insoluble protein was lower than that of soluble protein.

|            | rErum2540(s) |                 | rErum2540(i)  |     | rErum2590(i) |     | rErum2580(i)            |     | rErum2550(i) |     |
|------------|--------------|-----------------|---------------|-----|--------------|-----|-------------------------|-----|--------------|-----|
|            | $PL^{a}$     | EE <sup>b</sup> | PL            | EE  | PL           | EE  | PL                      | EE  | PL           | EE  |
| Polymers   | µg/mg        | %               | µg/mg         | %   | µg/mg        | %   | µg/mg                   | %   | µg/mg        | %   |
|            |              |                 |               |     |              |     |                         |     |              |     |
| RG502H     | 18.3         | 100             | n/d           | n/d | <b>9.5</b> ° | 100 | 3.4                     | 100 | 11.8         | 100 |
| RG504      | 29.8         | 100             | 12.1          | 48  | 8.4          | 100 | <b>6.1</b> <sup>c</sup> | 100 | 30.0         | 100 |
| PLGA 85:15 | n/d          | n/d             | <b>10.5</b> ° | 84  | 3.05         | 79  | 7.6                     | 87  | 9.1          | 100 |
| R203S      | n/d          | n/d             | 6.9           | 67  | n/d          | n/d | n/d                     | n/d | n/d          | n/d |
| RG752S     | 22.2         | 92              | n/d           | n/d | n/d          | n/d | n/d                     | n/d | n/d          | n/d |
| PLGA 80:20 | 59.3°        | 100             | n/d           | n/d | n/d          | n/d | n/d                     | n/d | n/d          | n/d |
| RG503H     | 7.6          | 94              | n/d           | n/d | n/d          | n/d | n/d                     | n/d | n/d          | n/d |

Table 2.2. The protein loading (PL) and Encapsulation Efficiency (EE) of protein loaded microparticles.

<sup>a</sup>PL = amount of protein loaded per milligram of microparticles.

<sup>b</sup>EE = expressed as the percentage of protein detected in the microparticles relative to the amount added in the internal water phase. <sup>c</sup>Bold values = best polymer to use for encapsulation of each rErum protein.

(i) = insoluble protein.

(s)= soluble protein.

n/d, not done because of too low concentration.

In addition, a variation of the size and surface morphology of the different polymers in combination with different rproteins used was observed. The R203S and RG504 (Figure 2.10 and 2.11 respectively) microparticles had a uniform size distribution while RG502H and PLGA 80:15 microparticles had a bimodal (two distinct particle sizes) size distribution (Figure 2.12 and 2.13). Polymer PLGA 85:15 when used to encapsulate the insoluble protein resulted in slightly bigger diameters than other polymers encapsulating insoluble proteins (Figure 2.13). RG502H gave a slightly rougher surface morphology when encapsulating insoluble rprotein (Figure 2.12). Most of RG502H microparticles were very tiny (in nanometer size range). They looked coarser as though they were glued together with no spaces between them. Bigger ones were hollow with a few microcaves.



When encapsulating insoluble protein a suspension of 1 mg/ml was found to be the maximum optimal suspension for formation of microparticles with required features. The internal aqueous phase volume has to be 1 ml or less preferably 500 µl. For soluble proteins, any polymer could be used for encapsulation to a maximum of 3 mg/ml. RG502H and RG504 could even be used to encapsulate 6 mg/ml provided PVA is incorporated in the inner phase.



**Figure 2.10.** SEM micrographs of R203S microparticles encapsulating rErum2540 insoluble protein representative of all insoluble rproteins prepared with this polymer. PVA was used as internal phase viscosity enhancer. Micrographs were taken at 3000 X magnification.



**Figure 2.11.** SEM micrographs of RG504 microparticles encapsulating rErum2540 insoluble protein representative of all insoluble rproteins prepared with this polymer. PVA was used as internal phase viscosity enhancer. Micrographs were taken at 3000 X magnification.





**Figure 2.12.** SEM micrographs of R502H microparticles encapsulating rErum2580 insoluble protein representative of all insoluble rproteins prepared with this polymer. PVA was used as internal phase viscosity enhancer. Arrows show two distinct particle sizes. Micrographs were taken at 3000 X magnification.



**Figure 2.13.** SEM micrographs of PLGA 85:15 microparticles encapsulating rErum2540 insoluble protein representative of all insoluble rproteins prepared with this polymer. PVA was used as internal phase viscosity enhancer. Micrographs were taken at 3000 X magnification.



# 2.3.4. Stability of protein loaded into and released from microparticles

The stability of BSA encapsulated into RG502H microparticles was tested before that of encapsulated rErum proteins. To achieve this, RG502H-BSA microparticles were alkaline digested or loaded directly onto the gel. BSA in the digestion mixture and directly extracted from microparticles was analyzed by SDS-PAGE gel electrophoresis. The electrophoretic mobility, the aggregation and fragmentation of encapsulated BSA was compared to that of native BSA (Figure 2.14). The microencapsulation process did not have any detrimental effect on the structural stability of BSA (Figure 2.14). Similarly, the estimated molecular weights of the rproteins remained the same following encapsulation into RG502H, RG504 and PLGA 85:15 polymers and over 6 months period of storage in desiccators kept at room temperature (Figure 2.15). No extra bands representing degradation or multimer formation were visible. Microparticles dissolved in SDS-PAGE sample buffer and loaded directly onto a SDS-PAGE gel (Figure 2.16) resulted in higher protein yields for gel analysis than alkaline digestion of DCM dissolution.



**Figure 2.14.** Integrity of BSA removed from microparticles as shown by Coomassie stained SDS-PAGE gel. Lane 1, native BSA. Lane 2, BSA removed from RG504 microparticles following alkaline digestion overnight. Lane 3, BSA directly extracted into the well following loading of 2 mg of RG504-BSA microparticles soaked in SDS-PAGE gel electrophoresis sample loading buffer. Lanes M, molecular weight markers.



**Figure 2.15.** Silver stained SDS-PAGE gel of r1H12 proteins before and after encapsulation into microparticles. Freeze-dried microparticles encapsulating rproteins were stored for 6 months in dessicators at room temperature. Lane 1, native rErum2590; Lanes 2-4, rErum2590 after encapsulation into RG502H, RG504 and PLGA 85:15 microparticles respectively and removal by alkaline digestion overnight. Lane 5, native rErum2580; Lanes 6 and 7, rErum2580 removed from PLGA 85:15; Lane 8, rErum2580 removed from RG504 microparticles. Lanes M, molecular weight markers. Arrows show bands of interest.



**Figure 2.16.** SDS-PAGE gel electrophoresis of soluble rErum2540 protein. Lane 1, native rErum2540 soluble protein. Lane 2, rErum2540 soluble protein extracted from RG504 microparticles directly loaded onto SDS-PAGE gel. Lane M, molecular weight markers.



To test the stability of BSA released from microparticles, samples (~20  $\mu$ l) of the *in vitro* release experiments were analysed by SDS-PAGE gel electrophoresis. BSA released from Float-A-Lyzer<sup>®</sup> was below the limit of detection by silver stain (results not shown). In order to detect the stability of released BSA, the collected supernatant had to be concentrated by acetone precipitation or by centrifugal filter devices before loading the gel. Released protein could be detected on day three. The centrifugal filter device was more effective than precipitation to yield detectable protein (Figure 2.17a). Detection of low concentration BSA was also achieved by adding small pieces of PVDF membrane into the bulk (PBS pH 7.4) release media a day before the sampling point. The membrane was able to trap the limited amount of BSA released by microparticles into the release media (PBS pH 7.4). Direct loading of PVDF pieces on SDS-PAGE gel showed that BSA degradation first observed in Figure 2.14 was increased by carrying out release experiment in PBS (Figure 2.17b). The single BSA sharp band observed previously at 66 kDa (Figure 2.14 lane 1 and Figure 2.17a) was spilt into two bands (Figure 2.17 b).



**Figure 2.17.** SDS-PAGE gel electrophoresis of BSA released from poly (D, L-lactide-co-glycolide) 50:50 (RG502H) microparticles following incubation in Float-A-Lyzer<sup>®</sup> in 1 x PBS, pH 7.4 at 37 °C: BSA released on day three of in vitro incubation and concentrated using the Amicon<sup>®</sup> Ultra centrifugal tubes (A) and loaded on the gel (Lane 1). Lanes 1-2, BSA released and trapped on PVDF strips (B) on day 14 and 21 (Lanes 1-2). Lane 3, BSA released on day 7. Lane M, molecular weight markers. Arrow shows BSA degradation.


Measurement of the pH of the release medium using pH test strips (SIGMA<sup>®</sup>) on the days of sampling indicated that the PBS pH dropped from 7.4 to 6. Therefore, the stability and *in vitro* release profile of rErum proteins were tested in release media ( $12 \text{ mM NaH}_2PO_4$ , 75 mM NaCl, 2 mM urea and 62 mM imidazole pH 8) supplemented with 0.02% sodium azide as a bacteriostatic agent and 5 mM SDS as a solubilizing agent to stabilize the pH. The stability of rproteins during a time release study from microparticles was determined using either the Float-A-Lyzer<sup>®</sup> (dialysis) or the sample and separate method (eppendorf). Generally, there were no obvious signs of protein aggregation and hydrolysis attributed to microencapsulation or release characterised by generation of bigger and smaller protein fragments respectively. The stability of rproteins was still intact for the duration of the release study irrespective of the method used (Figure 18). This means that the release media pH was constant even in the Float-A-Lyzer® where a small amount of fresh medium was added during the sampling time points (Figure 18b). Thus, this release medium was used in all ensuing studies unless stated otherwise. Only the sample and separate method was used to determine the stability of rErum2590 (Figure 18a) and 2550 (Figure 18c) due to the release of a low concentration of proteins from microparticles. Protein was constantly released starting from day 1 of *in vitro* incubation. However, lower protein concentrations were detected at week 2 (Day 14) when using the sample and separate method (Figure 2.18c lane 5 and 2.20b lane 5). Even at week 6 microparticles still released intact protein thus further indicating that both the encapsulation procedure and the release medium did not harshly affect the stability of released proteins (Figure 2.19a).

Microparticles were formulated using polymers with lower glycolide ratios (PLGA 80:20 and RG752S) because of their reported slow degradation rates. These microparticles also released proteins with unaltered structural integrity when incubated *in vitro* for 6 weeks (Figure 2.19a and b respectively). Even RG502H and RG503H microparticle (with fastest degradation rate) encapsulating soluble rErum2540 rprotein did not have a negative effect on the stability of rprotein (Figure 2.20). A high molecular weight polymer RG504 was also used to encapsulate rErum2540 insoluble protein (Figure 2.21). No significant



protein degradation was observed, because bands were identical for the native and entrapped rproteins.



**Figure 2.18.** *SDS-PAGE gel electrophoresis and silver staining of insoluble rproteins released from RG504* (A and B) and RG502H (C) microparticles following 37 °C incubation in eppendorf tube (A and C) and *Float-A-Lyzer*<sup>®</sup> (B) containing release medium (12 mM NaH<sub>2</sub>PO<sub>4</sub>, 75 mM NaCl, 2 mM urea and 62 mM imidazole pH 8) supplemented with 5 mM SDS and 0.02% sodium azide. Both gels: Lanes M, molecular weight markers. Lanes 1, native rErum2590, 2580 and 2550 respectively. Lane 2-8, rErum2590 released from microparticles on day 1, 7, 21, 28, 37, 42 and 49 respectively (A). Lane 2-6, rErum2580 released from microparticles on day 7, 14, 21, 28 and 42 respectively (B). Lane 2, rErum2550 removed from alkaline digestion of RG502H microparticles (C). Lanes 3-6, rprotein released from microparticles on day 1, 7, 14 and 28 respectively.





**Figure 2.19.** *SDS-PAGE gel electrophoresis and silver staining of rErum2540 soluble rproteins released from PLGA 80:20 (A) and RG752S (B) microparticles. Both gels: Lanes M, molecular weight markers. Lane 1, native rprotein. Lane 2, rprotein removed following alkaline digestion of microparticles. Lane 3-8, rprotein released from microparticles on day 1, 7, 14, 21, 28 and 42 respectively in a Float-A-Lyzer*<sup>®</sup> *suspended in release buffer kept at 37 °C.* 





**Figure 2.20**. SDS-PAGE gel electrophoresis and silver staining of rErum2540 soluble rproteins removed and released from RG502H (A) and RG503H (B) microparticles. Both gels: Lanes M, molecular weight markers. Lane 1, native protein. Lane 2, rprotein removed following alkaline digestion of microparticles. Lanes 3-9, rprotein released from microparticles on day 1, 7, 14, 21, 28, 37 and 42 respectively (A). Lanes 3-6, rprotein released from microparticles on day 1, 7, 14 and 28 respectively (B). RG502H (A) microparticles were incubated at 37 °C in Float-A-Lyzer<sup>®</sup> suspended in 25 ml of 12 mM NaH<sub>2</sub>PO<sub>4</sub>; 75 mM NaCl; 2 mM urea and 62 mM imidazole pH 8 supplemented with 0.02% sodium azide as bacteriostatic agent and 5 mM SDS at 37 °C. RG503H microparticles (B) were incubated at 37 °C in a 2 ml eppendorf tube.





**Figure 2.21.** SDS-PAGE gel electrophoresis and silver staining of rErum2540 insoluble rproteins released from RG504 microparticles following 37 °C incubation in eppendorf tube (A) and Float-A-Lyzer<sup>®</sup> (B) containing release medium (12 mM NaH<sub>2</sub>PO<sub>4</sub>; 75 mM NaCl; 2 mM urea and 62 mM imidazole pH 8) supplemented with 5 mM SDS and 0.02% sodium azide). Both gels: Lanes M, molecular weight markers. Lanes 2-7, rprotein released from microparticles on day 1, 7, 21, 28, 37, 42 and 49 respectively. Lane 8, rprotein extracted from microparticles following alkaline digestion (A). Lane 2, rprotein extracted from microparticles following alkaline digestion from microparticles on day 1, 7, 21, 28, and 42 respectively (B).



The integrity of the encapsulated and released rErum2540 protein was further evaluated by western blotting. According to western blots of both the protein extracted and released from microparticles, the microencapsulation procedure preserved the integrity of the encapsulated protein (Figure 2.22). This further confirms earlier analysis of the protein stability by SDS-PAGE (Figure 2.19a).



**Figure 2.22.** Western blot of treated and native rproteins. Lane M, His<sub>6</sub>-tag markers. Lane 1, the native Erum2540 soluble rprotein, Lane 2, rprotein removed from alkaline digested PLGA 80:20 microparticles while lanes 3 and 8, rprotein released from PLGA 80:20 microparticles incubated in Float-A-Lyzer<sup>®</sup> on day 1, 7, 14, 28, 37, and 42 respectively as shown in Figure 2.19a.

## 2.3.6. *In vitro* release kinetics of BSA and r1H12 protein from PLGA microparticles

The *in vitro* release experiments were done to identify those microparticles that release proteins in a manner that mimics the DNA prime/rprotein boost vaccination strategy. They should have a low initial burst release and the release should start on day 42 following *in vitro* incubation. RG502H-BSA microparticles were investigated first, because RG502H has a faster bio-erosion rate according to the literature (Feng et al 2006).

A Float-A-Lyzer<sup>®</sup> was used to analyze the release of BSA from microparticles (Figure 2.23) because a detectable amount of BSA was loaded into the microparticles as indicated by EE. This is because in the Float-A-Lyzer<sup>®</sup> proteins are released into a big volume of release media. To avoid BSA aggregation and degradation shown previously (Figure



2.17) the following release medium was used (12 mM NaH<sub>2</sub>PO<sub>4</sub>, 75 mM NaCl, 2 mM urea and 62 mM imidazole pH 8) supplemented with 5 mM SDS as solubilizing agent and 0.02% sodium azide as a bacteriostatic agent. Samples were taken from the release media at pre-determined time points. The corresponding determined protein concentrations were plotted against time and a sigmoidal like BSA release profile was observed (Figure 2.23). The RG502H microparticles released BSA in three phases. The initial protein burst release (day 1) was 30%, followed by lag phase (days 6-8) with little or no protein release and lastly the fast release phase (day 12-46) where 100% of BSA was released. Within 4 weeks of *in vitro* incubation, BSA was completely released from the microparticles. Before continuing with the incubation of rErum protein loaded microparticles, RG504-BSA microparticles were subjected to in vitro release under similar conditions as the RG502H-BSA microparticles for comparison. Again, a release profile characterised by three phases was observed. In contrast to RG502H-BSA, microparticles RG504-BSA microparticles had a higher initial burst release (~40%; day 1) and similar lag release phase (days 6-8; Figure 2.23). RG504-BSA released only 80% of BSA during the four weeks duration of the experiment. rErum loaded microparticles were evaluated under similar conditions.





**Figure 2.23.** The in vitro release kinetics of BSA from RG502H and RG504 microparticles. The microparticles were loaded in Float-A-Lyzer<sup>®</sup> kept in (12 mM NaH<sub>2</sub>PO<sub>4</sub>, 75 mM NaCl, 2 mM urea and 62 mM imidazole pH 8) supplemented with 5 mM SDS and 0.02% sodium azide at 37 °C for 6 weeks. Samples (1 ml) were taken 2-3 times a week.

Recombinant proteins released from microparticle formulations were polyphasic irrespective of the test method used (sample and separate method Figure 2.24 and dialysis or Float-A-Lyzer<sup>®</sup> Figure 2.25). The release patterns were characterized by an initial burst release (Day 0-7) due to protein adsorption and diffusion from microparticle surfaces, slow release phase (Day 7-28) and second release phase or the fast release phase (Day 28-42). Generally, the sample and separate method (Figure 2.24) showed the highest initial burst release ranging from 20-80% while the dialysis test method (Figure 2.25) had the lowest initial burst release of 10-45%. RG504 microparticles, encapsulating soluble rErum2540 protein showed the lowest burst release of 10% (Figure 2.25). The release of rErum2550 from RG502H was biphasic characterised by ~52% burst release and a further ~5% release from day 14-42 (Figure 2.24a). rErum2540 (insoluble) was released from RG503H in a triphasic manner (burst release ~65%, lag phase followed by a second release phase where ~10% of rprotein was released) (Figure 2.24a). Polymers with 50:50 monomer compositions had the lowest initial burst release (15-70%) (Figure



2.24a) in comparison to the polymers with low/no glycolic acid (Figure 2.24b) with initial burst release ranging from 45-80%. The highest initial burst release of 80% was from the homopolymer R203S encapsulating insoluble rErum2540 (Figure 2.24b).

The dialysis test method displayed a pulsatile (delivery of proteins in an initial burst followed by a trickle release or discontinuous) release profile of protein from RG752S, RG502H and PLGA 80:20 microparticles (Figure 2.25). RG504 (Figure 2.25) and RG502H (Figure 2.25) microparticles had the fastest release completed within 6 weeks of incubation in a dialysis membrane and on day 14 both the microparticles released a very minimal amount of rprotein.

From this data and based on the Float-A-Lyzer<sup>®</sup> of RG504-BSA (Figure 2.23) and sample and separate method of RG504-rErum2550 (Figure 2.24a), RG504 microparticles had protein release patterns that best met the criteria for DNA prime/rprotein boost immunisation. RG504 microparticles were still physically visible in the eppendorf tubes even after the termination of release experiments, thus showing signs of slower bio-erosion rate not observed with highly hydrophilic polymers. However, gel pictures shown in Figures 18 and 21 indicate that RG504 microparticles release rprotein in a continuous manner as determined by both *in vitro* release procedures.

In contrast, PLGA 80:20 and RG752S also met the requirements according to Float-A-Lyzer<sup>®</sup> release profiles. These microparticles still have protein to be released even after day 42 of *in vitro* incubation. SDS-PAGE gel pictures show that the release of rproteins from PLGA 80:20 (Figure 2.19a) and RG504 (Figure 2.21b) microparticles was similar. These microparticles seem to double their protein release during each week of incubation. The release of proteins from RG502H (Figure 2.19a) and RG752S (Figure 2.20b) microparticles appear to be constant after week 2 of incubation.





**Figure 2.24.** In vitro release profiles of insoluble (i) rErum proteins from PLGA microparticles formulated using polymers with different characteristics as indicated in the legend. The microparticles were incubated in (12 mM NaH<sub>2</sub>PO<sub>4</sub>, 75 mM NaCl, 2 mM urea and 62 mM imidazole pH 8) supplemented with 0.02% sodium azide and 5 mM SDS in an eppendorf tube rotated with Stuart<sup>®</sup>SB3 rotator at 37 °C for 6 weeks. Representative microparticles of polymers with 50:50 monomer compositions (A). Representative microparticles of polymers with lower glycolic acid monomer ratio (B). Samples (1 ml) were taken weekly.





**Figure 2.25.** In vitro release kinetics of rErum2540 soluble rprotein from PLGA microparticles formulated using polymers with different characteristics. The microparticles were loaded in Float-A-Lyzer<sup>®</sup> kept in (12 mM NaH<sub>2</sub>PO<sub>4</sub>, 7 mM NaCl, 2 mM urea and 62 mM imidazole pH 8) supplemented with 5 mM SDS and 0.02% sodium azide at 37 °C for 6 weeks. Samples (1 ml) were taken weekly.



#### 2.4. Discussion

Adequate delivery of antigen to APCs, antigen processing and presentation of the antigenic fragments to T-cells are the prerequisites for potent cellular immune responses against infectious diseases, tumours and intracellular bacteria (reviewed by Heit et al. 2008). DCs and macrophages are important role players in the presentation of antigen to T-cells. Because of their particulate nature, microparticles were shown to be capable of furnishing the APCs with the antigen for presentation to T-cells in a pulsatile manner, thus sustaining the immune response to that antigen (reviewed by Gupta, Singh & O'Hagan 1998; Audran et al. 2003). The use of FITC-BSA MP demonstrated the importance of the size of particle in order for that particle to be phagocytozed by APCs. Microparticles of less than 10 µm in diameter have been shown to be the most attractive to professional antigen presenting cells (reviewed by Jiang et al. 2005). It was shown that nanoparticles promote cellular immune responses while micron-sized particles induce antibody-mediated responses (reviewed by Jiang et al. 2005). In most cases, the two immune responses complement each other (reviewed by Gupta, Singh & O'Hagan 1998; Kanchan & Panda 2007). This context was the major focus of the microparticles formulations that we have developed. Most of the microparticles presented here have diameters less than 5  $\mu$ m with majority of the particles below 1  $\mu$ m in size. This was achieved by increasing the sonication time from 10 s to 1 min during encapsulation. The use of sonicators to prepare the primary water-in-oil emulsion has been reported to play a role in the final morphology of microparticles (Bilati, Allemann & Doelker 2005a). Microparticles with small diameters were developed encapsulating four E. ruminantium rproteins of interest. These microparticles therefore have the potential of stimulating the cellular immune system.

One of the morphological features of the microparticles that is emphasized most in literature is a smooth surface area (Kang & Singh 2001). Microparticles with smooth surface areas and limited number of surface pores are capable of releasing the antigens over a prolonged period depending on the rate of polymer degradation. These microparticles have low burst release of their entrapped materials. Our micrographs also allude to the achievement of this second goal upon which our formulations were focused.



This feature might be very useful when a DNA prime/rproteins boost vaccination strategy is used, where cationic microparticles that release pDNA earlier and microparticles encapsulating the rproteins released later for boosting is used.

In order to determine the EE of the rproteins, microparticles were initially disrupted by DCM and the protein extracted in PBS. This procedure yielded very low amounts of the protein from microparticles thus emphasizing earlier observations by (Feng et al. 2006) where they recovered more protein out of the microparticles following alkaline digestion than with DCM disruption. DCM extraction resulted in a cloudy layer of BSA in the interface of DCM and PBS. It was then decided to digest the microparticles at room temperature overnight using 0.1 M NaOH/5% SDS. This procedure resulted in release of most encapsulated protein from the microparticles thus making the calculation of encapsulation efficiency more accurate.

Another major purpose of the study was to form microparticles loaded with at least 150 µg rproteins per formulation. Using Resomer<sup>®</sup> RG502H and RG504 and soluble rErum2540 protein, encapsulation efficiencies were achieved that tripled the targeted amount. Microparticles formulated with polymer containing a low glycolide ratio (PLGA 80:20, PLGA 85:15 and RG752S) gave the highest encapsulation efficiencies and protein loading. The addition of 2.5% PVA in the inner water phase during encapsulation resulted in increased protein loading and EE irrespective of the polymer used. Similar results were reported by (Yang, Chung & Ng 2001) when encapsulating BSA into PLGA 65:35. These investigators suggested that this was caused by interaction of PVA with BSA. This interaction protected BSA from the solvent thus preventing its diffusion into the external aqueous phase (Yang, Chung & Ng 2001).

One objective of microencapsulation is the retainment of the stability of the encapsulated antigen after formation, storage and during *in vitro* release from the microparticles (Bilati, Allémann & Doelker 2005b). It is well known that stability of protein antigen is believed to determine the quality of the antibody immune response to that antigen (reviewed by Hanes, Cleland & Langer 1997). The integrity of the protein antigen is very important for



neutralizing antibody responses and antibody affinity maturation (reviewed by Hanes, Cleland & Langer 1997; Lu 2006; Pauyo et al. 2006; David et al. 2007). Thus if the protein released from microparticles is not of the same conformation as the native protein, affinity maturation will select for antibodies with lower affinity or different specificity for the native protein. This will result in the production of antibodies that cannot effectively neutralize the pathogen. However, in this study antibody immunity was not the main focus of these formulations but rather cellular immunity because it was demonstrated to play a protective role against heartwater infection (reviewed by Totté et al. 1999). Therefore, some degradation of protein during microencapsulation and release is permissable in this study as long as such degradation does not cause the antigenic epitope of the protein to be degraded. This is because T-cell receptors that activate T-cell responses recognise short linear sequences of amino acids. Stability would have been an issue if the main intention was to induce neutralizing antibody responses mediated by Bcell receptors that recognise the three dimensional structure (native form) of specific amino acid sequences (reviews by Hanes, Cleland & Langer 1997; O'Hagan Derek. 1998).

During microencapsulation especially when using the w/o emulsion solvent evaporation technique the protein of interest is exposed to harsh conditions such as organic solvents, the polymer itself and shearing forces that promote the denaturation or degradation of the antigen (reviewed by O'Hagan Derek. 1998). SDS-PAGE gel electrophoresis or western blotting techniques were used to monitor the stability of the protein following exposure of the protein to these unfavourable conditions. In our study, the stability of the rErum proteins was not affected by microencapsulation and prolonged storage in desiccators kept at room temperature. This is because proteins are protected within the microparticles. Western blots demonstrated that the integrity of proteins was not altered by the microencapsulation and *in vitro* release procedures as compared to the native protein. This means that the nature of the immune response elicited by the encapsulated protein may be similar to the one elicited by the native protein.



Another focus of this study was to formulate microparticles that are capable of releasing protein antigen in a controlled and sustained manner preferably with low initial burst release. The rproteins should only be released at day 42 of *in vitro* incubation thus mimicking the DNA prime/rproteins boost vaccine regimen. Based on the *in vitro* release profiles the protein-encapsulated microparticles were not capable of mimicking the 2 x DNA prime/1 x rproteins boost single dose vaccine. To our knowledge, no one has ever reported on an *in vitro* release study that resembles the 2 x DNA prime/1 x rproteins following a single administration. Only those microparticles with low initial burst release and incomplete release at day 42 of incubation could better mimic the DNA prime/rproteins boost single dose vaccine. Microparticles made of PLGA 80:20 and RG752S were still releasing rproteins even after the duration of the study as evaluated by Float-A-Lyzer<sup>®</sup>. However, all the microparticles could be successful in a 2 x DNA prime/1 x rproteins boost vaccination that administers DNA and rproteins separately (two immunisations).

Preferably, the *in vitro* release experiments should be carried out at pH 7.4 in PBS, which is nearest physiological condition (Crotts & Park 1997; reviewed by Giteau et al. 2008). Unfortunately, earlier experiments using BSA and rErum proteins demonstrated the instability of these proteins in those conditions. This shows that the buffering capacity of PBS is affected by acidic monomers from polymer degradation. The release medium (12 mM NaH<sub>2</sub>PO<sub>4</sub>, 75 mM NaCl, 2 mM urea and 62 mM imidazole pH 8) supplemented with 0.02% sodium azide as a bacteriostatic agent and 5 mM SDS as a solubilizing agent was demonstrated to be an optimal release buffer for insoluble rproteins. Urea and SDS detergents are capable of stabilizing the encapsulated protein during its transition from solid (freeze-dried state) to liquid state (reviewed by Giteau et al. 2008). They showed that urea (protein unfolding agent) increased release of lysozyme by more than 10%. In this study, polymer degradation did not lower the pH of the release medium (12 mM NaH<sub>2</sub>PO<sub>4</sub>, 75 mM NaCl, 2 mM urea and 62 mM imidazole pH 8) supplemented with 0.02% sodium azide as a bacteriostatic agent and 5 mM SDS as a solubilizing agent) but it did lower the pH of the PBS release medium to as low as 6. The main priority for the *in vitro* release experiments is the complete release of protein in its native state followed by



the release in near *in vivo* (physiological) conditions (reviewed by Giteau et al. 2008). This means that it might be a good idea to include a pH stabilizer to the encapsulated protein for *in vivo* studies.

Most of our microparticle formulations were characterized by high initial burst release with the exception of RG504 microparticles loaded with insoluble rErum2550 protein as evaluated by the sample and separate method. The initial burst release can be regarded as the measure of the amount of protein associated with the surface of microparticles. This means that the homopolymer R203S microparticles gave the highest initial burst release because they had most of the proteins on the surface, thus making these microparticles useful only when rproteins are needed to prime the immune response and when humoral immunity is a necessity. Humoral immunity requires the activation of B-cells, which in turn requires presentation of multiple copies of antigens on microparticles surfaces (reviewed by O'Hagan 2006). Therefore, R203S could be suitable for encapsulation of pDNA, because fast release kinetics will be needed for pDNA to prime the immune response.

According to the sample and separate method, RG502H, RG503H and RG504 microparticles did not release on week 2 (Day 14) as demonstrated by SDS-PAGE gel electrophoresis and *in vitro* release curves or profiles. This was due to the fact that the microparticles were in the lag phase of protein release as mentioned in chapter 1. Lag release depends on the rate of protein diffusion out of the inner core of microparticles. In this phase all the proteins initially associated with the surface of microparticles were already washed away during the initial burst. The lag phase is preferred in this study and further work is required to prolong this phase over 3 weeks, thus enabling the rprotein-loaded microparticles to be used to reduce the 3 x administrations of 2 x DNA prime/1 x rproteins boost vaccine to one administration. These results suggest that the sample and separate method is more sensitive to analyse burst release and lag release phase than the dialysis test method. This is because with the sample and separate tube method a small amount of release medium is used whereas larger volumes are used in dialysis method.



These results indicate that the choice of test method is very critical especially when the microparticles are intended for in vivo administration. It is therefore better to choose the test method that better mimics the *in vivo* conditions (Kostanski & DeLuca 2000). This is the reason why the dialysis method was used as an alternative in vitro release test method. The Float-A-Lyzer<sup>®</sup> method is more reliable than the tube method because the microparticles are not subjected to harsh treatment that can influence the release of proteins such as the resuspension of microparticles following centrifugation after each sampling time. There is also no accumulation of acidic monomers such as glycolic and lactic acids as these are washed away by the bulk release media in which the dialysis membrane is suspended. This also helps to maintain a constant pH. The pulsatile release manner displayed by most of the microparticles, means that microparticles can be useful for booster immunization needed to provoke secondary immune responses (Sah, Toddywala & Chien 1995). These release patterns characterised by high initial burst release followed by a sustained delivery of protein with lag time release of proteins mimics the priming of immune response followed by administration of small boosters given close to each other. This could lead to a prolonged supply of proteins to the immune system (Audran et al. 2003). The pulsatile release is regarded as the most effective regimen in the design of vaccine formulations (Sah, Toddywala & Chien 1995).

In conclusion, four r1H12 proteins were successfully encapsulated in microparticles made of different polymers. The inclusion of PVA as the internal phase viscosity enhancer resulted in the formation of microparticles with generally smooth surface morphology, less than 10 µm diameters, spherical shape, high protein loading and EE irrespective of the polymer used. The structural integrity of the protein was not altered by the encapsulation procedure. Microparticles could also be stored at room temperature in the desiccators for long periods with the retainment of protein stability. The release of rproteins were sustained and polyphasic. RG504 had the best release profile characterised by the lowest initial burst release, thus making it a polymer of choice for prime/boost vaccine trial. Future work needs to focus on ways to reduce the initial burst release of proteins. The pulsatile release manner means that the microparticles could make a good auto-booster vaccine. Because the ultimate goal of this work is to use microparticles to



improve the delivery of pDNA prime/rproteins boost vaccine, this study also focussed on the adsorption and encapsulation of pDNA onto/into polymeric microparticles as described in chapter 3.



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### Chapter 3:

# DNA preparation and encapsulation into and adsorption onto biodegradable microparticles

#### 3.1. Introduction

Genetic immunization has revolutionized the field of vaccinology. This is because of several advantages that are not often seen in other vaccine formulations such as the possibility of multivalent vaccines by combining different genes, ease of transport in freeze-dried form, simplicity of manufacturing and the ability of DNA vaccine to elicit desired cell mediated and antibody mediated responses (reviewed by Dhama et al. 2008). This immunisation strategy involves the delivery of genes encoding a protective protein antigen derived from a pathogen and its transcription and translation in the host cells. *In vivo* translation should result in a protein that resembles the original and would therefore be able to induce a specific immune response (reviewed by Dhama et al. 2008). The gene is transcribed with the help of a eukaryotic or viral promoter (such as cytomegalovirus); poly-adenylation signal sequence and termination sequences all of which are incorporated into the expression plasmid. In addition, the plasmid should also contain a bacterial origin of replication to assist replication of the plasmid in bacteria as well as an antibiotic resistance gene, which helps with the selection of transformed bacteria.

Once the protein is expressed in the mammalian host, posttranslational modification is required for folding into its native three-dimensional conformation which in turn is needed to provoke the immune responses especially the antibody response. Proteins that contain signal peptides (either naturally or added into the plasmid vector) would be exported from the host cell in its native state where the protein would be available for uptake by macrophages or dendritic cells. The protein is processed into antigenic peptides for presentation on either MHC II for CD4<sup>+</sup> helper T-cell recognition or/and activation and on MHC I for CD8<sup>+</sup> cytotoxic T-cell activation (Bowersock & Martin 1999). The helper T-cells secrete cytokines that help to activate the necessary immune cells.



Naked pDNA has been used successfully to provoke the necessary antigen specific immune responses to infectious human diseases (Díez & Tros de Ilarduya 2006) following administration via different routes such as the intradermal (i.d.), intramuscular (i.m), subcutaneous (s.c.) and intranasal (Pouton & Seymour 2001). Either way it is possible for DNA to reach APCs such as dendritic cells that can lead to elicitation of the CMI responses. Cell mediated immunity can be induced by addition of CpG motifs found in bacterial genomic DNA. CpG motifs can only induce pseudo-protective immune responses by activating DC IL-12 expression upon binding to host toll-like receptors (reviewed by Rothenfusser.et al. 2002). Adjuvants could be included in the genetic vaccination strategy to increase the magnitude and duration of antigen specific immune responses. So far, several adjuvants such as those based on alum salts and cytokines have been used with DNA vaccines. Alum salts mainly enhance the humoral immune response, while genetic adjuvants (cytokines) can stimulate both humoral and/or CMI responses depending on the type of cytokine administered. Cytokines are low molecular weight proteins often secreted in a transient manner with the ability to manipulate the duration and the intensity of immune response. Cytokine encoding plasmids used as DNA vaccine adjuvants can help manipulate differentiation and expansion of Th1 and Th2 cytokine producers. Th1 cytokines such as IL-2, IL-12, IL-15, IL-18 and IFN-γ promote CMI response while Th2 cytokines such as IL-4 will favour antibody response (Greenland, Letvin 2007). The major limitations of cytokine adjuvants are their short half-lives in vivo (Greenland & Letvin 2007; Dhama et al. 2008). In addition, if the cytokine concentration is too high or too low, it may induce a non-desirable effect (Atkins et al. 1997; Greenland & Letvin 2007). Thus, the choice of adjuvants as well as the method of delivery may help enhance a particular immune response (Shedlock & Weiner 2000).

DNA is often prone to degradation by DNAses and clearance by lysosomes in phagocytes (Díez & Tros de Ilarduya 2006). Thus, DNA carriers are often required to enhance DNA delivery. New generation DNA vaccines incorporate DNA carriers for optimal targeting to the appropriate host cells and immune response activation. These gene delivery systems can be either viral or non-viral. Viral vectors are most commonly used for this



purpose and retrovirus, adenovirus and herpes virus are some of the preferred viral vectors used for delivery of exogenous genes into mammalian cells (Zhang & Godbey 2006). They contain genes from other organisms and are engineered to retain the ability to infect cells, but not replicate after introduction into the host. These advances have lead to gene therapy, which involves injection of foreign genes that could cure disease into host cells such as human and animal cells. Gene therapy has been used to treat inherited disorders by replacing defective genes with copies of normal genes (Whittlesey & Shea 2004; reviewed by Luten et al. 2008).

Recently non-viral gene delivery systems such as cationic lipids and cationic polymers have been receiving much attention (Little et al. 2005). The main reason being that viral gene vectors are inherently immunogenic (reviewed by Luten et al. 2008) and can only take up a limited size of the DNA insert (reviewed by Dhama et al. 2008). Cationic polymers such as PLGA-CTAB (Singh et al. 2003) and PLGA-PEI have been used widely to deliver pDNA vaccine to mice and induced protective immune responses (Oster et al. 2005). Among the non-viral gene delivery systems, biodegradable polymeric carriers are the most preferred because of their reduced toxicity and the avoidance of accumulation of polymers in the site of administration. Another advantage is that more pDNA can be targeted to the APCs due to phagocytosis of particulate material in the micron to submicron range. PLGA and chitosan polymers are the ideal polymers to use when it comes to pDNA vaccine delivery because they are non-toxic, biodegradable, and can be formulated to be administered once and provoke the necessary immune responses over time. Microparticles can be formed using the microencapsulation technology to contain pDNA vaccine in their core or on their surfaces by addition of cations such as CTAB and PEI (reviewed by O'Hagan 2006).

Formulation of DNA vaccines by adsorption to cationic microparticles is a powerful means of increasing vaccine potency. Adsorption of HIV *gag* DNA (one of HIV vaccine candidates) to cationic microparticles increased CD8<sup>+</sup> T-cell and antibody responses in mice by 100 and 1000 fold respectively as compared to naked DNA immunisation. In another report *Ag85A* (DNA vaccine candidate against tuberculosis) DNA adsorbed onto



cationic PLG microparticles induced protective immune responses against aerosol challenged mice using doses of PLG-DNA two orders of magnitude lower than was required for naked DNA immunisation (Mollenkopf et al. 2004; reviewed by O'Hagan 2006). Moreover, single immunisation of mice with cationic microparticles adsorbing hepatitis B small envelope (HBsAg) DNA resulted in the availability of HBsAg expressing APCs in draining lymph nodes at 24 h and day 14 post inoculation (He et al. 2005).

The principal drawback of microencapsulation is the stability or integrity of vaccine antigen, which is at risk, especially during the homogenization and polymer degradation stages (reviewed by Hanes, Cleland & Langer 1997; Jilek, Merkle & Walter 2005). Similarly, heat generated during homogenization can denature DNA being encapsulated thus leading to conversion of super-coiled (SC) pDNA into a linear form which has the lowest transfection efficiency. By using agarose gel electrophoresis the integrity and stability of encapsulated DNA can be investigated (Bilati, Allemann & Doelker 2005). If the stability and integrity are affected by the microencapsulation process, coencapsulation of DNA with stabilizers such as NaHCO<sub>3</sub> can be included to preserve the stability of the DNA. Homogenization and sonication has to be carried out on an ice bath to keep DNA as cool as possible. The addition of an antacid such as magnesium hydroxide could overcome DNA degradation caused by polymer degradation induced pH fluctuations. Alternatively, adsorption of pDNA onto cationic microparticles can be used to avoid problems associated with microencapsulation such as: slow release rate of DNA from microparticles, low EE, DNA degradation and denaturation which limits the amount of DNA available for transfection of target cells and induction of immune response (O'Hagan et al. 2001; Kasturi, Sachaphibulkij & Roy 2005; Jilek, Merkle & Walter 2005; O'Hagan, Singh & Ulmer 2006). In this approach, blank microparticles were formed that contain cationic agents such as CTAB and PEI on their surfaces. These positive charges were then used to adsorb negatively charged pDNA. This enhances the amount of SC DNA immediately available for APCs after i.m. administration of microparticles (Takahata et al. 1998; Singh et al. 2000; Wischke et al. 2006).



The goal of this study was to develop microparticles encapsulating and adsorbing four 1H12 *E. ruminantium* ORFs cloned into a pCMViUBs mammalian expression vector (Collins et al. 2003). The adsorbed DNA was formulated in such a way that it is released within 24 h of administration and the encapsulated DNA released at day 21 after administration thus giving the possibility for inherent prime/booster effects without repeated inoculations.



#### 3.2. Materials and Methods

See appendix A for list of all materials used and buffer compositions

#### 3.2.1. Plasmid DNA expression

Four 1H12 ORFs (Erum2550, 2540, 2590 and 2580) were previously cloned into the pCMViUBs mammalian expression vector (Collins et al. 2003). Large scale production was done by transforming the pCMViUBs\_ORF constructs into TOP10 *E. coli* cells and the plasmids were amplified by the TOP10 cells overnight in 1000 ml LB/amp (50  $\mu$ g/ml) broth. The plasmids were purified using NucleoBond<sup>®</sup> Xtra Maxi Plus kit, according to the instructions of the manufacturer. A sample of each of the pCMViUBs\_ORF constructs were sequenced.

#### 3.2.2. DNA encapsulation

Microparticles were formulated using a modified double emulsion w/o/w solvent evaporation technique (Jeffery, Davis & O'Hagan 1993). Equal volumes containing (2.5% PVA, M.W.15,000, MP Biomedicals, Inc) in Tris-EDTA (10 mM Tris-HCl 1 M Na<sub>2</sub>EDTA pH 8.5) and 0.5-3 mg/ml pDNA of the *E. ruminantium* ORFs was added to an organic phase solution of 100 mg polymer in 5 ml DCM. The mixture was sonicated (Branson 250 micro tip sonifier) for 1 min on ice to obtain a w/o primary emulsion. The primary emulsion was added drop-wise into a 15 ml aqueous solution of 0.03% PVA before being homogenized for 1 min using the Ultra-Turrax<sup>®</sup> homogenizer (Janke and Kunkel, IKA-Labortechnik). The formed microparticles were hardened by stirring them overnight at room temperature in 100 ml solution of 0.03% PVA. Microparticles were collected by centrifugation at 10,000 x g for 10 min and washed three times in distilled water. The washed microparticles were freeze-dried overnight using the (Edwards High vacuum). The freeze-dried microparticles were stored in desiccators at room temperature until further use. Alternatively, the aqueous solution of pDNA was first mixed with an equal volume of 0.5% CTAB solution for 30 min at room temperature. The formed complex was used as internal aqueous phase and subjected to encapsulation as described above.



#### 3.2.3. DNA adsorption onto microparticles

Blank cationic microparticles were used to adsorb pDNA. The same double emulsion solvent evaporation technique described above was used to form microparticles with some modifications. Briefly, 200 mg of polymer dissolved in 10 ml methylene chloride was emulsified with ~500 µl of TE buffer (10 mM Tris-HCl, 1 M Na<sub>2</sub>EDTA pH 8.5) at a high speed using a Branson 250 sonifier to obtain a water-in-oil emulsion. To this emulsion, 50 ml of 0.5% CTAB solution was added. The mixture was stirred overnight at 300 rpm with a magnetic stirrer in an open beaker to evaporate the organic solvent. Hardened microparticles were washed twice in deionized water by centrifugation at 10,000 x g for 10 min using a Sorvall<sup>®</sup>RC-5B Refrigerated Superspeed centrifuge (Du Pont Instruments). Freeze-dried microparticles (100 mg) were incubated with 1 ml TE buffer (10 mM Tris-HCl, 1 M Na<sub>2</sub>EDTA pH 8.5) containing 500 µg pDNA at 4 °C for 6 h on platform shaker for adsorption (Oster et al. 2005). Following centrifugation at 13,000 x g for 10 min, the supernatant was removed and used in the determination of adsorption efficiency. The pellet was freeze dried and sent for electron microscope examination.

#### 3.2.4. Particle size and surface morphology by electron microscopy

A thin layer of Japan Gold Size (Winsor & Newton) artists resin was applied to an aluminium scanning electron microscope viewing stub and allowed to dry (air dry or oven dry) to a sticky, but not liquid consistency. Dried microparticles were then applied to the sticky surface of the stub using a fine (No. 1 or 2) artist paint's brush. The stub was then tapped (side on) to dislodge any loose particles. The sample was sputter coated with gold and viewed at 8 kV using a Hitachi S-2500 scanning electron microscope. Micrographs were taken at between 300 X and 3000 X magnifications.

#### 3.2.5. Encapsulation (EE) and Adsorption Efficiency (AE)

The percentage of pDNA adsorbed relative to the amount of pDNA used during adsorption was determined by measuring the  $A_{260}$  nm of the supernatant following adsorption and centrifugation at 13,000 x g for 10 min using the NanoDrop<sup>®</sup> ND-1000 Spectrophotometer. Alternatively, the amount of adsorbed and encapsulated DNA was



determined by using an extraction technique (Barman et al. 2000). In this method, dried microparticles (2.5-10 mg) were resuspended in equal amounts (200  $\mu$ l-500  $\mu$ l) of TE buffer (10 mM Tris-HCl, 1 M Na<sub>2</sub>EDTA pH 8.5) and DCM in a 2 ml eppendorf tube. After 90 min of end-to-end shaking at room temperature, the mixture was subjected to centrifugation at 13,000 x g for 5 min and the top aqueous layer containing the DNA was removed. The amount of pDNA in the supernatant was quantified by measuring A<sub>260</sub> nm using a NanoDrop<sup>®</sup> ND-1000 spectrophotometer.

## 3.2.6. Determination of *in vitro* release kinetics and stability of DNA associated with microparticles

Between 10-60 mg of microparticles were incubated in 1.5 ml of TE buffer (10 mM Tris-HCl, 1 M Na<sub>2</sub>EDTA pH 8) supplemented with 0.01% of Tween-80 as a wetting agent and 0.02% sodium azide as a bacteriostatic agent at 37 °C for 21 days. At predetermined time points (Day 1, 7, 14 and 21); the microparticles were subjected to centrifugation at 13,000 x g for 10 min to separate the supernatant. All of the release media were sampled and replaced with an equivalent amount of fresh media. Microparticles were further incubated with rotation at 10 rpm using the Stuart<sup>®</sup> rotary SB3 rotator until the next sampling time point. The collected supernatant was stored at -20 °C until further use. The supernatant sample from extraction procedure and *in vitro* release were used in agarose gel electrophoresis to compare the stability to that of untreated pDNA. Between 10-20  $\mu$ l of DNA sample mixed with loading dye was loaded onto 1% agarose gel containing 0.4  $\mu$ g/ml ethidium bromide in TBE Buffer (100 mM Tris, 90 mM Boric acid, 1 mM EDTA pH 8.4). The gel was run for 2 h at 80 V and 140 mA and visualized under AutoChemi<sup>TM</sup> UVP BioImaging system.



#### 3.3. Results

#### 3.3.1. Microparticles size and surface morphology

Microparticles have to be less than 10  $\mu$ m in diameter, have smooth surface morphology and be spherical in shape with highest encapsulation efficiency (EE) and DNA loading (DL) efficiency in order to qualify as a vaccine. Scanning electron microscopy was used to analyze the physical characteristics of the microparticles (Figure 3.1-3.5). In general, all polymers used here made microparticles with required morphological features. RG504 polymer made microparticles with slightly smaller diameter than the more hydrophilic polymer RG502H (Figure 3.1a). These microparticles had uniform size distribution while RG502H microparticles seem to have two distinct populations (Figure 3.1b).



**Figure 3.1.** SEM micrographs of RG504 microparticles encapsulating Erum2590 pDNA (A) and RG502H microparticles encapsulating Erum2590 pDNA (B) representative of all pDNA encapsulated into this polymers. Micrographs were taken at 3000 X magnification.

Encapsulation of CTAB-pDNA complexes with RG504 (Figure 3.2a) and RG752S (Figure 3.2b) polymers resulted in microparticles with diameters less than  $3 \mu m$ , with smooth and non-porous surface morphology. It is interesting to note that these microparticles are highly individualized (not aggregated) compared to those encapsulating naked DNA. However, RG752S microparticles were slightly bigger in size than RG504 microparticles but still within the required size range.





**Figure 3.2.** SEM micrographs of RG504 microparticles encapsulating 0.5% CTAB-Erum2550 pDNA complexes (A). RG752S 0.5% CTAB-Erum2540 pDNA complexes (B) representative of all pDNA encapsulated into this polymer. Micrographs were taken at 3000 X magnification.

Cationic microparticles (microparticles with 0.5% CTAB on their surfaces) made with RG203S (Figure 3.3), RG503H and RG502H (Figure 3.4) had similar physical features. These microparticles were denser and less than 1  $\mu$ m in diameter and spherical in shape. Adsorption of DNA onto RG504 cationic microparticles resulted in the increase in microparticles diameters from 1  $\mu$ m to 4  $\mu$ m which is still within the required size range (Figure 3.5). Figure 3.6 shows that the diameter can also go above 10  $\mu$ m suggesting that care should be taken when using this polymer to adsorb DNA.



**Figure 3.3.** SEM micrographs of R203S-0.5% CTAB adsorbed Erum2580 pDNA representative of all pDNA adsorbed onto this polymer. Micrographs were taken at 3000 X magnification.



**Figure 3.4.** SEM micrographs of RG503H-0.5% CTAB adsorbed Erum2540 representative of all pDNA adsorbed onto this polymer. Micrographs were taken at 3000 X magnification.





**Figure 3.5.** *SEM micrographs of RG504 cationic microparticles without pDNA (RG504 0.5% CTAB) (A) and adsorbing Erum2550 pDNA (RG504 0.5% CTAB Erum2550 (B). Micrographs were taken at 3000 X magnification.* 



**Figure 3.6.** SEM micrographs showing the increase in size of RG504 cationic microparticles after Erum2550 pDNA adsorption (RG504 0.5% CTAB). Micrographs were taken at 3000 X magnification.

#### 3.3.2. Encapsulation (EE) and Adsorption efficiency (AE)

The EE, which is a measure of the amount of pDNA incorporated into microparticles, was measured following dissolution of the matrix by organic solvent. The EE ranged from 0-95%, while the DNA loading expressed as the amount of pDNA per milligram of freeze



dried microparticles ranged from  $0-2 \mu g/mg$  (Table 3.1). In general, RG502H (hydrophilic polymer) was able to reach EE as high as 55.4% and DNA loading of 2 µg/mg without further manipulation of the protocol. Low glycolic acid polymer such as RG752S and PLGA 85:15 needed addition of excipients in the internal water phase to improve their DNA loading characteristics from 0% to as high as 95% and 7% respectively (Table 3.1). Complexing Erum2540 pDNA with 0.5% CTAB improve the EE of RG752S microparticles from 0% (not shown) to 22.1% (shown in bold) (Table 3.1). Preparation of PVA in 0.1 M NaHCO<sub>3</sub> dramatically improved EE of Erum2550 into RG752S microparticles from 0% (not shown) to 95% (shown in bold). The DNA loading increased from 0 to 440 ng/mg. EE of Erum2580 pDNA into R203S also improved when NaHCO<sub>3</sub> was added.

| Plasmid DNA |          |                 |          |      |          |                          |          |       |  |  |  |
|-------------|----------|-----------------|----------|------|----------|--------------------------|----------|-------|--|--|--|
|             | Erum2550 |                 | Erum2590 |      | Erum2540 |                          | Erum2580 |       |  |  |  |
| Polymer     | DL       | EE              | DL       | EE   | DL       | EE                       | DL       | EE    |  |  |  |
|             | µg/mg    | %               | µg/mg    | %    | µg/mg    | %                        | µg/mg    | %     |  |  |  |
|             |          |                 |          |      |          |                          |          |       |  |  |  |
| RG502H      | 2.0      | 22.7            | 1.03     | 55.4 | 0.06     | 30                       | 1.5      | 5     |  |  |  |
| RG504       | 0.03     | 22              | 0.06     | 30   | 0.06     | 14.5                     | 0.125    | 1.25  |  |  |  |
| R203S       | 0.17     | 0.56            | 0        | 0.33 | 0.015    | 0.46                     | 0.13     | 27.2* |  |  |  |
| RG752S      | 0.44     | 95 <sup>b</sup> | 0        | 0    | 0.35     | <b>22.1</b> <sup>a</sup> | 0        | 0     |  |  |  |
| 85:15       | -        | -               | -        | -    | 0.10     | 6.8                      | 0.10     | 7.1*  |  |  |  |

Table 3.1. DNA loading (DL) and Encapsulation Efficiency (EE %) of PLGA microparticles

<sup>a</sup>Complexing pDNA with 0.5% CTAB <sup>b</sup>Preparation of PVA in 0.1 M NaHCO<sub>3</sub>

The amount of pDNA adsorbed onto cationic microparticles (0.5% CTAB) was measured from supernatant that remained following adsorption of DNA and DCM dissolution of microparticles. It was found that DCM dissolution was less effective for AE determination, because of the accumulation of polymer residue on the interface of DCM and aqueous solution thus making sampling of supernatant containing DNA difficult. The results shown (Table 3.2) were based on the estimation of DNA in the adsorption supernatant. Erum2540 pDNA was used as a test pDNA. It was adsorbed onto several polymers and the one exhibiting highest loading efficiencies was chosen and used to



adsorb the other Erum plasmids. Once again, hydrophilic polymers RG502H and RG503H gave the best AE and DL. The homopolymer R203S had the lowest DL (0.04  $\mu$ g/mg), but interestingly high AE (99%). Because adsorption of all Erum plasmids onto RG502H gave satisfactory results (microparticle size, surface morphology and DNA loading efficiencies), there was no need to try other polymers with all the plasmids. RG502H microparticles were chosen for stability testing of all plasmids based on the highest EE exhibited following DCM disruption (Table 3.1) and analysis of DNA in adsorption supernatant (Table 3.2).

| Plasmid DNA |          |          |          |      |          |       |          |       |  |  |  |  |
|-------------|----------|----------|----------|------|----------|-------|----------|-------|--|--|--|--|
|             | Erum2540 |          | Erum2550 |      | Erum2580 |       | Erum2590 |       |  |  |  |  |
| Polymer     | $DL^{a}$ | $AE^{b}$ | DL       | AE   | DL       | AE    | DL       | AE    |  |  |  |  |
|             | (µg/mg)  | (%)      | (µg/mg)  | (%)  | (µg/mg)  | (%)   | (µg/mg)  | (%)   |  |  |  |  |
|             |          |          |          |      |          |       |          |       |  |  |  |  |
| RG502H      | 0.28     | 99.7     | 18.34    | 99.9 | 3.31     | 98.67 | 4.67     | 91.68 |  |  |  |  |
| RG503H      | 2.5      | 99.56    | -        | -    |          |       | -        | -     |  |  |  |  |
| RG504       | 0.47     | 51.3     | -        | -    |          |       | -        | -     |  |  |  |  |
| R203S       | 0.04     | 99       | -        | -    | 0.04     | 99    |          |       |  |  |  |  |

Table 3.2. DNA loading (DL) and Adsorption Efficiency (AE %) of PLGA cationic microparticles

<sup>a</sup>Amount of pDNA encapsulated per milligram of freeze-dried microparticles

<sup>b</sup>Percentage of p DNA encapsulated relative to amount added into internal water phase

#### 3.3.3. Stability of plasmid DNA

The structural stability of pDNA encapsulated into, adsorbed onto and released by PLGA microparticles was analyzed in comparison with that of non-treated native pDNA. Agarose gel electrophoresis was used to show the stability of pDNA following association with microparticles (Figure 3.7-3.14). Following encapsulation of pDNA into RG502H polymer, microparticles were stored for 6 months in dessicators at room temperature. Slight DNA degradation or smears were observed following examination of Erum2590 and Erum2550 pDNA extracted from microparticles (Figure 3.7). Almost equal proportions of open circular (OC) and super-coiled (SC) DNA were observed in both treated and non-treated pDNA (Figure 3.7, 3.8 and 3.10). This probably suggests that encapsulation is not the only factor behind conversion of DNA from SC to OC.


The initial release experiments were carried out in PBS pH 7.4. Degradation of Erum2550 pDNA was observed following DCM disruption of RG502H microparticles (Figure 3.7). This was elevated after *in vitro* release experiments in PBS (Figure 3.8 lanes 4 and 6.). Release experiments were thereafter carried out in TE buffer (Tris-HCl 10 mM, Na<sub>2</sub>EDTA 1 M pH 8) in an effort to reduce degradation by improving the buffer capacity of the media. However, this did not effectively reduce DNA degradation (Figure 3.9-3.10). RG502H microparticles with non-porous surface morphology (because of addition of PVA as internal phase viscosity enhancer) and therefore reduced initial burst release of encapsulated pDNA shows that TE buffer did not negatively affect the stability of pDNA (Figure 3.10). Complexing pDNA with 0.5% CTAB before encapsulation improved the stability of pDNA during encapsulation and release during the kinetic study as the DNA degradation smear disappeared (Figure 3.11). The long incubation resulted in most of Erum2540 pDNA existing in OC form after day 21 (Figure 3.11). The stability of DNA released on day 21 (Figures 3.8, 3.9 and 3.11) was difficult to judge because of mobility retardation possibly due to aggregation. Adsorption of Erum2580 and 2540 pDNA onto RG502H 0.5% CTAB microparticles did not significantly alter the stability of pDNA (Figure 3.12 and 3.13). Similar results were observed following adsorption of other Erum2540 pDNA onto RG504 0.5% CTAB microparticles (Figure 3.14). Erum2550 and 2590 pDNA also behaved the same when adsorbed onto cationic RG504 and RG502H microparticles (results not shown). The SC topology was the most dominant form after 24 h of in vitro incubation.



**Figure 3.7.** Agarose gel electrophoresis showing the stability of pDNA extracted from RG502H microparticles following encapsulation and 6 months storage at room temperature in a dessicators. Lanes *M*, Lamda DNA ladder. Lanes 1, 3, 5 and 7, non-treated Erum2540, 2590, 2550 and 2580 pDNA respectively. Lanes 2; 4 and 6, extracted Erum2540, 2590 and 2550 respectively. Lanes 8 and 9, Erum2580 pDNA extracted from RG502H microparticles. SC, super-coiled. OC, open circular.





**Figure 3.8.** Agarose gel electrophoresis showing the stability of Erum2550 pDNA released from RG502H microparticles following encapsulation and in vitro incubation for 28 days in PBS pH 7.4 supplemented with 0.01% Tween-80 and 0.02% sodium azide at 37 °C with rotating on Stuart<sup>®</sup> SB3 rotator. Lanes M, Lamda DNA ladder. Lane 1, non-treated pDNA. Lanes 2-6, pDNA released on day 1, 7, 14, 21 and 28 respectively. SC, super-coiled. OC, open circular.



**Figure 3.9.** Agarose gel electrophoresis of Erum2590 pDNA released by porous RG502H microparticles following encapsulation and in vitro incubation for 22 days in TE buffer(Tris-HCl 10 mM, Na<sub>2</sub>EDTA 1 M pH 8) supplemented with 0.01% Tween-80 and 0.02% sodium azide at 37 °C with rotating on Stuart<sup>®</sup> SB3 rotator. Lanes M, Lamda DNA ladder. Lane 1, non-treated pDNA. Lane 2, pDNA extracted from microparticles. Lanes 3-6, pDNA released on Day 1, 7, 14 and 22 respectively. SC, super-coiled. OC, open circular.



**Figure 3.10.** Agarose gel electrophoresis showing the stability of Erum2590 pDNA released from nonporous RG502H microparticles following encapsulation and in vitro incubation in TE buffer (10 mM Tris-HCl Na<sub>2</sub>EDTA 1 M pH 8) supplemented with 0.01% Tween-80 and 0.02% sodium azide at 37 °C with rotating on Stuart<sup>®</sup> SB3 rotator. Lanes M, Lamda DNA ladder, Lane 1, non-treated pDNA; Lanes 2-9, pDNA released on day 1, 7, 14, 21, 28, 35, 42 and 45 respectively.SC, super-coiled. OC, open circular.



**Figure 3.11.** Agarose gel electrophoresis of Erum2540 pDNA-0.5% CTAB complexes released from RG752S microparticles following encapsulation and in vitro incubation in TE buffer (10 mM Tris-HCl, 1 M Na<sub>2</sub>EDTA pH 8) supplemented with 0.01% Tween-80 and 0.02% sodium azide at 37 °C with rotating on Stuart<sup>®</sup> SB3 rotator. Lanes M, Lamda DNA ladder, Lane 1, non-treated pDNA and Lanes 2-7, pDNA released on day 1, 7, 14, 21, 28 and 35 respectively. SC, super-coiled. OC, open circular.



**Figure 3.12.** Agarose gel electrophoresis showing the stability of Erum2580 pDNA released from RG502H-0.5% CTAB microparticles following adsorption and in vitro incubation in TE buffer (10 mM Tris-HCl, Na<sub>2</sub>EDTA 1 M pH 8) supplemented with 0.01% Tween-80 and 0.02% sodium azide at 37 °C with rotating on Stuart<sup>®</sup> SB3 rotator. Lane M, Lamda DNA ladder, Lane 1, non-treated pDNA. Lanes 2 and 3, pDNA released on day 1 and 7 respectively. SC, super-coiled. OC, open circular.



**Figure 3.13.** Agarose gel electrophoresis showing the stability of Erum2540 pDNA released from RG502H-0.5% CTAB microparticles following adsorption and in vitro incubation in TE buffer (10 mM Tris-HCl,1 M Na<sub>2</sub>EDTA, pH 8) supplemented with 0.01% Tween-80 and 0.02% sodium azide at 37 °C with rotating on Stuart<sup>®</sup> SB3 rotator. Lane M, Lamda DNA ladder, Lane 1, non-treated pDNA and Lanes 2 and 3, pDNA released on day 1 and 7 respectively. SC, super-coiled. OC, open circular.



**Figure 3.14.** Agarose gel electrophoresis showing the stability of Erum2540 pDNA released from RG504H-0.5% CTAB microparticles following adsorption and in vitro incubation in TE buffer (10 mM Tris-HCl,1 M Na<sub>2</sub>EDTA pH 8) supplemented with 0.01% Tween-80 and 0.02% sodium azide at 37 °C with rotating on Stuart<sup>®</sup> SB3 rotator. Lane M, Lamda DNA ladder, Lane 1, non-treated pDNA. Lanes 2 and 3, to pDNA released on day 1 and 7 respectively. SC, super-coiled. OC, open circular.

#### 3.3.4. In vitro release kinetics of pDNA

The *in vitro* release kinetic experiments of pDNA by microparticles were done in 2 ml Eppendorf tubes. The Float-A-Lyzer<sup>®</sup> was used first, but it was later realized that the pDNA molecular size could not enable it to go through the dialysis membrane because of small MWCO, thus rendering detection of released DNA difficult. Only microparticles that exhibited detectable EE and DNA loading were used in the release kinetics experiments. Because pDNA from all four ORFs was similar in size, microparticle formulations were assessed under assumption that they all probably display similar release behaviour. This implies that the release profile of Erum2540 in RG7525 (Figure 3.15a) could be regarded as the representative profile of all Erum ORFs. Encapsulation of pDNA into smooth RG502H microparticles reduced the burst release of pDNA by microparticles (Figure 3.15a). This is seen by 20% release of pDNA observed during the first 2 weeks of *in vitro* incubation using the sample and separate method. The release of DNA encapsulated into microparticles was generally biphasic characterized by low burst release phase (Day 0-7) followed by the fast release phase (Day 7-28).

Adsorption of DNA onto cationic microparticles resulted in the highest burst release as well the fastest DNA release (Figure 3.15b). The microparticles released between 50-90% of adsorbed DNA within 24 h of *in vitro* incubation. Microparticles encapsulating pDNA-0.5% CTAB complexes (RG752S-Erum2540) only started to release on day 7 and



completed on day 21 of *in vitro* incubation (Figure 3.15a). Thus, adsorption resulted in the fastest release of pDNA from microparticles, completed in week 3 of *in vitro* incubation. DNA encapsulated into microparticles was not completely released from microparticles during 28 days of *in vitro* incubation. These findings are supported by agarose gel electrophoresis (Figures 3.9-3.14). The results show that encapsulation of pDNA could be manipulated to control the release of pDNA from RG502H microparticles by inclusion of PVA as internal phase viscosity enhancer. PVA inclusion resulted in lower burst release profile as shown by low detection of pDNA (Figure 3.10 lane 2-4) while adsorption of pDNA onto cationic microparticles resulted in the highest burst release (reaching 90%) of DNA (Figures 3.12-3.14).





**Figure 3.15.** In vitro cumulative release patterns of pDNA from PLGA microparticles incubated in TE buffer (10 mM Tris-HCl, 1 M Na<sub>2</sub>EDTA pH 8) supplemented with 0.01% Tween-80 as a wetting agent and 0.02% sodium azide as a bacteriostatic agent. The microparticles were rotated on Stuart<sup>®</sup> SB3 rotator kept at 37 °C: microparticles encapsulating pDNA (A) and cationic microparticles with pDNA adsorbed onto surface and encapsulating CTAB-pDNA complexes (B). p. porous. s, smooth non-porous surface.



#### 3.4. Discussion

A single dose of microparticles should be able to replace several booster immunizations needed to achieve protective immune responses in sheep challenged with virulent E. ruminantium. The aim of this study was to formulate microparticles adsorbing and encapsulating plasmid E. ruminantium DNA. These microparticles should be small with diameters below 10 µm, spherical in shape and able to release pDNA within 24 h and on day 21 after inoculation. Based on these objectives and the results obtained in this study the microparticles formulated here met the requirements for a microparticle-based DNA vaccine that has the potential to replace the conventional DNA vaccine that requires several administrations to achieve protection. In general, the *in vitro* release experiments indicated that microparticles were formed that released pDNA during each week of incubation using RG502H polymer. Non-porous RG502H microparticles encapsulating pDNA had low burst release and only released 100% of DNA on day 21 of incubation. Adsorption of pDNA onto RG502H-0.5% CTAB made microparticles that released 50% of DNA during day 1 while complexing pDNA with CTAB followed by encapsulation into copolymer RG752S made microparticles that released 40% pDNA on day 7 and 20% on day 14. We also observed that RG502H microparticles could be manipulated to release encapsulated pDNA on day 21 and 28 of *in vitro* incubation by addition of PVA as internal phase viscosity enhancer. Thus, a 2 x DNA prime could be achieved using a mixture of microparticles releasing DNA at different time points.

One concern for DNA vaccine production is the nature of the immunogenic pDNA (Cooke et al. 2004). pDNA exists in one of the three main forms namely the SC, OC and the linear form. As the SC pDNA is very compact and less prone to enzymatic degradation in host cells, it is the most effective of the three forms when it comes to transfection efficiency. A study done in mice for determination of biological activity of SC and OC forms of pDNA revealed that i.m. injections resulted in three times higher transfection effectivity of SC pDNA than with OC pDNA when priming a MVA-boosted CD8<sup>+</sup> T-cell responses (Pillai et al. 2008). Another report speculated that the higher transfection efficiency of SC pDNA might be due to ability of this form of pDNA to enter the perinuclear region more effortlessly than OC and linear forms. This results in SC



pDNA entrapped in the nuclei of cells and translation of the mRNA can be initiated (Remaut et al. 2006). Therefore, the proportion of the three forms of pDNA in a vaccine may be used as a measure of the efficiency of that particular vaccine and can be visualized with agarose gel electrophoresis. Agarose gel electrophoresis measurement of the nature of pDNA in this study following adsorption/encapsulation showed that the microparticles preserved the SC and OC nature of pDNA. Adsorption of pDNA resulted in more of the SC form than obtained with encapsulation. These findings suggest that the use of these microparticles as vaccines will probably also yield transfection efficiencies that will result in the expression of antigenic protein with the ability to provoke the necessary immunity.

The micrographs further demonstrated that most of the microparticles were less than  $10 \,\mu\text{m}$  in diameter and were evenly dispersed in Eppendorf tubes during the *in vitro* release study, thus showing no signs of aggregation. These two features make these microparticles easy to manipulate with a syringe and susceptible to APC uptake once administered, especially to macrophages that target particulate materials. A study about the effect of microparticle size on transfection efficiency of pDNA demonstrated that size plays an important role during transfection. Transfection of COS-7 cells with smaller-sized and larger-sized microparticles showed that smaller-sized nanoparticles have a transfection rate of 27-fold higher than that of larger-sized nanoparticles (Prabha et al. 2002). In a review on vaccine delivery systems, it was demonstrated that virus-sized nanoparticles are more effective at inducing cellular and humoral immune responses (Scheerlinck & Greenwood 2008). As microparticles prepared in this study also possess the above-mentioned qualities, it may be an indication that immunisation of sheep with these microparticles will yield similar results which include the improvement of the protective efficacy of a DNA vaccine to challenge in the field.

Microencapsulation did not significantly change the integrity of pDNA as indicated by agarose gel electrophoresis of the encapsulated pDNA. However, pDNA degradation was observed during pilot *in vitro* release experiments when PBS pH 7.4 was used as release medium. This observed degradation could be indicative of changes in the pH of release



medium due to polymer bio-erosion. The release study was repeated using *in vitro* release medium (PBS) with higher pH (pH 8) in order to try to preserve the SC nature of pDNA. Again, pH measurement of the *in vitro* aliquots using pH test strips indicated a general decrease in pH when PBS was used as a release medium. In contrast, TE buffer (10 mM Tris-HCl, 1 M Na<sub>2</sub>EDTA pH 8) maintained a constant pH that dropped slightly over time but was maintained within the physiological pH range. However, the use of pH higher than physiological range does not affect the ability of the *in vitro* release study to predict the in vitro-in vivo correlation. This is because when inside the cell or in targeted area the pH is controlled and maintained constantly and does not fluctuate as much as was found in vitro. It is therefore easier to predict the in vivo behaviour of a protein if the vaccine antigens are stable and not degraded during the *in vitro* release study (Giteau et al. 2008). In their review on how to achieve sustained and complete release of protein from PLGA microparticles they argued that although PBS pH 7.4 is said to be near physiological pH range, one cannot study the *in vitro* release of lysozyme in this buffer because it causes the protein to aggregate. Instead these researchers used glycine buffer with pH 2.5 in which lysozyme was stable which made prediction of its *in vivo* release behaviour as a microparticles-based protein easier (Giteau et al. 2008).

Microparticles made from RG752S, R203S, and PLGA 80:20 and PLGA 85:15 generally met most of the required features. However, DNA loading and encapsulation efficiency as well as adsorption efficiency of pDNA in these microparticles was very low. It is reported that hydrophobicity of these polymers might be the major cause of poor DNA loading and EE. The same results were observed by Díez and coworkers when pDNA was encapsulated into microparticles made of the same polymers (such as RG752S, RG504, RG502H and RG503H) (Díez & Tros de Ilarduya 2006). In their study, hydrophilic polymers captured more pDNA as compared to encapsulation into RG752S, which is highly hydrophobic in nature. Based on these observations the hydrophilic polymer RG502H and the polymers with highest molecular weight (RG504) were used to encapsulate the other Erum pDNA. These two polymers gave the highest encapsulation efficiences in comparison to other polymers. Co-encapsulation of PVA with DNA did not improve the EE and DL of low glycolic acid polymers as observed with protein



encapsulation in the previous chapter. Future work may benefit from using the more hydrophilic polymer RG502H when encapsulating pDNA in both the naked and complexed form especially when high EE is required.

Encapsulation of pDNA-0.5% CTAB complexes improved the EE when using RG752S microparticles. This might be because this procedure condensed the DNA into a compacted form. This was observed using PEI that causes polymers to easily wrap around it (Zhou et al. 2008). Another method that could be used to confer extra protection to the pDNA is the process of DNA condensation (Dunne et al. 2003). Pre-condensation of DNA with protamine sulphate was shown to increase the EE of pDNA into PLGA microparticles and increase the protection of DNA against DNase 1 degradation. DNases are among the many obstacles that pDNA encounters in the extra-cellular spaces as well as inside the cell before it is transcribed and translated. These barriers often reduce the amount of pDNA available to express enough protein antigen for provoking protective immunity (Pouton & Seymour 2001; Remaut et al. 2007). In our study, microparticles formed encapsulating 0.5% CTAB-pDNA complexes were able to protect the pDNA from possible DNase degradation for the six months period of storage and duration of *in vitro* release study.

Adsorption of pDNA onto cationic surfaces of microparticles improved the DL and AE. Again microparticles made of hydrophilic (RG502H) and high molecular weight polymers (RG504) attracted more pDNA than microparticles made of hydrophobic polymers. The high molecular weight and viscosity of RG504 as well as the presence of negatively charged carboxylic groups on RG502H polymers might be the fundamental cause of this increase in AE (Díez & Tros de Ilarduya 2006). The RG502H carboxylic end groups attract more cations on their surfaces, which in turn attracts the highly anionic DNA to the surface of microparticles. DNA adsorption avoids exposure of pDNA to extreme conditions such as the sonication, organic solvent etc (reviewed by Hanes, Cleland & Langer 1997; Jilek, Merkle & Walter 2005). This is the reason why cationic microparticles had the highest proportion of SC pDNA than pDNA encapsulating



microparticles. These results support the fact that adsorption of pDNA is better than encapsulation in terms of preservation of pDNA stability as well as improving EE.

Surfactants such as PVA as well as the cations PEI and CTAB preserve the stability of pDNA (Gebrekidan, Woo & DeLuca 2000). The major drawback of this method is the formation of aggregates between pDNA and CTAB or PVA. These aggregates affect the electrophoretic mobility of pDNA in agarose gel as observed during encapsulation of naked DNA and complexed DNA in our study. This was also pointed out by Gebrekidan and coworkers when complexing pDNA with polylysine (Gebrekidan, Woo & DeLuca 2000). They observed a small band at the spot well following electrophoresis of pDNA-PLL complexes and suggested formation of high molecular weight aggregates as the main cause. Nonetheless, complexion of pDNA with CTAB and the use of PVA in our study resulted in preservation of SC form of pDNA. However, we observed lack of electrophoretic mobility of pDNA released from microparticles on day 21. This indicates that not all of the pDNA is affected by this problem. These pDNA-CTAB complexes could be disrupted by the addition of highly negatively charged heparin to the release medium (Howard et al. 2004). Heparin will displace the negatively charged DNA for positive charged CTAB thus resulting in easy detection of free DNA by agarose gel electrophoresis in the release medium. A 6-fold increase in free pDNA level was detected in the PEI/DNA solution following the addition of heparin sulphate to the sample (Howard et al. 2004). However, this DNA-CTAB aggregation is not expected to be problematic for protein expression in the host cells. The DNA-PVA aggregation is not a covalent interaction and is expected to be reversible inside the cell where the buffering system is well regulated. Investigators complexed DNA with PEI before microencapsulation and found that these complexes are more penetrative to immune cells than uncomplexed DNA (Zhou et al. 2008). These PEI-DNA complexes even induced serum antibody responses 2-3 orders of magnitude greater than naked DNA (Zhou et al. 2008). Recent reports showed that DNA complexed with cations such as PEI before encapsulation was the most effective way of provoking potent immune response to prime-boost vaccine (Zhou et al. 2008). A study conducted in mice compared traditional biodegradable microspheres, which release naked DNA with microspheres releasing



PEI/DNA complexes. This study found out that PEI/DNA complexes induced potent CTL responses at low doses (Zhou et al. 2008). They suggested that these complexes are more penetrative to cytoplasm than naked DNA. In our study, it was demonstrated that DNA-CTAB complexes preserved the SC nature of pDNA over a 2 week period of *in vitro* release.

Adsorption of pDNA onto cationic microparticles did not only preserve the structural integrity of pDNA but also resulted in 50% release of DNA within 24 h of *in vitro* incubation. This makes these microparticles suitable for priming an immune response in a DNA prime/rprotein boost vaccination. In contrast, encapsulation of pDNA into RG502H microparticles reduced the burst release of pDNA especially if the formed microparticles are non-porous. Porous microparticles (RG502H) released encapsulated pDNA as early as the first day of *in vitro* incubation. This was observed by running an aliquot of release medium on the agarose gel, which shows the stability as well as the time of release of pDNA. Porous microparticles were generally not aggregated and released encapsulated pDNA from day 1 of *in vitro* incubation. It might be that they had DNA attached to their surfaces or that their porous surfaces allowed quicker penetration of release medium to the core of the matrix thus contributing to faster diffusion of encapsulated pDNA and hence higher burst release. Nonetheless, it was demonstrated that encapsulation of DNA can also result in microparticles with low burst release.

In conclusion, the microparticles produced in this study possess the features of singledose microparticles-based vaccine that can be used in place of a multi-dose DNA vaccine. The EE and AE% show that one formulation can provide more than 220  $\mu$ g of DNA that is stable and with controllable release. The more hydrophilic polymer RG502H adsorbed and encapsulated more pDNA than other polymers. This polymer also shows that it is possible to manipulate the release of pDNA associated with microparticles.



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# Chapter 4: Concluding Discussion

In this study, the formulation of microspheres for use as a single dose vaccine to mimic a 1H12 DNA prime, DNA 1<sup>st</sup> boost and recombinant protein 2<sup>nd</sup> boost was investigated. A recombinant vaccine should induce potent protective immunity better than the currently used infection and treatment method where sheep blood is used infected with the E. ruminantium Ball3 stock (Oberem & Bezuidenhout 1987). Since its inception about 50 years ago, attempts based on the inactivated (Martinez et al. 1994) and attenuated live vaccines (Jongejan 1991; Shkap et al. 2007) gave some success but not good enough to replace it. The hope for an effective and stable vaccine shifted to genomics and proteomics to deliver a non-living vaccine (Mwangi et al. 2002) that can also be used in rural areas where infrastructures are limited. Such a vaccine will not need cold storage for stability preservation, will be cost effective and would reduce the use of chemical tick control (reviewed by Purnell 1984). The latter often led to increased chemical resistance in ticks as well as the accumulation of health risking residues in meat and milk. Biodegradable microparticles have been shown to improve the immunogenicity of recombinant vaccines of important diseases such as diphtheria (Singh et al. 2006), hepatitis (Bharali et al. 2008), HIV and veterinary diseases such as brucellosis (Estevan et al. 2006) and Salmonella enterica serovar Abortusovis (Estevan et al. 2006). Administration of a single shot of microparticles encapsulating the major membrane antigen from abortusovis in mice conferred protection comparable to that attained following administration of attenuated commercial available Rv6 (Estevan et al. 2006). Thus, in order to improve on the current 1H12 recombinant vaccine, biodegradable polymeric microparticles were employed to encapsulate or adsorb pDNA and their respective rproteins.

Using biodegradable PLGA polymers with different physico-chemical characteristics, the double emulsion solvent evaporation technique and the incorporation of 2.5% PVA in the internal water phase, resulted in microparticles with heterogeneous size distribution. These microparticles were spherical in shape with a smooth and non-porous surface



morphology and were less than 5 µm in diameter. Microparticles formulated using polymers with low glycolide ratios released 80% of the encapsulated proteins within the first week of *in vitro* incubation with most of the proteins being released on day one. Microparticles formulated using polymers with 50:50 monomer ratios released the recombinant proteins during week one and three of in vitro incubation. These microparticles did not release any protein in week 2 (day 7-14). Microparticles with 0.5% CTAB on their surfaces adsorbed DNA and released more than 40% of DNA on day 1 with 100% release by day 14. RG502H microparticles formed with PVA as the internal phase viscosity enhancer released intact DNA only from day 12 to day 21. In addition, the integrity of both pDNA and rprotein antigen were not affected adversely by the microencapsulation procedure and prolonged storage at room temperature. This was revealed by the intact SC nature of pDNA seen on agarose gel electrophoresis of encapsulated and adsorbed DNA as well as the SDS-PAGE gel electrophoresis of encapsulated rproteins following over 6 months of storage at room temperature. This protocol can therefore be easily applied for future work that might need the induction of antibody responses.

The size of microparticles formed here could result in the induction of the desired cellular immune response. It has been shown that the particulate nature of microparticles is the key in the induction of such immune responses (reviewed by Bowersock & Martin 1999). It is speculated that small sized microparticles will activate DCs, which are the primary immune response cells that help in the fragmentation and presentation of antigens to T-cell receptors leading to elicitation of cellular immune responses. It is reported that macrophage phagocytosis of particular molecules such as inert 2-micron-diameter polystyrene beads induced IL-12 p40 mRNA expression. This cytokine will then in turn activate IFN- $\gamma$  and thus Th1 immunity (Fulton et al. 1996). Recently, it was confirmed by using polystyrene microparticles, that phagocytosis depends on the particle size (Champion, Katare & Mitragotri 2007). Rat alveolar macrophages exhibited maximal phagocytosis and attachment of microspheres with 2-3  $\mu$ m diameters (Champion, Katare & Mitragotri 2007).



Based on the observation of the physico-chemical features of the formed microparticles, such as PL, DL, EE, size and morphology coupled with the knowledge that the vaccine antigens used here had been shown to induce protective immunity, it could be suggested that immunization of sheep with these particles will enhance the induction of protective immunity in the laboratory as well as in field challenges. Future work should include *in vitro* transfection of encapsulated and adsorbed pDNA by using cell line cultures such as COS-7 and the HEK-293 to demonstrate transfection efficiency. Microparticles with encapsulated pDNA could also be exposed to nucleases to determine the protective ability of the polymer. This will prove that pDNA will be protected against nucleases *in vivo* (Díez & Tros de Ilarduya 2006).

One future goal will be to use the biodegradable microparticles as a vaccine to mimic a DNA prime-rprotein boost vaccination strategy with a single administration dose. In order for this microparticle-based vaccine to serve this purpose, it should be able to release pDNA on day 1 and 21 and release rproteins on day 42 following a single injection. However, the release profiles of microparticles reported in chapter 2 would not allow that because of the inability to stop the initial burst release or any protein release before day 42 of *in vitro* incubation. Further work needs to be done to reduce the initial burst release of protein from microparticles. Too high initial burst release or uncontrolled release of proteins from microparticles is one of the main challenges of this immunisation method. A high initial burst release rapidly depletes the microparticles of the protein thus reducing the effective lifetime of the system (Ahmed, Dashevsky & Bodmeier 2008). The uncontrollable early release of vaccine antigens may lead to immunological tolerance of the antigen. Immunological tolerance results in the inability of the an immune system to respond to an antigen. Immune tolerance can also be caused by small doses of vaccine antigens that are below the dose required for induction of immune response (reviewed by Hanes, Cleland & Langer 1997). However, tolerance induction was not observed when an antigen was presented in particulate form, thus microparticle-based vaccines can be formulated to release the vaccine antigen in a sustained and prolonged manner with the ability of provoking immune responses specific to that antigen (reviewed by Bowersock & Martin 1999).



One way of reducing the initial burst release of protein is to add excipients such as gelatin, sucrose and trehalose during encapsulation. Microparticles encapsulating BSA that contained gelatin as internal water phase viscosity enhancer exhibited a slow and sustained release profile compared to microparticles produced without gelatin (Yushu & Venkatraman 2006). The extraction of proteins close to the surface of microparticles or leaching the microparticles in buffer before administration is another way of getting rid of high burst release, but this procedure reduces the total amount of protein that will be released (Whittlesey & Shea 2004). The use of double walled microspheres or polymer coating may also be used to reduce initial burst release (Huang & Brazel 2001). The surface extraction method depends on the fact that during encapsulation some of the protein is entrapped in the inner core of the microparticles and can only be removed by diffusion following matrix hydration and during polymer degradation. The remaining protein will be on the surface of the microparticles and because it is loosely bound, it could be easily removed by washing the microparticles several times before in vivo administration. This protein is mainly responsible for initial burst release. Increasing the concentration of polycations such as PEI, CTAB and pLL can also prevent the initial burst release of DNA from microparticles. In a study in which pDNA was adsorbed onto cationic (CTAB) microparticles, the investigators found out that increasing the CTAB concentration above 0.6% (w/w), increased the loading efficiency of pDNA and decreased the *in vitro* release (Singh et al. 2003). Another study comparing the *in vitro* release profile of complexed and uncomplexed pDNA from microparticles indicated that microparticles containing free pDNA had a more rapid initial in vitro release than microparticles with polylysine complexed plasmid DNA (Gebrekidan, Woo & DeLuca 2000).

Recently, it was reported that the incorporation of glycerol monooleate (GMO) into microparticles encapsulating phosphorothioate also resulted in the reduction of burst release of PLGA microparticles. Because of the viscous nature of GMO, it was mixed with water and co-solvent. This mixture makes the formulation syringeable and injectable. GMO is non-toxic and biodegradable (Ahmed, Dashevsky & Bodmeier 2008). In another study gelatin microparticles encapsulating bone morphogenetic protein-2



(BMP-2) were compared with PLGA microparticles encapsulating BMP-2 (Patel et al. 2008). The study showed that gelatin microparticles exhibited a minimal burst release of BMP-2 while PLGA microparticles exhibited a moderate burst release. The rate of *in vitro* release was dependent on the extent of gelatin cross-linking. For our formulation it might be better to try mixing rproteins with gelatin prior to encapsulation. Another study found that encapsulation parameters such as homogenization, volume of external water phase and covering of double emulsion during solvent evaporation, play a role in the reduction of initial burst release (Luan & Bodmeier 2006). It was found that by decreasing the volume of the external water phase and the homogenization speed the burst release could be decreased. The addition of 0.5 M NaCl to the external water phase as well as covering of the preparation beaker delayed polymer precipitation and resulted in the formation of non-porous microparticles with a low initial burst release (Luan & Bodmeier 2006). Another important initial burst release reducing variable was increasing concentration of emulsion droplets stabilizer (PVA) in the external water phase.

However, a DNA prime-rprotein boost vaccination strategy is still possible using these microparticles. The reason being that beside the unwanted initial burst release, most of these microparticles (protein loaded) still contain enough antigens to provoke an immune response even after 42 days of in vitro incubation. Because microparticles are capable of increasing the magnitude and the duration of antigen specific-immune response it is speculated that immunization with these particles will induce better immune responses than the one induced by naked DNA prime/rprotein boost using adjuvant (Pretorius et al. 2008). Furthermore, the fact that microparticles were highly individualized (not aggregated) and are in a free flowing powdery state, they could be easy to administer using a syringe. Future work needs to follow the so-called top-to-bottom approach of gene delivery system rather than the bottom-to-top approach followed in this study in general (chapter 2 and 3). The bottom-to-top approach starts with the synthesis of microparticles encapsulating or adsorbing pDNA, which are tested for physico-chemical properties in buffer and subsequently its biophysical behaviour in cell culture. Remaut and co-workers suggested the top-to-bottom approach, which first studied body distribution of microparticles upon intravenous administration of the molecules (Remaut



et al. 2007). This approach will guide or point out which features (size and shape) microparticles need to possess for them to reach their intended target *in vivo*. Those microparticles with features that enable them to reach the *in vivo* target are then subjected to delivery phase. This phase is concerned with the interior of microparticles, the quality of nucleic acid released, the release rate or profile (sustained or pulsed etc). This is because the intensity of immune response depends on the size of particles. In this study the particles formed fall within the recommended size range needed for phagocytosis (less than 10  $\mu$ m). Nevertheless, this does not mean that they will all help the antigen to provoke immune responses of the same intensity. The possibility of performing the *in vitro* measurements in proper biological samples such as serum, vitreous, and sputum need to be explored also in order to get a better understanding of *in vitro-in vivo* correlation of microparticles release behaviour (Remaut et al. 2007).

This study has among other things widened our knowledge of microencapsulation technology, vaccine antigen delivery and optimized microencapsulation procedures that can be used in future to encapsulate heartwater vaccine antigens (rproteins and pDNA) that are stable at room temperature and capable of released intact in a sustained and controlled manner.



### 4.1. References

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# Appendix A:

# Materials, Buffers, Media and Solutions

# 1. Materials

| Product No: | Product name                                  | Manufacturer and     |
|-------------|---|----------------------|
|             |   | distributor          |
| UFC810024   | Amicon <sup>®</sup> Ultra Centrifugal filter  | Millipore            |
|             | devices Ultra-4 Centrifugal filter            |                      |
|             | unit 100 k                                    |                      |
|             | Anti-His <sub>6</sub>                         | Roche                |
| BCA1-1KT    | Bicinchoninic acid protein                    | SIGMA-ALDRICH        |
|             | determination                                 |                      |
| B9643-1L    | Bicinchoninic acid solution                   | SIGMA-ALDRICH        |
|             | Bovine Serum Albumin (BSA),                   | Roche                |
|             | Fraction V                                    |                      |
| 70584-4     | BugBuster <sup>®</sup> Protein Extraction     | Novagen <sup>®</sup> |
|             | Reagent                                       |                      |
| 420450250   | Cartridge desiccant, 250 mm x 2               | Scienceware®         |
|             | Criterion <sup>™</sup> XT Precast gel, 4-     | BIORAD               |
|             | 12%, Bis-Tris                                 |                      |
|             | Go-taq <sup>TM</sup>                          | Promega              |
|             | pET Trx Fus F and T7R primers                 |                      |
|             | BL21 Star <sup>TM</sup> DE <sub>3</sub> Cells |                      |
| 52365       | Hexadecyltrimethyl-ammonium                   | Fluka                |
|             | bromide (CTAB)                                |                      |



Japan Gold Size artists resin

Winsor and Newton, London

|           | 1 x XT reducing agent                      | BIORAD                  |
|-----------|--|-------------------------|
|           | MSB <sup>®</sup> Spin PCRapace kit         | Invitek                 |
| 740416.50 | NucleoBond <sup>®</sup> Xtra MaxiPlus      | Macherey-Nagel          |
| P-4411    | pH Test strips 7.0 -14,00                  | SIGMA                   |
| 71491     | Overnight Express <sup>TM</sup> instant TB | Novagen <sup>®</sup>    |
|           | medium                                     |                         |
| K0681     | PageSilver TM Silver staining kit          | Fermentas life sciences |
|           | Proof reading Polymerase, Pfu              | Promega                 |
| K102-01   | pET Directional TOPO                       | Invitrogen champion TM  |
|           | Expression kit                             |                         |
|           | SuperSignal West Pico                      | Pierce                  |
|           | Chemiluminescence                          |                         |

# 2. Preparation of buffers, media and solutions

# Ampicillin

Prepare a stock solution of 50  $\mu$ g/ml by dissolving 0.05 g ampicillin in 1ml Millipore water. Aliquot and freeze (-20 °C).

# 1 M IPTG

1.19 g isopropyl- $\beta$ -D-thiogalactopyranoside was dissolved in 50 ml dH2O, sterilized by filtration, aliquoted and stored at -20 °C.

# LB Agar plates

Dissolve 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl and 15 g bacto-agar in 1000 ml dH<sub>2</sub>O and sterilize by autoclaving. Allow the medium to cool at 55 °C and before pouring the plates add the appropriate amount of antibiotics.



# LB broth

Dissolve 10 g bacto-tryptone, 5 g yeast extract and 10 g NaCl in 800 ml dH<sub>2</sub>O. Adjust volume to 1000 ml with dH<sub>2</sub>O. Aliquot into 100 ml per bottle and sterilize by autoclaving. Before use add 50  $\mu$ g/ml ampicillin final concentration and store at 4 °C for 1 month.

# 10x PBS

The buffer consisted of 137 mM NaCl (8 g), 27 mM KCl (2 g), 8.8 mM KH2PO4 (1.2 g) and 73 mM Na2HPO4.2H2O (11.4 g) were dissolved in 900 ml dH<sub>2</sub>O, pH 7.5 The solution was made up to a final volume of 1 L, autoclaved and stored at room temperature.

# PBS-T

Dulbecos PBS with 0.05% Tween-20.

# 10x TBE

Buffer contains 889 mM Tris base, 889 mM boric acid and 25 mM EDTA.

# SOC medium

8.5 mM NaCl (0.5 g), tryptone (20 g), yeast extract (5 g) and 1 mM KCl (2.5 ml). 970 ml of  $dH_2O$  was added and NaOH was used to adjust pH to 7.5. The final volume was adjusted to 1000 ml, following autoclaving 1 M MgCl<sub>2</sub> and I M glucose were added.

# **Transfer buffer**

38 mM Tris base (9.1 g), 288 mM (43.25 g) glycine was dissolved in 1700 ml dH2O and 300 ml methanol was added, pH ~8.3-8.4. It was stored at room temperature.

# Wash buffer for Western Blot (PBS- Tween, 0.05%)

Dissolve 500 µl Tween<sup>®</sup>-20 in 1000 ml 1x PBS.