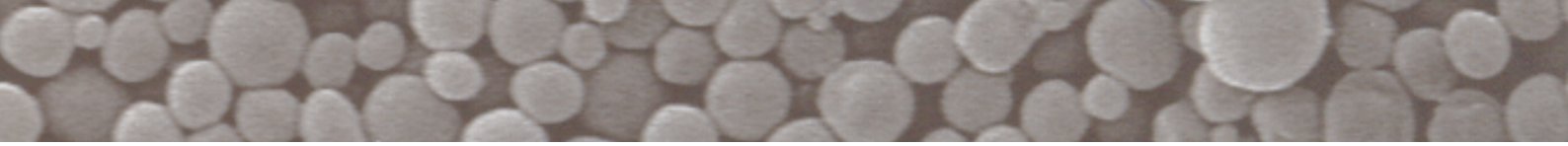

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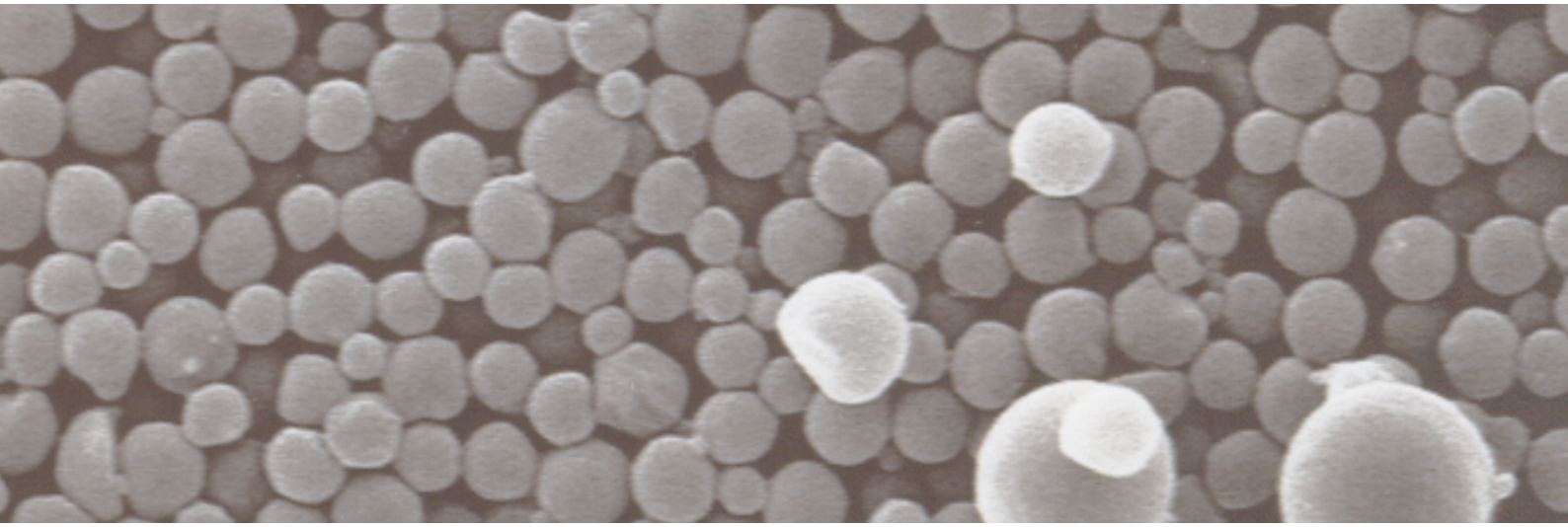

Development of alternative micro and nanoparticulate polymeric systems for mucosal delivery of Streptococcus equi antigens

Helena Isabel Fialho Florindo

DOUTORAMENTO EM FARMÁCIA
(Tecnologia Farmacêutica)

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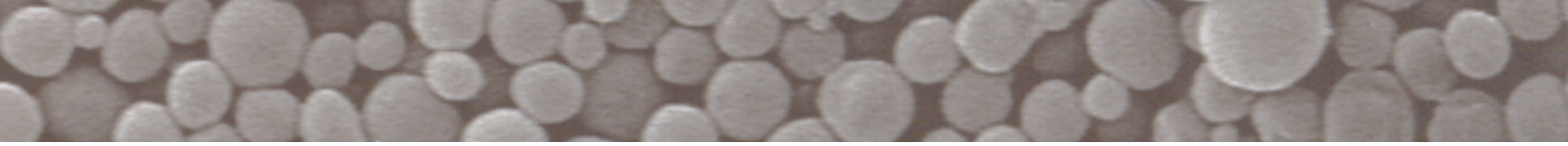

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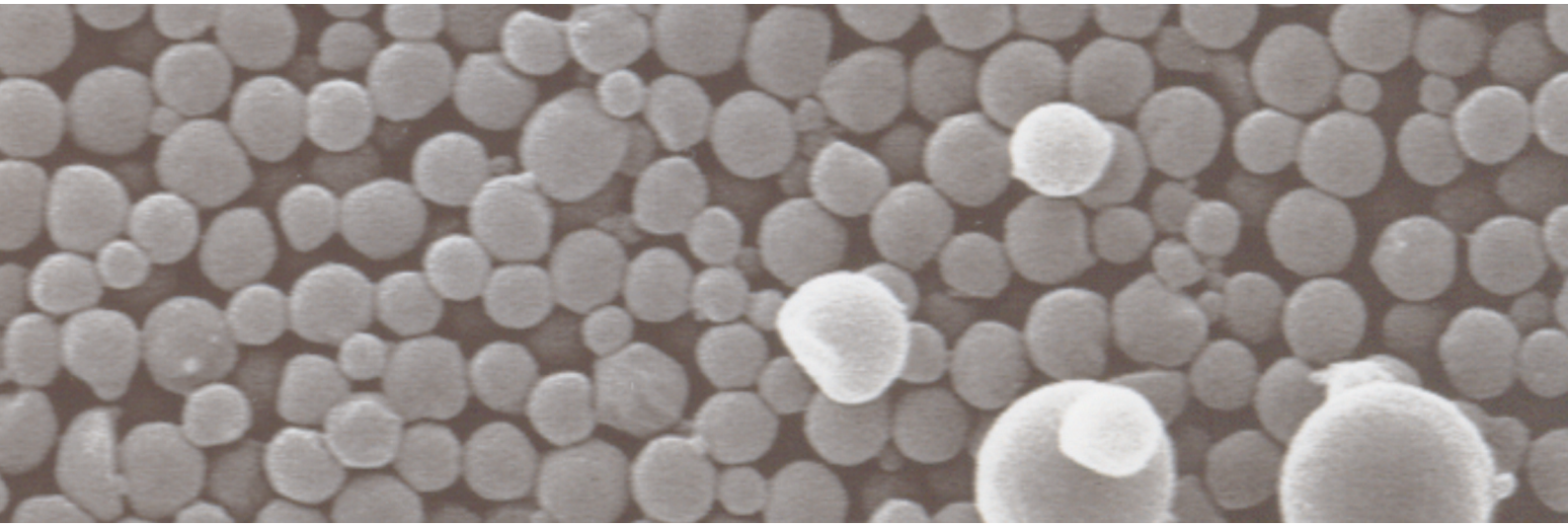

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The picture presented in the first page of each chapter was adapted from a scanning electron micrograph of poly-ε-caprolactone microspheres obtained during their study in this thesis.

For my parents and
grandmother

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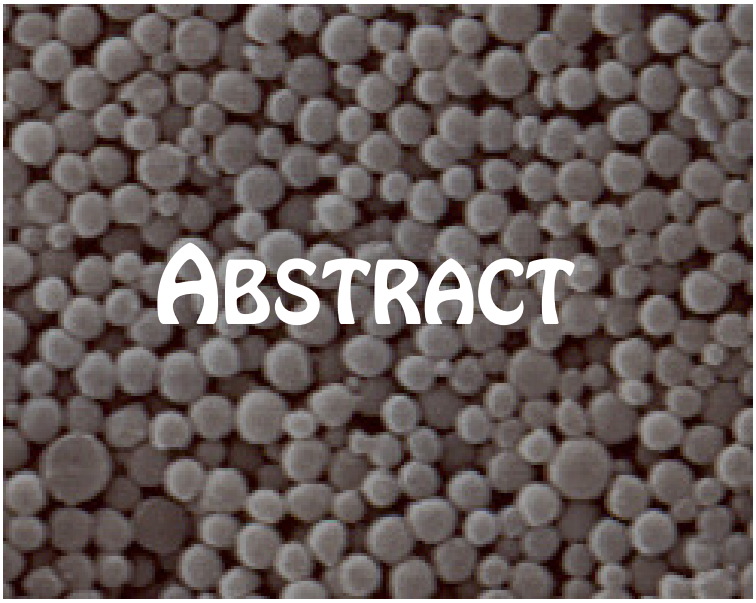

Abstract

S. equi is considered the causative agent of strangles, a very contagious disease of the upper respiratory tract of the *Equidae*. Although *S. equi* is sensitive to some antibiotics, most of the current treatments are ineffective. During the recovery period, horses develop a protective immune response mostly against the antiphagocytic cell wall-associated M-like protein SeM, which encourages the development of efficient vaccines.

The main purpose of this thesis was to develop and characterise stable polymeric particles and study their potential as mucosal *S. equi* antigens adjuvant.

Non-aggregated and easily dispersible micro and nanospheres based on poly-ε-caprolactone (PCL) and poly(lactic acid) (PLA) polymers were formulated by the double emulsion (w/o/w) solvent evaporation method and fully characterised. BALB/c monocyte macrophages (cell line J774.1A) were used in toxicity tests and cellular uptake studies. The influence of mucoadhesive polymers (chitosan (CS), glycolchitosan (GCS) and alginate (ALG)) and absorption enhancers (spermine (SP) and oleic acid (OA)) in particle physicochemical characteristics and consequently in the humoral, mucosal and cellular immune responses was as well evaluated.

The non-toxic cholera toxin B subunit (CTB) is a potent mucosal adjuvant, and the oligodeoxynuleotide with repeating C and G motives (CpG) can also be used to up-regulate mucosal and cellular immune responses, increasing preferentially the T helper type 1 (Th1) cell activity. Therefore, those adjuvants were co-administrated with soluble antigen and non-modified PLA and PCL particles, in order to assess their eventual synergic effect. Inspite of administration route (i.n. and i.m.), *S. equi*-loaded polymeric systems were able to significantly increase systemic and cellular immune responses, when compared with free antigens, isolated or co-admixed with CTB or CpG.

PLA-loaded particles generally induced higher mucosal antibody levels when compared with the correspondent PCL. Similar results were attained when the immune responses induced by *S. equi* antigens-adsorbed particles were

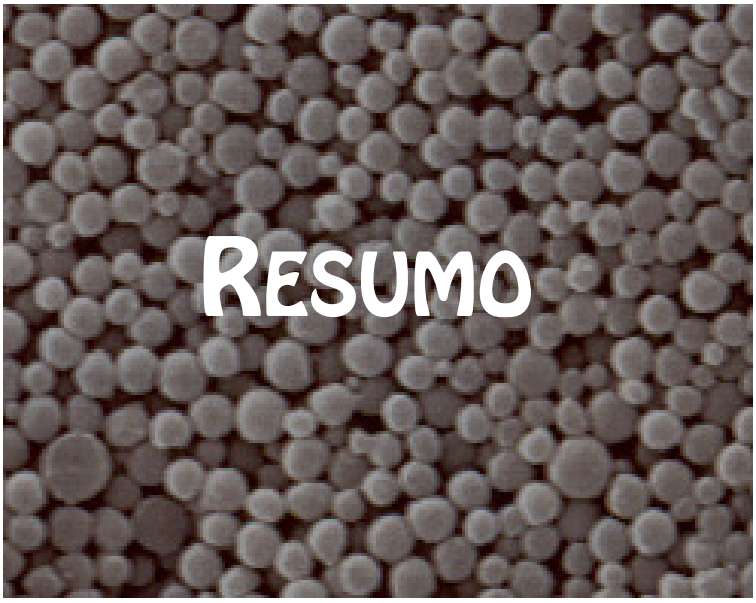
compared with those elicited by the correspondent entrapped ones. As per PCL nanospheres, those formulated with ALG and GCS, seem to induce a more balance Th1/Th2 immune response.

This study also confirmed the potential adjuvant of *S. equi* antigen-loaded PLA nanospheres, and again the mucopolysaccharide GCS induced the most prominent immune response.

SeM recombinant proteins were entrapped in PLA nanospheres and their adjuvant potential after i.m. administration in a mouse model was compared with that achieved with *S. equi* extract. In fact, it was possible to observe that PLA-GCS and PLA-OA nanospheres are alternative cost-effective preparations, able to induce a balanced IgG2a/IgG1 immune response.

These studies bring new insights into the strangles prevention field as the particulate carriers developed during this PhD thesis, mainly those containing GCS, are promising adjuvants for a safe vaccine against strangles, with no toxicity issues associated to their utilisation, in contrast to other adjuvants that have been associated to *S. equi* antigens. Therefore, the main goal of this thesis was accomplished but challenge studies must be done in order to support their future utilisation.

Keywords: poly-ε-caprolactone, poly(lactic acid), polymeric particles, *Streptococcus equi* subs. *equi*, mucosal adjuvant, vaccine


Resumo

A gurma é uma doença infecciosa causada pela estirpe altamente virulenta *Streptococcus equi* subsp. *equi* (*S. equi*), que afecta os equídeos e se encontra disseminada por todo o mundo. Trata-se de uma infecção que afecta o tracto respiratório superior, mais especificamente a nasofaringe e a drenagem dos gânglios linfáticos.

O *S. equi* atravessa as mucosas oral e nasofaríngea, aloja-se nos gânglios linfáticos localizados na região da faringe, onde leva à formação de abcessos. De um modo geral, a ruptura dos abcessos pode ocorrer internamente, o que se traduzirá num corrimento nasal purulento para a bolsa gutural ou para a faringe; ou externamente, no caso de abcessos localizados a nível subcutâneo. Embora aparentemente saudáveis, muitos animais podem alojar a bactéria durante meses, verificando-se elevado risco de contágio durante esse período.

A resistência do *S. equi* à fagocitose constitui a principal característica da patogénese desta infecção e os factores que mais contribuem para esta resistência são a cápsula do ácido hialurónico, a proteína M (SeM) e as proteínas segregadas IdeE e Se18.9.

O combate à gurma passa pelo desenvolvimento de uma vacina eficaz, uma vez que, no período de convalescença, 75% dos animais apresentam imunidade protectora, que se mantém durante longos períodos de tempo, dirigida maioritariamente à SeM. Esta proteína induz a produção de anticorpos opsonogénicos IgG e IgA específicos, presentes no soro e nas secreções nasais, que vão sucessivamente reconhecer e inactivar a bactéria.

No entanto, os resultados obtidos com as vacinas actualmente comercializadas são decepcionantes, para além de induzirem reacções adversas graves. Embora o mecanismo de protecção não se encontre completamente esclarecido, o fracasso das vacinas disponíveis parece estar associado à ausência da estimulação da resposta imunitária local, ao nível da nasofaringe.

A utilização de micro e nanopartículas poliméricas no desenvolvimento de vacinas é actualmente uma das mais promissoras estratégias de luta contra as

doenças infecciosas e uma das áreas mais investigadas em Tecnologia Farmacêutica. Os antigénios responsáveis pela indução de uma imunidade protectora contra vários agentes patogénicos podem ser imobilizados ou associados a sistemas de partículas tendo em vista a libertação prolongada e/ou pulsada do antigénio, mantendo uma resposta imunológica durante semanas a meses. Assim, protegem o antigénio da degradação prematura no organismo, permitem a estimulação prolongada do sistema imunitário e promovem a interacção entre o antigénio e as células apresentadoras de antigénios (APCs).

Está comprovado que micro- e nanopartículas de PLA e PLGA são bons transportadores de antigénios proteícos ou DNA, encapsulados ou adsorvidos. Estudos preliminares realizados pelo nosso grupo de investigação demonstraram que microesferas de PLGA são potenciais transportadores de antigénios do *S. equi*, uma vez que a incorporação e administração intranasal (i.n.) e intramuscular (i.m.) de bactérias mortas ou lisadas resultaram na protecção total de ratinhos contra a infecção experimental com uma estirpe virulenta do microrganismo. No entanto, tratando-se de uma vacina destinada a animais, o polímero PLGA pode comprometer a sua futura comercialização, já que se trata de um produto de elevado custo.

Pelo acima exposto, o objectivo desta tese prende-se com o desenvolvimento de partículas transportadoras do antigénio destinadas à administração pelas mucosas, formuladas com excipientes capazes de induzir respostas imunitárias protectoras contra a infecção.

O polímero utilizado nestes estudos, poli-ε-caprolactona (PCL), apresenta grande potencial pela sua biodegradabilidade, biocompatibilidade, baixa velocidade de degradação, hidrofobicidade, estabilidade *in vitro*, baixa toxicidade e baixo custo. A PCL foi utilizada na preparação de micropartículas pelo método da emulsão dupla (a/o/a) seguida da evaporação do solvente, cuja superfície foi modificada utilizando diferentes concentrações e pesos moleculares do CS ou ALG, de modo a avaliar a

influência das características da superfície das partículas nas propriedades imunoadjuvantes destes transportadores poliméricos (Capítulo 3). Produziram-se partículas esféricas, com carga positiva e negativa, cujo diâmetro médio variou entre 1,4 e 2,7 μm. A eficiência de adsorção obtida para as partículas com carga positiva ou negativa foi muito semelhante (53% e 54%, respectivamente), não tendo o processo afectado a estabilidade ou a integridade estrutural das proteínas constituintes do extracto, o que é um pré-requisito para que o antigénio possa desencadear a resposta imunológica adequada.

Como a utilização de solventes orgânicos constitui uma das principais desvantagens da utilização do método da emulsificação com evaporação de solventes, os resíduos de diclorometano presentes nas microesferas foram avaliados por ressonância magnética nuclear, sendo inferiores aos 600 ppm permitidos pela legislação aplicável às especialidades farmacêuticas. Além disso, em culturas celulares não se observou qualquer toxicidade associada a estas partículas.

Foram estudadas as características físico-químicas das partículas, tais como a forma, diâmetro médio, carga eléctrica superficial, capacidade de carga, toxicidade, integridade da estrutura proteíca e preservação das propriedades antigénicas. As partículas que apresentavam as características físico-químicas adequadas foram seleccionadas para estudos de imunização *in vivo*, de modo a avaliar o efeito da composição polimérica, do tipo de associação dos antigénios (adsorção ou incorporação), da via de administração (i.n. ou i.m.), da natureza do antigénio (extracto enzimático do *S. equi* ou proteína SeM recombinante), assim como da co-administração de diferentes adjuvantes (subunidade B da toxina colérica (CTB) e CpG-oligodesoxinucleótidos).

Seguidamente procedeu-se à caracterização das respostas imunológicas humoral e celular induzidas pelos antigénios do *S. equi* incorporados ou adsorvidos à superfície de micro- e nanopartículas de PCL ou PLA, de modo a avaliar a influência das características físico-químicas das partículas nas

propriedades imunoadjuvantes destes transportadores poliméricos. A estes polímeros associaram-se polímeros naturais, mucoadesivos e igualmente biocompatíveis, tais como o quitosano (CS) e o alginato (ALG), ou promotores da absorção, como a espermina (SP) e o ácido oleico (OA).

De início, foi investigada a resposta imunológica desenvolvida em ratinhos BALB/c vacinados pela via subcutânea (s.c.) com uma única dose de partículas formuladas por adsorção, correspondente a 10 μg de SeM. Este estudo decorreu durante 300 dias, não se tendo observado quaisquer efeitos adversos, nos grupos tratados com microesferas, durante todo o período experimental. Mesmo 300 dias após uma administração única de antigénios de *S. equi* adsorvidos à superfície das microesferas de PCL, os grupos vacinados com partículas induziram títulos de anticorpos IgG específicos mais elevados do que os observados no grupo vacinado com o antigénio em solução. Obteve-se uma resposta Th1/Th2 mista, nomeadamente para as formulações de PCL com carga superficial positiva. Para além disso, a imunização com as microesferas PCL resultou num aumento geral dos níveis de citocinas produzidas pelas células do baço, nomeadamente de IFN-γ.

Após ter sido confirmado o potencial adjuvante das microesferas de PCL, foi estudado o efeito da redução do tamanho das partículas e foram comparadas as respostas imunológicas sistémicas, locais e celulares induzidas após a adminsitração i.n. de duas doses de extracto enzimático do *S. equi* adsorvido e incorporado em nanopartículas de PCL (Capítulo 4). A baixa solubilidade do CS a pH fisiológico determinou a sua substituição por um derivado hidrossolúvel, o glicolquitosano (GCS), o qual foi dissolvido na fase interna da emulsão a/o. Tal como observado no Capítulo 3, preparou-se um outro lote de nanopartículas de PCL modificadas pelo ALG, de modo a prolongar o tempo de residência das mesmas à superfície da mucosa nasal. Formularam-se igualmente partículas de PCL associadas a promotores da absorção (SP e OA), a fim de se avaliar o seu efeito nas propriedades adjuvantes destes transportadores poliméricos. Todos os sistemas de

partículas apresentaram diâmetros médios inferiores a 500 nm. No entanto, tanto o diâmetro médio como a captura por macrófagos da linha celular J774A.1, foram influenciados pela composição das partículas. As nanoesferas contendo antigénios adsorvidos ou encapsulados induziram um aumento dos níveis de anticorpos locais e sistémicos, mesmo 12 semanas após a administração da primeira dose. As citocinas quantificadas nos sobrenadantes de culturas dos esplenócitos dos animais vacinados indicaram o desenvolvimento de uma resposta mista Th1/Th2. Tal como esperado, os níveis de anticorpos induzidos pelos antigénios incorporados nas partículas foram mais baixos do que aqueles quantificados nos fluidos biológicos dos animais imunizados com os antigénios adsorvidos à superfície das nanoesferas de PCL, o que aliás está de acordo com os perfis de libertação *in vitro* obtido para ambos os sistemas. Ainda que a interpretação global e integrada das respostas imunológicas sistémica, local e celular seja complicada, é possível verificar que a composição polimérica e o tipo de associação do antigénio às partículas determinaram a natureza da resposta obtida. Deste modo, é interessante verificar que, embora todos os sistemas tenham induzido respostas imunológicas contra os antigénios do *S. equi*, os polissacáridos parecem ter um potencial adjuvante adicional relativamente a este sistema de nanoesferas, quando comparados com os promotores da absorção estudados. Ainda assim, as nanoesferas de PCL são potenciais adjuvantes para os antigénios em estudo, uma vez que não se verificou um efeito adicional na co-administração do adjuvante CTB e das nanoesferas PCL-PVA.

No Capítulo 5 descreve-se a comparação do potencial adjuvante das nanoesferas de PLA relativamente às nanoesferas de PCL. Este estudo confirmou as propriedades adjuvantes das nanoesferas de PLA quando associadas às proteínas do extracto do *S. equi*, sendo de destacar a resposta obtida após administração i.n. das partículas formuladas com o GCS. Os níveis de anticorpos IgA doseados na solução de lavagem dos pulmões dos animais vacinados com as nanoesferas de PLA foram mais elevados do que os

quantificados nos correspondentes grupos que receberam as partículas de PCL, tendo-se observado a mesma tendência relativamente aos anticorpos sistémicos. Estes resultados poderão dever-se à menor velocidade de libertação das proteínas de *S. equi* a partir das nanoesferas de PCL, uma vez que o estudo não foi prolongado para além das 12 semanas após a administração da primeira dose.

Finalmente, procedeu-se à incorporação da proteína SeM recombinante em nanoesferas de PLA. Tratando-se do primeiro estudo do potencial adjuvante de uma proteína do *S. equi* recombinante purificada associada a nanopartículas poliméricas, optou-se pela administração i.m. de duas doses de modo a comparar com os resultados obtidos após a imunização dos animais, pela mesma via, com nanoesferas de PLA contendo o extracto de *S. equi*. Após as 12 semanas, foi possível concluir que as nanoesferas PLA-GCS e PLA-OA contendo o extracto de *S. equi* constituem uma alternativa viável ao antigénio recombinante de elevado custo. Tal como observado para a CTB, o CpG administrado com as nanoesferas de PLA-PVA não induziu um aumento dos títulos de anticorpos relativamente aos obtidos nos grupos de animais vacinados apenas com as nanoesferas, com a excepção das PLA-PVA.

De uma maneira geral, é possível concluir que o principal objectivo desta tese foi atingido, uma vez que foram desenvolvidos e caracterizados sistemas alternativos de micro- e nanopartículas capazes de induzir respostas imunológicas sistémicas e locais. As partículas formuladas com GCS, constituem potenciais adjuvantes a considerar no desenvolvimento de uma vacina segura contra a gurma, sem sinais toxicidade associada, contrariamente ao verificado no caso dos restantes adjuvantes geralmente utilizados com os antigénios de *S. equi*. Ainda assim, importa referir que a composição polimérica adequada à resposta imunológica desejada depende do tipo de antigénio de *S. equi* utilizado.

Infelizmente não foi possível avaliar a protecção dos animais vacinados contra a infecção pela estirpe virulenta *S. equi*, nomeadamente pelas nanoesferas

formuladas com GCS, o que poderia ter confirmado e reforçado os resultados obtidos e descritos ao longo desta tese. Juntamente com os ensaios em cavalos, esses estudos farão parte do trabalho a ser realizado no futuro, uma vez que deles certamente dependerá a aplicação futura destes sistemas.

Palavras-chave: poli-ε-caprolactona, poli(ácido láctico), partículas poliméricas, *Streptococcus equi* subs. *equi*, adjuvante, vacina.

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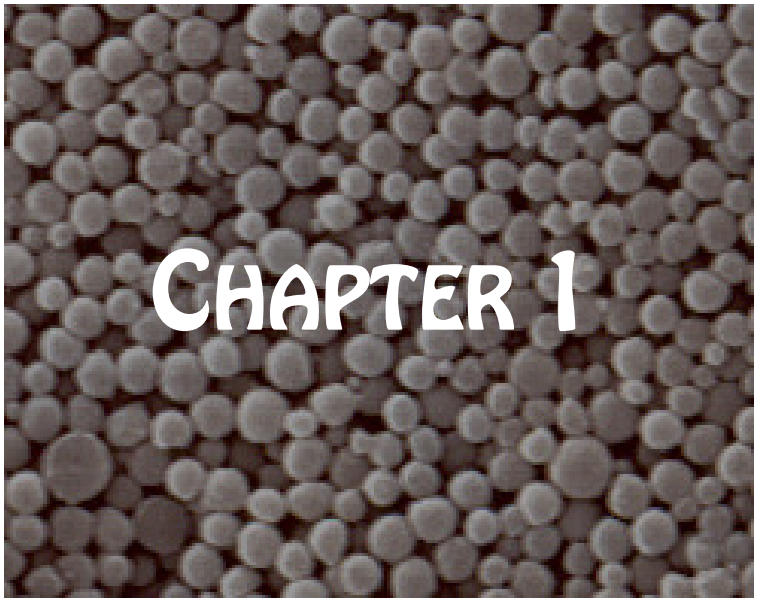
List of Abbreviations

ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ALG	Alginate
ANOVA	Analysis of variance
APCs	Antigen presenting cells
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl-1-phosphate
BSA	Bovine serum albumin
CD4 + T-lymphocytes	T-lymphocytes with CD4+ receptors
CD8+ T-Lymphocytes	T-lymphocytes with CD8+ receptors
CMIS	Common mucosal immune system
CpG-ODNs	Oligodeoxynuleotide with repeating C and G motives
CS	Chitosan
CT	Cholera toxoid
CTAB	Cetyl trimethylammonium bromide
CTB	Cholera toxin B subunit
CTL	Cytotoxic T lymphocytes
DCM	Dichloromethane
DMEM	Dulbecco`s modified Eagle's media
DT	Diphtheria toxoid
E.E.	Encapsulation efficiency
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC-BSA	Bovine serum albumin fluorescein isothiocyanate
GCS	Glycol chitosan
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAP-GST	Hyaluronate associate protein
HIV	Human immunodeficiency virus
HMW	High molecular weight
i.d.	Intradermic

i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
IFN-γ	Interferon gamma
Ig	Immunoglobulins
IL2	Interleukin 2
ISCOMS	Immunostimulating complexes
L.C.	Loading capacity
Lgt	Prolipoprotein diacylglyceryl transferase
LMW	Low molecular weight
Lpp	Lipoprotein acid phosphatase enzyme
LPS	Lipopolyssacharide
Lst	Lipoprotein signal peptidase
LT	*Escherichia coli* heat-labile toxin
LTB	*E. coli* Subunit B heat-labile enterotoxin
MALT	Mucosal-associated lymphoid tissue
MBL	Metal binding lipoprotein
M-cells	Nonciliated macro-fold cells
MDP	Muramil-dipeptide
MHC	Major histocompatibility system
MMW	Medium molecular weight
MPL	Monophosphoril lipid A
MTP	Muramil-tripeptide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NALT	Nasal-associated lymphoid tissue
NBT	Nitro blue tetrazolinum
NMR	Nuclear Magnetic Resonance
OA	Oleic acid
OD	Optical density
OVA	Ovalbumin
PAHs	Hyaluronate (capsule)-associated protein

PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% (v/v) Tween 20
PCL	Poly-ε-caprolactone
PCS	Photon correlation spectroscopy
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PLA	Poly(lactic acid)
PLG	Poly(glycolic acid)
PLGA	Poly(lactic-co-glycolic acid)
PMSF	Phenylmethanosulfonyl fluoride solution
PVA	Polyvinyl alcohol
s.c.	Subcutaneous
SAF	Syntax adjuvant formulation
SAIB	Sucrose acetate isobutyrate
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SeM	*S. equi* M-like protein
SEM	Scanning electron microscopy
SIgA	Secretory immunoglobulins IgA
SP	Spermine
Th1	T helper type 1
Th2	T helper type 2
TNF-β	Tumor necrosis factor β
TT	Tetanus toxoid
VLPs	Virus-like particles
VMD	Volume mean diameter



Chapter 1

Literature review

STRANGLES

Strangles is an infectious disease that affects the upper respiratory tract, mainly the nasopharynx and lymphatic nodes of the *Equidae*. Foals up to five years old are more susceptible to this disease [1, 2]. Nevertheless, it was already reported a strangles infection in two humans [3, 4] and one dog [5].

Streptococcus equi subsp. *equi* (*S. equi*) and *S. equi zooepidemicus* (*S. zooepidemicus*) are the two subspecies of *Streptococcus equi*, a Lancefield group C Streptococcus. The latter are commensal bacteria, frequently found in the upper respiratory tract of a variety of animals, such as horses, pigs, cows, dogs and cats. However, they can develop a secondary infection, consequence of a viral infection or stress. Symptoms of the resulting pathogenic situation are similar to those presented by animals suffering of strangles, caused by the virulent strain *S. equi*.

This pathogenic agent was firstly identified by Sand and Jenson, in the 19th century (1888), and by Schitz in the same year [6, 7].

Figure 1.1 – Horse infected with *S. equi*, which presents swallowed pharyngeal lymph nodes (adapted from [11]).

Strangles, from latin *strangulina*, was reported for the first time by Giordano Ruffo, between 1251 and 1262, in his manuscript *De Medicina Equorum*, and by Alberto Magno, in *De Animalibus*, in the period 1258 and 1262 [7]. It is a highly contagious worldwide infection, believed to be responsible for 30% of all reported equine infections [2, 8, 9].

Healthy horses are easily infected after ingestion or inhalation of environment particles contaminated with *S. equi*. These bacteria cross the oral and nasopharynx mucosae, lodge in the lymphatic nodes of pharynge and head regions, mainly in retropharyngeal and submandibular lymph nodes, causing abscess formation that, with disease progression, will be extremely painful (Figure 1.1) [2, 9-11].

Infected animals present depression (Figure 1.2), high fever, swollen lymph nodes and acute pharyngitis and rhinitis. These abscesses can frequently result in airway obstruction, cough and respiratory sounds. The drainage of exudate occurs after abscess disruption and this mucopurulent liquid contains a high amount of infectious *S. equi*. Therefore, at this phase, there is a high risk of infection of other horses in contact with the infected animal [2, 13] (Figures 1.3 and 1.4).

Figure 1.2 – *S. equi* infected horse (adapted from [12]).

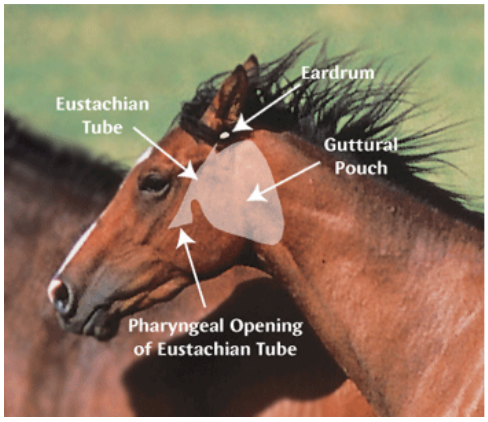


Figure 1.3 – Schematic localisation of guttural pouch in *Equidae* (adapted from [11]).

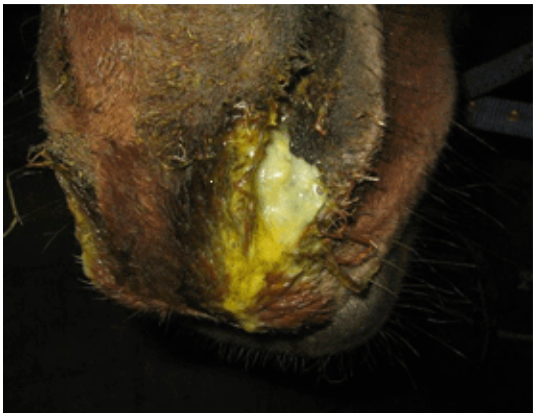


Figure 1.4 – Nasal secretions characteristically presented by *S. equi* infected animals (adapted from [12]).

Subcutaneously located abscesses may burst externally. These animals will present lesions in the skin that can be confused with a simple injury (Figure 1.5). In most cases, four to six weeks after the rupture and drainage of abscesses, starts up the healing of skin lesions and total recover of the disease, with complete elimination of *S. equi* of animal body [2, 9, 10, 14-16].

The *S. equi* is transmitted through various mechanisms, which, although may involve direct contact with nasal secretions or exudate drainage following rupture of abscesses, can be often associated with the use of contaminated

equipment and utensils, since the bacteria can survive in the environment for weeks [2, 17].

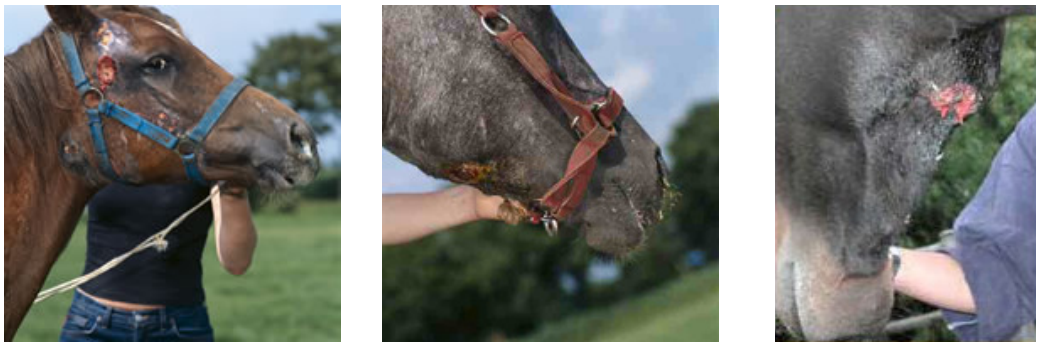


Figure 1.5 – Cutaneous lesions observed in the active phase of the disease as a consequence of subcutaneously located abscess rupture (adapted from [11]).

The infected animals have a long period of convalescence and a high level of morbidity, leading to a state of chronic illness or even death [1, 2, 10, 13, 17]. Indeed, although most cases are benign, about 5% of infections may result in more advanced states of the disease, consequence of the spread of the bacteria by the systemic circulation, with abscess formation in other parts of the body, particularly at the abdomen, chest and joints. The rupture of these abscesses (Figure 1.6) will lead to the release of its purulent contents, causing the pathological situation called 'bastard strangles' that, in most cases, leads to animal death [8].

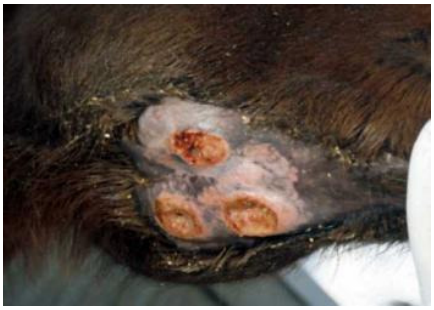


Figure 1.6 – Submandibular abscess rupture (adapted from [18]).

The words 'bastard strangles' first appeared in European veterinary literature in the seventeenth century, during which the words 'bastard', 'false' and 'imperfect' were used to refer atypical forms of diseases. However, the poor understanding of the etiology of the disease, made difficult the consensual use of the words 'bastard strangles' among authors, being then reserved for cases involving the formation of abscesses in the retropharyngeal lymph nodes, with drainage of its purulent content into the pharynx or the guttural pouch [7].

At the end of the eighteenth century, the term disappeared, being replaced by "irregular strangles", even if expressions like 'abscesses metastases', 'anomalous strangles' and 'supressed strangles' were also used. The word 'bastard strangles' was again used in the twentieth century, by Hayes and Wortley, both in the year 1906, but continued to be applied to denominate different situations, such as abscess spreading, disease at a middle stage or abscess formation in the retropharyngeal lymph nodes and parotid gland. There were still authors who preferred to refer to these cases as "atypical strangles","metastatic strangles" and "irregular strangles". Even today the use of that expression is not consensual, existing scientists who disagree with its use in the veterinary scientific literature [7].

DIAGNOSTIC

Animals infected with *S. equi* have an incubation period of 3 to 15 days, followed by abscess maturation and rupture, resulting, in most of the cases, in the total recovery of animals. However, the content of abscesses formed in the region of the pharynx can lodge in the guttural pouch (Figure 1.7), which may occur without any signs of the disease and, apparently, with full recovery of the horse [2, 9, 10, 14 -16]. The guttural pouches are unique in *Equidae* and consist in two isolated compartments, placed to the left and right in the throat, and communicate with the pharynx by a hole (Figure 1.3). This aperture opens intermittently, allowing the release of bacteria into the throat of these animals, and hence for the environment (beds, pastures, forage, drinking water, feed, utensils). This state of the disease occurs in 10% of infected animals and appears to be a consequence of an incomplete drainage of purulent liquid after disruption of abscesses present at the guttural pouch or in the lymph nodes [19]. As a matter of fact, though apparently healthy, these animals can accommodate *S. equi* for months, constituting a high risk of contamination of other horses during that period [2, 9, 10, 14-16, 20].

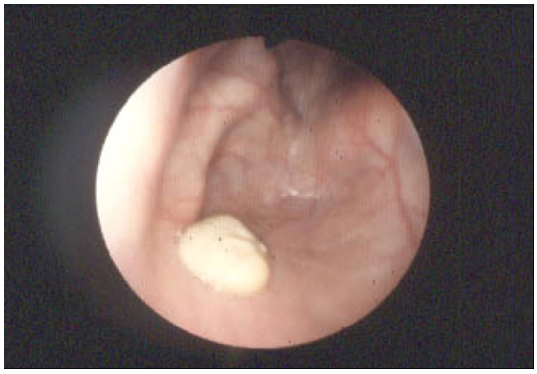


Figure 1.7 – Chondroid in the guttural pouch as a consequence of mucopurulent liquid accumulation and hardening with time (adapted from [2]).

The anti-*S. equi* antibodies can be identified in the serum of horses by a variety of methods, such as radioimmunoassay, bactericidal indices, mouse protection test and gel diffusion precipitin test [2, 9, 10, 14-16]. However, these techniques hardly detect the *S. equi* housed in the guttural pouch, which means that animals are identified as healthy for long periods of time, since they do not show any clinical signs of disease. Thus, the horse carrying the infectious *S. equi*, harbouring and preserving the organism for months, is the most important cause for the maintainance of the disease for prolonged periods of time, since the *S. equi* only survives for some weeks in the environment [10, 14]. Consequently, strangles remains an endemic disease worldwide and is of great economic importance since, after diagnosis, the infected animals will have to be isolated from other horses living in the stable and undergo a disabled and controlled mobility, until three negative results are obtained, during a period of two weeks. Still, to prevent any outbreaks, it is necessary to monitor the animals that have recovered from the disease for extended periods of time, since there are cases of animals carrying *S. equi* for 15 months without showing any clinical signs during the last 12 months [20]. As mentioned, the isolation of animals is particularly important since the risk factors associated with the development of this sub-clinical state of disease are not known. Epidemiological studies can help to clarify the reasons why some animals become carriers of *S. equi*, while others wholly eliminate [2].

Currently, the detection of animals carrying the infectious agent is carried out by the culture of secretions from the nasopharynx or, preferably, the upper respiratory tract and guttural pouches obtained by endoscopy. The latter can assess the tissues and the surrounding area, as well as collect samples from the washing of these pouches, which will be analysed by laboratory culture methods and amplification polymerase chain reaction (PCR) [16, 21]. However, the time needed to obtain the results and the cost of diagnosis, associated with the variety of mechanisms of *S. equi* transmission, make it difficult to implement the utilisation of these tests on a large scale in the event of an outbreak, as well as the rapid identification of animals that have been in contact with the infected horse and therefore may develop the disease [22]. Thus, it is essential the development of efficient and practical diagnostic tests, capable of direct use in the habitat of animals and able to quickly detect the presence of bacteria in infected animals, especially those who are in the prolonged period of convalescence and those that are true reservoirs of the bacteria. Indeed, these two cases are directly related with the main limitations of novel diagnostic tests. For example, results obtained in healthy and convalescent animals with the latest commercially available enzyme-linked immunosorbent assays (ELISA) [23], based on the detection of the main virulence factor of these bacteria (*S. equi* M protein, SeM), were not conclusive [2, 9, 14, 15].

The development of these tests requires a complete identification of quantitative and qualitative differences between the immune responses of animals carrying the *S. equi* and those identified in animals that have fully recovered from the disease, mainly for the absence of clinical signs and the elimination of bacteria. This information, in addition to result in a greater knowledge of risk factors in the development of persistent infection, may help in the prevention or resolution of the problem underlying the horses, apparently healthy, that constitute carriers of the bacteria, which will certainly contribute to reduce the incidence of this disease [2 , 9, 14].

Overall, strangles prevention is based on the quarantine of infected animals and in the identification of which may have been in contact with them, to enable

their isolation for weeks, preventing recurrent outbreaks of the disease. Having in mind the mechanisms of transmission, it is easily expected that the cleaning conditions of places where animals are kept, as well as persons who have contact with them, are particularly important.

TREATMENT

Strangles treatment is not consensual in the veterinary community because, although *S. equi* is sensitive *in vitro* to some antibiotics such as penicillin, erythromycin, lincomycin, tetracyclines and chloramphenicol, some prefer to allow the progression of the disease, drain the abscesses and eliminate the bacteria from animal body, while others may choose to prevent its development through the use of antibiotics [1, 2, 24].

Most of the treatments are not effective when the animal is already presenting the clinical signs of disease and a considerable number of animals recover completely within a few weeks without treatment. Moreover, some veterinarious believe that treatment with antibiotics contributes to delayed abscesses maturation, favouring the systemic spread of the infectious microorganism, which is the most serious stage of the disease, mainly resulting in death [10, 18]. Thus, although each strangles outbreak has individual characteristics, particularly regarding the financial condition of owners, the type of animal accomodation, the number of horses infected or in contact with the infectious agent and the degree of disease progression, its prevention seems to be the priority. The quarantine period should be adopted as soon as it is suspected or diagnosed the presence of the pathogen. To help the recuperation of animals, usually quite weak, analgesics and anti-inflammatories may be administered so that they can again eat and therefore ingest an adequate nutritional support, promoting the recovery process.

S. equi VIRULENCE FACTORS

Although this is a worldwide endemic disease, little is known about the virulence factors of *S. equi*, although most of the work done in this field has been targeted for the study of substances secreted or present at the surface of the bacterial cell [8, 25- 27].

Different isolated *S. equi* strains showed a great uniformity within their genetic composition, in contrast to the high variability showed by *S. zooepidemicus* strains. Recent studies also confirmed that the *S. equi* is a clone that has evolved from *S. zooepidemicus* [2].

The pathogenesis of *S. equi* infections is not fully understood, although the study of its genome has contributed for its better understanding [27, 28].

The infectious mechanism occurs in three different phases:

Phase I – *S. equi* adhesion to the upper respiratory tract mucosa;

Phase II – *S. equi* respiratory epithelium invasion;

Phase III – *S. equi* resistance to phagocytosis by polymorphonuclear cells.

PHASE I – *S. equi* ADHESION TO UPPER RESPIRATORY TRACT MUCOSA

The animals may be contaminated by a variety of transmission mechanisms, which seem to be dependent on the ingestion or inhalation of *S. equi*. The microorganism starts by adhering to the upper respiratory tract epithelium and then crosses the oral and nasopharyngeal mucosae. Overall, this first step is based on the binding of bacteria to the mucosal membrane and appears to be critical for the infection development and bacteria colonisation. It involves the participation of a series of adhesins already identified, such as the following:

Fibronectin binding proteins

During this first phase, fibronectin binding proteins seem to assume particular importance and the proteins SFS, ZAG and FNE have been already identified in *S. equi* strains [6, 27, 29-31].

The binding of bacterial surface proteins to the fibronectin, a dimeric glycoprotein found both in plasma and in the extracellular matrix, appears to be one of the mechanisms used by *S. equi* to establish their association with the animal cells and further internalisation. Thus, the identification of *S. equi* proteins involved in these connections has been the subject of several studies in order to better understand and possibly control the transmission of these bacteria [32-34]. These protein constituents of Gram-positive bacteria, as *S. equi*, able to establish links with fibronectin, have the characteristics usually attributed to the bacterial cell wall proteins, of which it is important to highlight the signal peptide, the C-terminal domain, that contains the anchor motif (e.g. LPXTG) that binds these proteins to the bacterial surface, and the hydrophobic domain, followed by a series of positively charged residues. In the C-terminal domain there is a region consistituted by repeated residues, responsible for the connection to the fibronectin N-terminal fragment (29 kDa), created after its digestion with trypsin. Other proteins can also connect through a domain not repetitive to another region of the fibronectin molecule [6, 30]. The subspecies *S. equi* bind to the native fibronectin. However, unlike *S. zooepidemicus,* does not recognize the 29 kDa fragment of this protein.

Lipoproteins

From the study of the bacterial genome it was possible to assess that the lipoproteins constitute about 2% of the proteomes of the genomes of Gram-positive bacterial strains. These proteins, after suffering a lipid modification, remain anchored in bacterial capsules and participate in important functions related to the input of nutrients, cells adhesion, protein maturation and transmission of signals in the cell. So, it is possible to predict that they are likely to play a key role in the interaction between the bacterial and host cells.

The bacterial lipoproteins are synthesised with type II signal peptides that direct them to the protein export route and later to a pathway where they will be modified, which requires the involvement of two enzymes and is

characteristic of prokaryots. The first is the enzyme prolipoprotein diacylglyceryl transferase (Lgt), which transfers a diacylglycerol group of membrane phospholipids to a cysteine residue, located in the cleavage region of the type II signal peptides. The prolipoprotein signal sequence, situated before the modified cysteine, will then be cleaved by lipoprotein signal peptidase (Lsp), leaving the lipid anchor unit in the N-terminal of this amended lipoprotein. This route appears to occur in Gram-positive bacteria in general, and both enzymes Lgt and Lsp are major targets of antimicrobial drugs, since they are exclusive of prokaryots and probably play important roles in the bacteria virulence and colonisation. Although Lsp and Lgt are not essential for the bacterial growth, several studies have shown that they are vital for the virulence of various bacterial agents, such as *Listeria monocytogenes*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*, in the case of Lsp, and *Streptococcus pneumoniae*, for Lgt [8].

Three lipoproteins were already characterised: lipoprotein acid phosphatase enzyme (Lpp) [35], metal binding lipoprotein (MBL), which is PsaA of *Streptococcus pneumoniae* homologous involved in the uptake of manganese [36], and hyaluronate (capsule)-associated protein (PAHs), that seems to be implicated in the capture of oligopeptides [37]. Indeed, the capsule hyaluronic acid of *S. equi* participates in the bacterial adhesion to the host cells, being an important factor in the first phase of the *S. equi* pathogenesis. It was observed that mutant strains without capsule had lower virulence in mice and horses [38, 39].

These features attracted the attention of Hamilton *et al.* (2006) to study the importance of these proteins in the virulence of *S. equi* and, if confirmed, if the effect was due to a particular lipoprotein or to the path of production of these proteins [8]. They studied the role of a lipoprotein in the *in vitro* colonisation of equine epithelial tissues by *S. equi*, and the development of the disease in a mouse model and ponies. The virulence of a mutant strain, deficient in lipoprotein maturase, was evaluated, in order to better understand the contribution of this lipoprotein for the pathogenesis of this microorganism. As a

result, the colonisation of the airway epithelial tissues was reduced after their inoculation with the mutant strain above referred, compared with the bacterial colonies seen after adding a virulent strain or a mutant strain to the cell culture, in which the enzyme Lgt was not synthesised (*S. equi*Lip) in order to identify whether the possible effect would be due to the virulent lipoprotein maturase in study or the lipoproteins in general, since the Lgt catalyses the first step of the lipid modification cascade. In this case, it was observed a decrease of purulent mucus production and none of the five infected ponies have shown clinical signs of disease, unlike those infected with the virulent strain or inoculated with the mutant strain *S. equi*Lip. These results suggest that lipoprotein maturase is a component to be considered in the formulation of vaccines for the prevention of strangles, since it seems to significantly contribute to *S. equi* virulence [8].

PHASE II – *S. equi* RESPIRATORY EPITHELIUM INVASION

The second phase of the infection mechanism involves the invasion of epithelium respiratory tract by *S. equi* bacteria and their transport to the lymph nodes and blood vessels present in *lamina propria*. The bacterium produces a series of degradation enzymes, such as hyaluronidase [40] and cytolitic toxins as streptolisin-like hemolysine, which are thought to have an important role in this particular phase [41].

PHASE III – *S. equi* RESISTANCE TO PHAGOCYTOSIS BY POLYMORPHONUCLEAR CELLS.

The resistance to phagocytosis is the main characteristic of the pathogenesis of this infection, essential for abscess development. SeM (FgBP with 58 kDa) and SzPSe (40 kDa) are bacterial cell wall antiphagocytic proteins called *S. equi* M proteins. They are fibrillar α-helicoidal molecules showing genetic homology only between their signal and cell wall associated sequences [42]. These proteins are antiphagocytic and opsonogenic, as antiserum is opsonic for their strain. Moreover, they have the ability to bind both to IgG and fibrinogen,

therefore being designated as M-like fibrinogen binding proteins. They also limit the C3b complement factor deposition on bacterial cell surface, which is the basis for their antiphagocytic action, as *S. equi* will not be identified by phagocytic cells as a foreign element, [42-44]. The SeM and the SzPSe, when isolated from different *S. equi* geographically located in different places, have the same molecular weight.

The role of SzPSe, the second *S. equi* M protein, in the virulence of *S. equi* is not yet known. In addition, it was found that SeM is responsible by 70% for fibrinogen binding, so that this mechanism can not be predominantly attributed to SzPSe M protein. The low homology between cellular wall external domains of these proteins suggests that the binding to fibrinogen seems to be dependent on the participation of other molecules beyond the linear sequence of amino acids [42].

It is important to mention that *S. equi* ability to resist killing by neutrophils also depends on the expression of the hyaluronic acid capsule [39]. Moreover, the factors that most contribute for phagocytosis resistance are:

- hyaluronic acid capsule;
- SeM - which requires the capsule for its function;
- the secreted proteins: IdeE, which blocks the phagocytosis mediated by C3b factor, and Se18.9 that binds to the complement regulatory factor H [27].

SeM

Taking into consideration the genetic homology observed between *S. equi* strains, it is easy to see that this bacterium produces a single type of SeM, with a molecular weight of 58 kDa [2, 42]. However, Chanter *et al.* (2000) and Menhan *et al.* (1998) found that SeM is not entirely uniform, which was surprising given the recognition of different strains of *S. equi* by serum obtained from convalescent horses [45, 46]. These authors observed genetic differences in sequences located immediately after the N-terminal, between 37 and 183 amino acids, which appear to be subject to great variability [45, 46]. Forty three SeM

alleles were identified, apparently related to geographical distribution, since some appear to be typical of North America and Japan, while others have been isolated in outbreaks occurred in Europe, Brazil and Australia. Therefore, the genetic sequencing of SeM of bacteria isolated in some outbreaks may help to better understand the epidemiology of this disease, allowing to identify any transmissions occurred during animal transportation around the globe and to develop new strategies for controlling the disease [2].

The SeM proteins are the main protective antigen. They can stimulate the production of anti-SeM specific antibodies, which are the basis of the so-called bactericidal reaction of the immune serum. It is not known the influence of N-terminal sequence variation of SeM of different *S. equi* strains in the protection conferred by vaccines that target this protein, although it may explain the modest results obtained in animal protection after being infected with these bacteria [11, 36, 42]. Although SeM presents an antiphagocytic activity similar to group A M proteins, repetitive regions A and B characteristic of this group of proteins are not found in SeM genetic sequences, even if their secondary strucuture is similar. The SeM can be called M protein because it satisfies the required opsonogenicity typically assigned to this family of proteins [42]. In conclusion, SeM is essential to the persistence of the disease, not only because it plays an important role in the mechanism of resistance to phagocytosis, but also because, apparently, the protective immunity requires specific anti-SeM antibodies [42].

Indeed, previously infected animals develop a protective immune response against SeM, presenting immunocomplexes of IgG and IgA anti-SeM, both in serum and in nasal secretions. The development of this immune response, verified especially during the active phase of the disease, appears to result from the continuous inflammatory responses triggered in animals and contribute to the genetic variability of SeM, which results from insertion of amino acid residues in the genetic sequence. Some authors think that this variation is the key for the persistence of the disease and bacteria virulence, since it changes the antigenic epitopes and is thus not recognised by the animal body. However,

further studies are needed to prove this hypothesis [2]. Combating and preventing the serious complications of the disease depend on the development of an effective vaccine [1, 36]. The response reflected in the production of anti-SeM antibodies has motivated the search for a vaccine based on the units of these M proteins [10]. However, taking into account the variability previously mentioned, regarding the development of vaccines, an interesting approach would be the use of subunits characteristics of strains of the area where the outbreak occurred, although this does not constitute a practical approach [6, 25]. The commonly used vaccines are composed by several subunits, not subject to significant variations from strain to strain, or attenuated *S. equi* bacteria that will eventually stimulate immunity against various antigens, resulting in a more efficient protection against different *S. equi* strains. The globalisation of its use might allow greater control of this endemic disease.

Other bacterial cell surface proteins may be subject to such changes and also involved in the *S. equi* virulence [6, 25, 47, 48].

In summary, while it is possible to identify and study each of these three phases, much remains unclear, particularly the mechanisms underlying the invasion and binding by *S. equi*, and their interaction with the phagocytic cells.

Other antigens important for *S. equi* resistance to phagocytosis

Apparently, *S. equi* strains have a range of antigens that bind to both major histocompatibility system (MHC) class II molecules and T cells, resulting in their stimulation and immune response direction, with the release of cytokines IL1, IL2, IL6 and tumor necrosis factor β (TNF-β), developing the characteristic symptoms of the acute phase of flu disease [2].

Only the antiphagocytic proteins Se18.9, SeM, SePE-I and SePE-H are exclusively expressed by *S. equi*. The latter two are pyrogenic exotoxins, capable of inducing strong mitogenic responses in mononuclear cells present in horse blood, although only the last one has the ability to cause high fever in these animals [49]. Similarly to protein SeM, sera of convalescent horses have antibodies specific to those two mitogens, the animal being protected against

the effect of SePE-I [39, 49]. These agents may also be important for abscess formation and thus for *S. equi* evolution and ability to redirect the immune response triggered upon infection. However, it is unknown if this mitogen will be able to induce protection against strangles because, despite the vaccination with SePE-I has protected animals against its pyrogenic effect, their infection with *S. equi* was not further evaluated. In addition, there are no published results supporting anti-SePE-I antibody production after administration of this particular protein [27].

Two other *S. equi* mitogenic toxins, SPEL and SPEM, were recently identified in 100% and 72%, respectively, strains of *S. equi* studied by Waller *et al.* (2007) [2]. These toxins can enhance the immune response induced by SePE-I, being necessary to develop opsonogenic antibodies against the three mitogens previously mentioned, to confer animal protection [50, 51].

Currently accepted, the main *S. equi* virulence factors are the capsule hyaluronic acid and the *S. equi* cell wall antiphagocytic M proteins.

PREVENTION OF THE DISEASE

Strangles combat seems to be dependent on the development of an effective vaccine since 75% of animals in the convalescent period have a protective immunity that is maintained for long periods of time, which can go up to 5 years, directed mostly against the protein SeM. The IgG and IgA specific anti-SeM present in serum and nasal secretions, will successively recognise, connect to and inactivate the *S. equi* that invade the animal organism. Thus, this protective immune response developed during infection supports the idea that a vaccine will be able to prevent strangles infection [1, 2, 9, 10, 17, 36].

S. equi isolated from different organisms are genetically and antigenically similar, and their protective immunity seems to be specific of this particular subspecies. Thus, vaccination with *S. zooepidemicus* will not give protection for a subsequent infection with *S. equi*, although the two subspecies have a DNA homology of more than 92% [42].

It is recommended the regular vaccination of horses against strangles and despite the development of many research projects since the 80s, directed to the development of an effective vaccine, the results obtained are disappointing, including the widely used commercially available vaccines. These vaccines, consisting of inactivated microorganism or SeM rich extracts, isolated or associated to adjuvants, do not have effectively contributed to control the disease and to protect horses against *S. equi* infection, causing serious adverse reactions [2, 9, 10, 25, 28, 42, 52].

The use of vaccines associated to efficient diagnostic tests seems to be the key to prevent and control strangles outbreaks [2, 9]. In Portugal no vaccine is available and despite the extensive ongoing research, there are only three vaccines marketed over the world [14].

Strepguard® with Havlogen® (Intervet Ltd), Equivac® S and Equivac 2 in 1 (Pfizer Animal Health Pty Ltd) vaccines are *S. equi* enzymatic extracts administered by intramuscular route (i.m.) [25, 53, 54]. The former is co-administered with the adjuvant Havlogen® in order to reduce adverse reactions usually assigned to this type of vaccine, and is currently marketed in Canada. The use of Equivac® 2 in 1, an association between tetanus toxoid (TT) a *S. equi* extract, and Equivac® S vaccines are approved in Australia and New Zeland.

Although the mechanism of protection is not fully known, the failure of the vaccines currently available seems to be associated with the lack of stimulation of local immune responses at the nasopharynx, and therefore to low levels of mucosal IgA and IgG subclasses, which are protective antibodies similar to those found in convalescent horses [9, 10, 13, 55].

Generally, the immune response developed locally plays a predominant role in protecting the upper respiratory tract against the infection by bacterial agents. Furthermore, although the immunity to *S. equi* is linked to the production of high levels of systemic anti-SeM antibodies, it is accepted that these antibody titres are not necessarily associated with the protection afforded by their

immune response, being predominant the response developed locally [2, 10, 36]. These facts suggest that these IgA antibodies produced in the respiratory tract are extremely important for the prevention and control of this disease, particularly at the ports of entry of this bacterial agent, and their action will be complemented by systemic antibodies [2, 9, 10, 36, 56, 57].

In order to distinguish the types of immune responses, Galan and Timoney (1985) conducted a study with ponies, which were treated with proteic supernatants obtained in *S. equi* cultures, associated with the adjuvant aluminium hydroxide [24]. In order to verify the protection given to animals, these authors induced the disease in ponies and then evaluated their response against *S. equi* extracted proteins, particularly assessing the levels of IgA and IgG in serum and in the nasal discharge. Differences were observed at the level of specificity of the antibodies produced in serum or at the nasopharyngeal mucosa. The IgA was the bacterial isotype predominantly present in nasal secretions. The systemic antibodies recognised a variety of proteins present in both the extract and supernatant of bacterial culture, not only of *S. equi*, but also of *S. zooepidemicus equi*. In contrast, the immunoglobulins IgA and IgG of mucus recognised only two proteins with molecular weights of 41 kDa and 46 kDa present in the enzymatic extract, with no differences between the proteins recognised by IgG or by IgA. Moreover, the seric immunoglobulins recognised the smaller hydrolytic products of the M protein (29 kDa and 37 kDa). Thus, these authors found some specificity for the proteins recognised by serum antibodies and those produced at the nasopharynx. This divergence in the specificity of the nasal and systemic antibodies may explain differences in local and systemic immune responses of horses to *S. equi*, and supports the independence between them [24]. The protection conferred to convalescent horses after new infection with *S. equi* was higher than that developed after the first infection, so the authors concluded that the protective immune response appears to be associated with nasopharyngeal antibodies. Possibly, the immunoglobulins IgG and IgA produced locally not only help to prevent the

development of the disease, but also to reduce the entry of the bacterial agent at the pharynx.

Since 1997, it has been approved in the United States of America (USA) the use of a vaccine (Pinnacle®, Fort Dodge) based on a genetically modified *S. equi* strain, without capsule and no virulent [58]. This vaccine is administered through the nasal route and prevents or reduces the severity of clinical signs attributed to this disease. It was developed in order to stimulate systemic and mucosal responses, similar to the protective immunity shown by animals during the convalescence period. However, although it allowed the stimulation of antibodies produced locally at the nasopharynx similar to those found in convalescent animals, horses treated with this vaccine showed severe adverse reactions, mainly mucopurulent nasal discharges and abscess formation mostly in local of injection, but also elsewhere in the animal body, which contributed for its rejection by the European authorities [13, 55, 59]. Moreover, animal protection was limited to a short period, and therefore the administration of repeated doses of the vaccine was necessary [22, 27].

In 2004 Equilis StrepE®, (Intervet Ltd) was introduced in the United Kingdom (UK) and USA. It is a vaccine based on a live attenuated *S. equi*, which parenteral administration conferred protection to all treated animals, but also resulted in important reactions at the injection site [60]. Alternatively, an intradermal administration (i.d.) in the upper lip mucosa was used (Figure 1.8), and conferred only a short duration of protection in 50% of treated animals. Consequently, it is advised the administration of this vaccine every three months only to animals with high or moderate risk of *S. equi* infection, since there were reported adverse reactions, mainly swelling of the site of infection, fever and, more occasionally, the formation of abscesses in the lymph nodes, which disappeared after 3 weeks [2, 14, 26, 27]. Some treated animals also developed the disease, which may be due

Figure 1.8 – Equilis StrepE® vaccine (adapted from [11]).

to infection with the virulent bacteria through contact with infected animals or the environment, and not induced by the vaccine itself [22]. However, all batches of vaccine were withdrawn and are sold only in the African market [60]. Even in countries where the vaccine was used with regularity, strangles remained as an extremely infectious and endemic disease.

Taking into account the virulence factors previously mentioned, some researchers believe that any resolution of the problem is dependent on the stimulation of multiple epitopes in order to induce high levels of antibodies and provide greater protection for animals by the administration of vaccines based on immunogenic *S. equi* cell surface proteins [2, 61]. Other authors have tried to clarify the molecular mechanisms underlying the pathogenesis of *S. equi* and thus find other virulence factors that may be potential candidates for the development of alternative vaccines [1, 6, 29, 31, 59].

Flock *et al.* (1999) have been investigating the formulation of a vaccine made by subunits based in several *S. equi* surface proteins previously identified, as it was found that vaccination with surface proteins of other Gram-positive bacteria, such as *Staphylococcus aureus*, in their recombinant form, have provided encouraging results in experimental models of infection [62, 63]. The specific antibodies produced against these proteins seem to have a double protective action, as they inhibit the bacterial cell wall adhesion to cell membranes and also by present opsonogenic activities [1, 59, 62, 63]. Three *S. equi* recombinant proteins were used for immunisation of mice and horses: SAE, a protein that binds specifically to α_2-macroglobulin, serum albumin and IgG of the host [64]; SPS, a fibronectin-binding protein [6], and FNZ, which is also a fibronectin-binding protein of the *S. equi zooepidemicus* [31], and that occurs in the truncated form (FNE) in *S. equi*. These proteins were studied alone or in combination, resulting in a significant protection, which was enhanced by the co-administration of FNZ and SPS proteins [1].

In another study, the same group assessed the level of protection conferred by three other types of proteins (15-20 μg per dose) located in the cell surface,

recently described as antigens in a mice strangles model: CNE, a protein that is the main factor responsible for *S. equi* binding to collagen; SclC (24 kDa), which presents a sequence of amino acids similar to the collagen [47]; and FNEB, a fibronectin-binding protein recently discovered (Figure 1.9) [51, 59, 65]. Animal protection was obtained after the administration of the first two above mentioned proteins, although the same result was not observed with FNEB (40 kDa). Co-administration of CNE (67 kDa) and EAG (19 kDa) led to a synergistic effect, contrary to the weak immune response obtained after the administration of EAG alone, which seems to be a result of the immunostimulatory action of CNE over EAG [59]. However, the CNE alone gives a significant immune response and protection, so that the synergistic effect may be due to the development of a response directed to two bacterial targets. Therefore, these observations allow predicting that the addition of a third antigen may also increase the protection level, although this has not yet been studied. CNE, SAE, FNZ, SPS and SclC induce the production of specific antibodies in animals infected by *S. equi*, which is a pre-requisite for their use as potential vaccine candidates [1, 47]. Probably, these antibodies may also be opsonogenic, although this has not yet been confirmed.

In summary, this group obtained promising results, suggesting that CNE, SclC and SAE are, among the recombinant proteins, the most promising for future studies of strangles vaccines, being potential alternatives to the use of live attenuated bacteria, even if their effectiveness and safety in horses has yet to be tested [1, 22, 59].

According to Waller and Jolley (2007), all currently available vaccines are not ideal in terms of safety, efficiency and practical use in the field. Thus, it remains necessary to develop a safe and effective vaccine against strangles, able to induce a long lasting immune response, which seems to be dependent on the concomitant stimulation of serum and mucosal antibodies [2, 9, 10, 27].

The development of this vaccine is limited by the incomplete knowledge of *S. equi* protective antigens and mechanisms of humoral and cellular mediated

immunity associated with this protection. However, the possibility to consult the gene sequences of *S. equi* and *S. zooepidemicus equi* allowed the identification and expression of immunogenic proteins, which are potential vaccine candidates [26].

Timoney *et al.* (2007) studied the protection conferred by associations of *S. equi* recombinant proteins, present at its surface or secreted, when administered to ponies. The proteins identified in the *S. equi* genetic library by the serum of a pony previously infected, were SeM, recSzPSe, Se73.9(CNE), Se51.9 (adhesion protein), Se44.2 (bind to IgG and platelets), Se46.8, Se75.3 (bind to multiple ligands), Se110.0, Se42.0 and 18.9 (antiphagocytic). All these proteins are present in the bacterial cell surface, except the last two, which are secreted by *S. equi* (Figure 1.9) [27]. This reinforces the idea that the proteins exposed at the bacterial surface appear to play a greater role in horse immune response, than those which are in the cytosol.

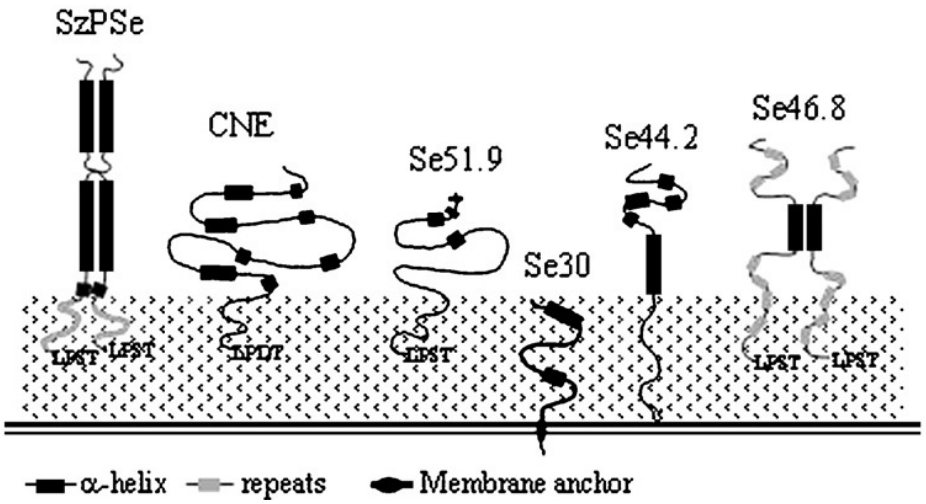


Figure 1.9 – Different types of *S. equi* cellular surface protein presentations (adapted from [27]).

It is also required additional studies involving the administration of these proteins through the mucous membrane and co-administration with different types of adjuvants, including delivery systems, to enhance immune response developed by their isolated administration.

ADJUVANTS USED WITH *S. equi* ANTIGENS

GENERAL ASPECTS

An effective immune response leads to the production of high levels of antibodies and the stimulation of cytotoxic T lymphocytes (CTL). Vaccines have the ability to induce the immune system so that, if a second contact with the antigen will occur, there is a rapid and extensive immune response, avoiding the effect of the pathogenic microorganism [66].

The immune system can recognise antigens by:

→ **Antibodies or immunoglobulins (Ig)** - expressed and secreted by B lymphocytes, and have the ability to bind to foreign structures, preferably those who are in the extracellular environment. This type of recognition depends on the native structure of the antigen;

→ **T lymphocytes –** recognise antigens captured by other cells and presented by the complexes formed between them and proteins of the MHC. The mechanism depends on the sequence of antigen units, rather then its three-dimensional structure. The T lymphocytes are divided in two groups, depending on the type of receptors expressed at their surface, $CD8^+$ ($CD8^+$ T-lymphocytes) and $CD4^+$ ($CD4^+$ T-lymphocytes), which determine the class of MHC system molecules involved in the complex formation. Thus, $CD8^+$ receptors recognise complexes formed between fragments of antigen, usually synthesised by the cell (such as viruses), and MHC class I molecules, while the $CD4^+$ T-lymphocytes will bind to complexes of peptides, extracellular or internalised by cells, and MHC class II molecules [67-69].

The cytokines play a key role in the activation, growth and differentiation of B and T cells, which interact with each other, and secrete factors that will stimulate other types of cells and possibly themselves. The $CD4^+$ T-cells can be divided into two subclasses, depending on lymphokines secreted after their stimulation and activation. Thus, the cellular immune response is

mediated by Th1 (T helper type 1) cells, secreting interleukin 2 (IL2), interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β), that will increase the expression of genes in antigen presenting cells (APCs) and activate B cells differentiation and IgG2a secretion. The production of cytokines after Th1 cells activation is extremely important for the eradication of *S. equi* pathogen, providing a useful vaccine for the immunisation of *S. equi* carriers. The humoral immune response is mediated by subclass Th2 cells, producing IL4, IL5, IL6, IL10 and IL13, being the IgG1 isotype primarily secreted after B cells activation [69, 70].

Wu and Russell (1997) showed that systemic and local immune responses are able not only to prevent infection, but also induce an immune response at the mucosa by the production of immunoglobulins IgA (SIgA) directed to specific pathogens [71]. This is of extreme importance especially when this mucosa is the port of entry to bacteria, such as *S. equi*, since the SIgA can block their binding to the mucosal surface and stimulate a protective immunity, usually more efficient than the systemic IgG antibodies [71, 72].

The extensive investigation of mechanisms involved in microorganisms recognition and biological interaction between them and immune system cells, such as macrophages, dendritic cells and epithelial cells (in the intestine), has allowed researchers to predict the adjuvant potential of certain substances and thus openned new perspectives for the development of alternatives vaccines to conventional forms [56, 73]. These vaccines must be secure, stable, easy to produce, store and administer, allowing efficiency at least equal to that obtained with their conventional vaccines, when available, and should not elicit serious adverse reactions [74].

Recent techniques of molecular biology, immunology and biotechnology have improved the development of a large number of specific and harmless antigens, including recombinant proteins, being one of the current trends for the formulation of new vaccines. However, these antigens are weakly

immunogenic, requiring multiple administrations and their association with adjuvants [56, 66].

Hence, vaccine adjuvants are needed. Adjuvants can improve the immune responses by different mechanisms [66, 68, 74]:

- → **Immunomodulation –** re-direction of the type of cytokines produced;
- → **Antigens presentation** – usually triggered by molecules or complexes that interact with the antigen in its native form;
- → **Transport of antigens –** usually achieved by the formulation of vehicles associated with the antigen, with the ability to interact with the APCs;
- → **Cytotoxic T lymphocytes induction –** antigens associated to particles or to water-in-oil (w/o) emulsions, that bind to MHC classe I molecules or brake the cellular membranes;
- → **Antigen depot –** micro and nanoparticles, emulsions (w/o) and aluminium salts associated to antigens.

The recognition of antigens by APCs is extremely important for their transport from peripheral regions to organised lymph tissues. The maturation of phagocytic cells leads to their migration to lymph nodes where they can then stimulate the B and T lymphocytes [75]. If the lymphatics are not reached, the antigen will not be recognised by the immune cells, with no induction of immune response [76]. The maturation and activation of phagocytic cells, the type of antigen and its presentation to APCs determine the type of immune responses induced by these cells [74-78].

Adjuvanticity depends on the administration routes, since they determine the type of APCs that will be stimulated. The intraperitoneal (i.p.) administration induces phagocytosis mostly by macrophages, while the i.d. injection led to a preferential activation of dendritic cells [66, 75].

Some antigens in its soluble form are not recognised by APCs and therefore do not induce the immune response [56, 74]. Consequently, antigens responsible

for the stimulation of these responses against certain pathogens can be imobilised or associated to controlled release particulate systems. These are captured by APCs, processed and transported to the lymph nodes and other organs, by the action of these adjuvants, based on a combination of some of the mechanisms previously mentioned [1, 66, 79-81].

Particulate antigen carriers such as polymeric microspheres and liposomes, seem to be the most suitable adjuvant to fulfill the main objectives: reducing the number of doses or the amount of antigen; increasing stability; administration by mucosal routes and eliciting an appropriate or stronger immune response, especially in the case of purified antigens or those more recently obtained by recombinant techniques [66, 82].

In general, adjuvants can be associated according to their form of presentation (Table 1.1), and different types have already been identified for human use, while others are in pre-clinical or clinical trials [83-85]. Even so, adjuvants already licensed (aluminium salts derived adjuvants in the USA and Europe, and emulsion-based adjuvant MF59 and antigen contained into immunostimulating reconstituted influenza virosomes (IRIVs) only licensed in the European market) do not allow the stimulation of cellular immunity, which justifies the continuous research directed to the development and utilisation of new adjuvants [56, 84-86]. Despite the variety of adjuvants already identified (Table 1.1), some of which stronger than the aluminium salts, their use in humans is limited by local or systemic toxicity [66, 83].

The choice of an adjuvant depends on the type of antigen, animal species, route of administration and adverse reactions often associated [66]. Recently, Merck and GlaxoSmithKline have developed two vaccines against human papilloma virus, composed by virus-like particles (VLPs) associated, respectively, to aluminium or AS04 adjuvant, which is based on aluminium and bacterial lipids already approved in Europe. The VLPs are genetically produced antigen-containing particles, whose size and shape are similar to those

presented by viruses [66]. The vaccine developed by Merck was approved in 2006 by the US Food and Drug Adminsitration (FDA) [66].

Table 1.1
Adjuvant examples*

ADJUVANT CLASSES	EXAMPLES
Particulate adjuvants	Aluminium hidroxide Aluminium or calcium phosphate oil/oil (o/o) or w/o emulsions as MF59 and Syntex adjuvant formulation (SAF) Liposomes Micro and nanoparticles (poly(latic-co-glicolic acid) (PLGA); chitosan (CS)) Immunostimulating complexes (ISCOMs®) Virosomes (IRIVs) Non-ionic surfactant vesicles (NISV)
Non-particulate adjuvants	Muramil-dipeptide (MDP) Muramil-tripeptide (MTP) and derivates Monophosphoryl lipid A (MPL) Trehalose dimicholate (TDM) Attenuated bacterial toxins (cholera toxin B subunit (CTB), *Escherichia coli* heat-labile toxin (LT)), subunits and mutant toxins (LTK63 e LTR72) Polyphosfazen Polynucleotides C3b complement factor CpG-oligodeoxinucleotides (ODNs) Saponin derivates (Quil-A, Quil-21) Non-ionic co-polymers (POE, POP) Cytokines (IL1, IL2, IL4, IL12, IL15, IL18) Granulocyte-macrophage colony-stimulating factor (GM-CSF) Interferon-gamma (IFN-γ) Carbohydrates (manan, glucan) Co-stimulating (CD80, CD86) and MHC system molecules *Streptococcus* cell wall components Virus and bacteria alive transporters Bacterial DNA *Mycobacterium vaccae* inactivated *Bordetella pertussis*
Adjuvant association	Freund adjuvant (complete and incomplete)

* Adapted from [66, 69, 83, 87]

A detailed approach to each type of adjuvants mentioned in Table 1.1 is beyond the scope of this manuscript. Consequently, the different types of adjuvants

already associated to *S. equi* antigens will be briefly referred, discussing in more detail polymeric micro and nanoparticles, since these are the adjuvants chosen to deliver *S. equi* antigens in a controlled manner and therefore to induce full protection after their mucosal administration.

ALUMINIUM SALTS

The aluminium salts were the first adjuvants to be approved for human use, being the only adjuvant regulated in the USA at the present [66, 74, 83, 87]. Aluminium hydroxide and aluminium phosphate can be good adjuvants when associated with antigen that by itself allow the stimulation of effective immune responses (eg tetanus (TT) and diphtheria (DT) toxoids, cell surface hepatitis B antigen), although the adjuvant effect obtained with the aluminium hydroxide is not sufficient to confer protection in many cases [56, 69]. Their mechanism of action is not completely known, though it seems to be due to the antigen depot effect in the site of administration, or to the macrophage or eosinophil activation, mainly stimulating antibody mediated immunity [66, 69, 83].

Aluminium phosphate and hydroxide have been the most used adjuvant in vaccines for human use, for the last 80 years. However, regulatory agencies defined some criteria that have to be fulfilled previously to the use of any adjuvant in humans, which would prevent the regulation of aluminium salts nowadays for use in human beings, because of their associated toxicity and side effects (Table 1.2) [66, 69, 83].

Aluminium is excreted by the kidneys, however, in cases of renal failure, it may trigger neurotoxicity resulting from their accumulation in the body, which is also often associated to Alzheimer's disease. The local toxicity of these adjuvants limits their administration through i.m. route, although the i.p. and s.c. routes are more effective in stimulating the immune response [66, 88-90].

The aluminium hydroxide association to other adjuvants, such as TT-entrapped microspheres, have been reported by various groups, and induced higher levels of antibodies [73, 92, 93]. It is important to mention that the amount of antigen

released *in vitro* from these carriers in the presence of aluminium hydroxide, was always lower than that obtained by those microspheres alone [73]. This adjuvant has been often associated with *S. equi* antigens, by several groups (Table 1.3) [23, 24, 49, 94, 95].

Table 1.2
Disadvantages presented by aluminium-based formulations

DISADVANTAGES
Failure to stimulate cellular immune responses
Limited activity when associated to recombinant proteins or other low immunogenic antigens
Variable absorption
Difficult freeze-drying
Repeted dose administrations
Non-stimulation of CTL and mucosal immune responses
Adverse reactions:
→ IgE stimulation - allergenic
→ Granuloma formation
→ Neurotoxicity

FREUND'S ADJUVANT (COMPLETE AND INCOMPLETE)

It is presented in the form of emulsions and its mechanism of action is due to the formation of an antigen depot in the local of administration, able to slowly release the antigen and stimulate the antibody production by B cells. Their use in humans has been limited by its associated toxicity, including the development of inflammatory reactions, granulomas and ulcers. However, its application is considered for the prevention of severe diseases [66].

In addition to aluminium salts, Freund's adjuvant is one of the most used adjuvants associated with *S. equi* antigens, being mainly administered to animals for the production of antiserum for further analysis (Table 1.3).

SUCROSE ACETATE ISOBUTYRATE

Sucrose acetate isobutyrate (SAIB) is a nonpolymeric, cheap adjuvant able to be aerosolised for intranasal (i.n) administration. It can be dissolved in an appropriate solvent, which will evaporate at the mucosal level, resulting in the formation of a biodegradable and biocompatible film able to release the antigens eventually dissolved in this polymeric solution [96].

Nally *et al*. (2001) studied the intranasal delivery properties of SAIB associated to a strong immunogenic SeM peptide (SEMF3), confirming the mucosal adjuvant property of this biocompatible excipient [96].

MONOPHOSPHORIL LIPID A

Monophosphoryl lipid A (MPL) is obtained by acidic hydrolysis of lipid A, and is the main responsible for the adjuvant effect of the Gram-negative bacteria cell wall lipopolissacharide (LPS) [66, 97]. It has the ability to induce Th1 immune responses and has been formulated as emulsions, in order to increase its activity [87]. This compound presents the lipid A adjuvant properties, but with lower toxicity, being already associated to a *S. equi* fibrinogen binding protein (FgBP) in order to assess their immunogenic potential (Table 1.3).

HAVLOGEN®

It is a polymer of acrylic acid cross-linked with polyallylsucrose, patented by the Intervet Ltd Company, and was associated with the *S. equi* enzymatic extract in the Strepguard® vaccine formulation (Intervet, Ltd) (Table 1.3) [53].

CHOLERA TOXIN B SUBUNIT (CTB) AND *E. COLI* HEAT-LABILE TOXIN B SUBUNIT (LTB)

Cholera toxin (CT) and *Escherichia coli* thermo-labile toxin (LT) are important adjuvants for systemic and mucosal immune responses (IgG and IgA), which may be administered by different routes, including oral, i.n., intragastric and

intravaginal [97-100]. However, in literature there are referred a few cases where the Cholera toxin (CT) adjuvant action was not verified when associated to certain antigens, such as Group B *Streptococcus* [101] and *Naegleria fowleri* lysates [102].

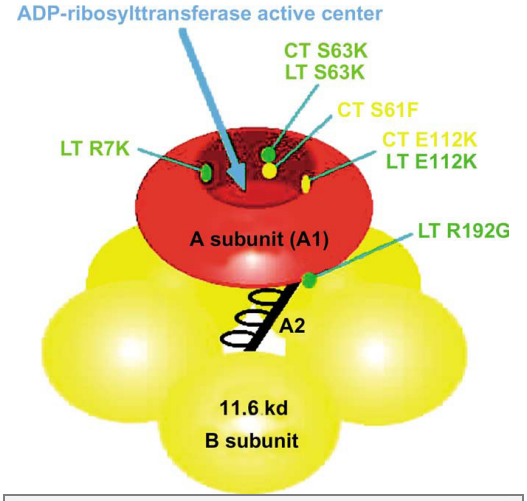


Figure 1.10 – *Vibrio cholerae* CT and *Escherichia coli* LT mutant properties, showing the affected aminoacids (adapted from [104]).

The toxicity and eventual accumulation in the central nervous system limit their use in humans and resulted in the development of genetically modified toxins, such as LTK63. Even so, CT safety problems were not solved by its mutant forms (Figure 1.10), as it was already found its transport by olfactory nerves to the brain and severe inflammatory responses [97]. However, the induction of a strong mucosal immune response has justified its association with recombinant *S. equi* proteins (Table 1.3).

CT has an A and B subunits, being the first involved in the modification of an adenylate cyclase regulatory protein, which is associated to the intestinal irritations observed in patients with cholera, while the B subunit (CTB) is a non-toxic component. CTB has five subunits that support the A subunit, being involved in the bacteria binding to cells, inducing a conformational modification that allows the toxic component (A subunit) to be introduced into the cell (Figure 1.10).

It was observed that the recombinant non-toxic CTB presents an adjuvant activity, even if the administration of higher doses were required. Even so, contradictory results have been obtained and therefore further experiments are needed in order to determine wether CTB can replace CT as an adjuvant [69, 97, 98, 100, 103].

IMMUNOSTIMULATING COMPLEXES (ISCOMS® AND ISCOMATRIX®),

ISCOMs®, described for the first time by Morein (1984), are strong immunostimulatory systems used as antigens carriers, improving their presentation to B cells and uptake by APCs [66, 105]. They are composed by saponins, phospholipids, cellular membrane antigens and cholesterol, among which are established hydrophobic interactions, allowing the formation of spherical particles with about 40 nm, which may incorporate hydrophobic or amphipatic antigens [66, 87].

Local reactions such as erythema and pain are attributed to the haemolytic activity of free saponin or triterpenoides glycosides QS21, used in ISCOMs® [105]. The QS21 is one of the 23 constituents of the natural QuilA, a saponin from *Quillaja saponaria*, which high toxicity precludes its application in humans.

The QS21 has the ability to improve humoral and cellular immune responses and has been included in different veterinary vaccines. Among human utilisation, its use is reserved for cancer prevention, because in such cases the risk-benefit assessment makes it acceptable [66, 87].

ISCOMs® maintain the saponins adjuvant action, but avoid adverse reactions triggered by those excipients, since the QS21 is linked to cholesterol, which is an important approach regarding the formulation of saponin-associated adjuvants [66, 87].

Unlike ISCOMs®, the ISCOMATRIX® does not have the antigen embedded in its formulation, being thus the later added. In addition to its great versatility, some researchers found that its use induces preferably a Th2 immune response, while the system ISCOMs® induced a mixed Th1/Th2 response [66, 106]. The use of these systems was discussed recently by Cox *et al.* (2006) and Pearse *et al.* (2005) [106, 107].

A vaccine based on ISCOMs® for the protection of horses against influenza is licenced since 1998 (Equip™, Schering-Plough) [87].

Table 1.3
Adjuvants associated to *S. equi* antigens

ADJUVANT	ANTIGEN	IMMUNISATION ROUTE	SPECIE	REFERENCES
Aluminium hidroxide	M protein	s.c. i.m.	Rabbits Horses	[94]
	S. equi supernatant cultures	i.m.	Ponies	[24]
	S. equi enzymatic extract and supernatant cultures	s.c.	Mice	[95]
	SePE-H and SePE-I recombinant (rec) proteins	i.m.	Ponies	[49]
	Rec SzPSe, Se73.9, Se51.9, Se44.2, Se46.8, SeM, Se75.3, Se42.0, Se110.0 and Se18.9.	s.c.	Ponies	[23]
Freund adjuvant (complete and incomplete)	M protein (antiserum)	s.c.	Rabbits	[108]
	Inactivated *S. equi*	i.p.	Rats	[109]
	SeM (antiserum)	s.c.	Rabbits	[42]
	rec Hyalutonate associate protein (HAP-GST)		Mice	[37]
MPL/DMT	FgBP	s.c.	Mice	[46]
	FgBP	s.c.	Mice	[110]
Havlogen®	SeM	i.m.	Horses	[111]
Cholera toxin (CT)	SeM	i.n.	Ponies	[52]
Sucrose acetate isobutyrate	Péptido da SeM (SeMF3)	i.n.	Horses	[96]
E. coli B Subunit heat-labile enterotoxin (LTB)	rec FNZ, SFS, e EAG	i.n. s.c.	Mice Horses	[1]
ISCOMs® (Matrix-S®)	rec CNE, SclC, EAG, FNEB	i.n.	Mice	[59]
ISCOMATRIX® (AbISCO)	rec CNE, SclC, EAG (19kDa)	i.n. i.m.	Ponies	[2]
PLGA microspheres	*S. equi*	i.n. i.m.	Mice	[61]

POLYMERIC MICRO AND NANOPARTICLES

The therapeutic use of particulate carriers for the development of an immune response is currently one of the most promising strategies to combat infectious diseases and, hence, one of the areas most investigated in pharmaceutical technology [56, 112]. There are many publications that justify its application in the induction of local and systemic immune responses, capable of protecting animals against certain pathogens [56, 68, 70, 84, 91].

ANTIGENS

The antigen entrapped in a polymeric carrier will be captured, processed and presented to APCs, such as macrophages or dendritic cells, and then to T lymphocytes ($CD4^+$ and $CD8^+$), in association with MHC molecules class I or II, depending on the nature of the antigen [69, 87].

Antigens responsible for the induction of a protective immunity against various pathogens may be detained or associated to particulate systems, which have the capacity to be modified and associated with other adjuvants in order to achieve a prolonged and/or pulsed release of the entrapped antigen [13 , 42, 57]. This approach maintains an immune response for periods much longer than those observed for antigen release from aluminium salts [66] or Freund's adjuvant [52, 113]. Thus, these polymeric carriers protect antigens from proteolytic enzymes, allow the co-entrapment of multiple antigens, promote antigen interaction with APCs, directing the delivery of vaccines to a specific target [66, 91, 113, 114, 115].

The prolonged and controlled release of antigens entrapped and/or adsorbed onto particles surface is fundamental to prevent a considerable loss of antigen associated with particles before they are taken up by APCs [73]. These carriers can mimic the prime and boost doses normally used in the conventional programmes of vaccination, reducing the frequency of administration needed to confer an extended protection to animals, which has been extensively demonstrated in the literature [42, 56, 74, 114, 116, 117]. Polymeric micro and nanoparticles allow the use of new therapeutic agents, such as macromolecules,

when compared with other controlled release systems, as implants, patches, injections or oral formulations.

Regardless of the type of antigen used, the vaccine will only be effective if it is administered by a desirable route and simultaneously is able to overcome the biological barriers and circulating antibodies present in animal's serum.

Several studies have shown that polymeric particles are an important type of antigen carriers with adjuvant properties. Their particulate nature along with the depot effect will determine the induction of the immune response [13, 42, 57, 66, 113, 114]. However, biodegradable and polymeric particles may present some problems, such as low loadings, loss of antigen activity during the entrapment process and difficulty in controlling the release of the active protein, especially when encapsulating larger and fragile molecules [118, 119, 120].

Soluble antigens are internalised by macropinocytosis, while antigens entrapped in polymeric particles are phagocytosed and therefore more efficiently presented by the MHC molecules, as demonstrated by several studies, in which there was even the stimulation of an immune response mediated by cells [68, 75]. Antigen presentation and the way it is processed are the main points to consider, defining the type and duration of an immune response, elicited by the administration of a single dose vaccine [66, 73]. The dendritic cells are of great importance within the immune system as they have the ability to phagocytose viruses, bacteria, antigens and particles. These cells are found in the blood, skin and mucosal surfaces, being the most important phagocytic cells of the immune system as they are able to process and display antigens to T lymphocytes, through the MHC class I and II molecules, and determine the type of immune response (humoral, cellular or tolerance) [75].

It is important to mention that the characteristics of each delivery system will define the type of immune response. While the neutralizing action of antibodies is dependent on protein conformation and structure, the recognition of antigens by the CTL epitopes is greatly affected by macromolecule degradation [75].

METHODS OF PRODUCTION

The development of new vaccine carriers is highly dependent on the reduction or even prevention of degradation, denaturation and aggregation of antigens, in order to ensure the maintenance of their antigenic properties. The extent and type of immune responses stimulated depend mainly on the stability of the antigen, particularly in the case of single dose vaccines, since inactive antigens do not contribute to the preservation of the immune response for long periods of time.

Different antigens present distinctive properties when entrapped in polymeric vehicles, so that each system needs to be developed in order to achieve a specific immune response [91].

There are several methods available to produce polymeric particles and their choice has mainly to do with the type of polymer and antigen to be used, and with the particle size desired. The methods more frequently used are the double emulsion water/oil/water (w/o/w) solvent evaporation technique, spray-drying and coacervation, which are modified accordingly to the polymer and antigens characteristics [75].

As it is generally accepted, amphipatic substances present the ability to surround a drop surface and spontaneously form micelles in aqueous systems, due to the intra and/or intermolecular interactions with hydrophobic residues, promoting stability by reducing free energy in the interface between the two phases, preventing flocculation and coalescence of droplets dispersed in the emulsion, resulting in smaller particles with lower polydispersity indices. The resultant carriers present a hydrophobic core and can be used as drug depots [121-123].

The double emulsion (w/o/w) solvent evaporation technique requires mechanical agitation and the use of organic solvents, such as dichloromethane (DCM), which normally does not affect the antigen structure and its immunogenic properties, although these aspects have to be confirmed in all systems formulated [120]. Protein denaturation can be displayed by sodium

dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), by the presence of higher or lower molecular weights bands, due to protein aggregates or fragments, respectively [120].

Several alternative methods have been developed to reduce the production costs, to maintain antigen structure and activity, to avoid the use of organic solvents, to obtain a sterile final product and to scale-up the process [56]. However, some limitations have risen as it is necessary the entrapment of high amounts of macromolecules, without change of their structural integrity or antigenic activity. As a result, the adsorption of important proteins in the biomedical field onto polymeric particles surface has been studied, providing a technical and promising alternative to entrapment, since it has been able to induce strong immune responses [56, 66, 87, 116, 124]. The proteins loading by adsorption prevents their contact with organic solvents, stress conditions, consequence of mechanical agitation, and low pH, resultant from the degradation of some polymers, such as poly(lactic acid) (PLA) and PLGA [56, 91]. This phenomenon is the product of the sequential adsorption of proteins with less affinity for adsorbents, and their displacement by those who have greater affinity for that surface. The interactions between different types of proteins in a mixture and these proteins with the adsorbents surfaces need to be clarified for a complete understanding and control of the absorption phenomena [66, 75, 87]. Proteins such as TT and albumin adsorb perfectly to the surface of PLGA particles, as previously described in the literature [124, 125]. Other researchers have chosen the adjustment of process production parameters, such as the one used for microparticles preparation by Kazzaz *et al.* (2000), in which they have conducted the replacement of non-ionic polyvinyl alcohol (PVA) polymer by anionic sodium dodecyl sulfate (SDS), resulting in the development of immune responses, including CTL, by the soluble antigen adsorbed onto particle surface [126]. Radiation can be used to sterilise these particles, prior to antigen adsorption [91].

The stability of the final product, regarding those produced with synthetic polymers, will be acceptable if kept at temperatures below their melting point.

The modification of the traditionally used polymers is also an interesting approach, for example by the use of different ratios of polymers having distinct degradation rates and molecular weights [56]. The polymeric carrier's degradation depends on erosion, which will be determined by the polymeric composition and molecular weight, porosity, size and loading capacity of particles, release rate of entrapped agent, interaction between polymers and antigen, and the method of entrapment used in its preparation. The burst release can not be too extensive as it reflects the amount of antigen released into the extracellular matrix and therefore not available to be taken up by APCs [75]. On the other hand, the *in vitro* characterisation of these polymeric systems is not standardized, which constitutes the major obstacle to their approval by the regulatory organisations such as the European Medicines Evaluation Agency (EMEA) and the FDA.

One of the most important characterisation assays is related with the determination of the amount of antigen actually associated to particle carriers and the confirmation that its structure remained intact, throughout the production method. Here, a possible aggregation makes it extremely difficult to complete the extraction of the protein actually entrapped, in addition to possible change of antigen activity and structure during the extraction process. The presence of interferents, such as sugars and the commonly used PVA, makes it difficult to obtain reproducible and accurate results for protein assays, and, thus, these aspects must be confirmed for each system of particles produced [91]. Consequently, FDA included the *in vitro* release tests in the guidelines for the approval of vaccines intended for parenteral administration, assuming mandatory the detection of at least 80% of the entrapped antigen [83, 91].

Vaccines to be used in healthy animals will only be accepted if minimal local and systemic side effects result from their administration. Adjuvants to be included in veterinary vaccines need to demonstrate the absence of serious adverse reactions, indicate the period of accumulation at the site of injection, and their elimination from the animal body. In addition, similarly to what is

observed for adjuvants to be used in humans, the preparation of those formulations should be easy and economic, as well as applicable to a variety of vaccines and result in a stable product [87]. Besides the characterisation usually performed to pharmaceutical products, the modification of gene expression induced by these polymeric carriers should be studied to better understand their impact on the molecular mechanisms of recognition and response of the immune system cells [56].

Polymeric composition, size, loading capacity, route of administration and the presence of other adjuvants will influence the particles uptake, their transport to the lymph nodes and interaction with surrounding tissues [70, 73, 75, 127-129].

Katare *et al.* (2005) performed the first experiment to evaluate the effect of particle loading in the type and duration of immune responses elicited by their i.m. administration [73]. The higher amount of protein entrapped in PLA particles led to increased levels of antibodies and the number of particles administered did not appear to have a role in the immune system stimulation. Systems presenting lower loadings will require a greater amount of particles in order to administer the same dose. Therefore, the APCs may become saturated, due to their limited capacity to transport the antigen-loaded particulate systems to the lymphatic tissues, although this could also result in a sustained antigen release, and consequently in higher antibody titres [70, 73]. On the other hand, a higher antigen loading, besides decreasing the number of particulate carriers needed to achieve a desired dose, will protect the antigen as it will be less susceptible to a possible inactivation due to particle degradation. Moreover, it is generally accepted that a larger amount of antigen initially presented to APCs promote a sustained immune response and therefore the activation of T lymphocytes [73].

Among the aforementioned characteristics, particle size seems to be the most important factor, although contradictory results have been presented in the literature [70, 127-129]. For the same size, the route of administration will

determine the type of APCs that will be stimulated and, consequently, the resultant immune response [73]. The particles size is particularly important as generally microparticles smaller than 10 μm seems to be more immunogenic than those with larger diameters [75, 120]. Some studies have indicated that microparticles with 10-15 μm are not phagocytosed [70, 73], although there are reports showing that carriers bigger than 20 μm have successfully induced immune responses, which shows that the antigen uptake and processing by APCs is not completely dependent on particle phagocytosis [70, 73, 127, 130, 131]. However, smaller particles or even polymeric fragments can be founded between these larger vehicles and can be taken up by APCs [70, 73]. In addition, larger particles usually present lower release rates, and therefore contain higher amount of antigen to present to the APCs, which will consequently express a higher number of MHC molecules at their surface. Other authors think that these larger particles will bind to macrophage surface, constituting a depot that will continuously release the antigen throughout time [66, 73]. However, particles with mean diameters between 50 and 100 μm will present reduced adsorption onto phagocytic cells and release the antigen in a limited manner due to smaller macrophage size (10-15 μm) [73].

Particles with mean diameters of 1 μm are more efficiently presented by MHC class I molecules at APCs surface and stimulate the $CD8^+$ T-lymphocytes, although particles with 0,04-0,05 μm elicited humoral and cellular immune responses, in contrast to the aluminium salts [66].

Katare *et al.* (2005) found that particles with 2-8 μm led to a humoral immune response for 250 days, significantly higher than that obtained in groups immunised with particles larger and smaller than 8 μm and 2 μm, respectively [73]. Regarding submicrometric particles, the results were not expected, and authors indicated that this could have been due to a more pronounced exocytosis [132] or a lower efficiency in antigen processing and presentation [133], when compared with the immune response induced by the micrometric particles. The release of the smallest particles from the endosomic vesicles to the

cytosol contributes to the stimulation of cell mediated immune responses, competing therefore with the production of antibodies, consequence of the antigen presentation by MHC class II molecules. As a result, the administration of a single dose of nanoparticles can result in lower levels of antibodies than those stimulated by microparticles [73, 129].

Generally, antigens carried by polymeric particles have the ability to escape the degradation in the endosomes, reach the cytosol in higher concentrations, than those antigens administered in soluble form, being presented by MHC class I molecules more effectively and for longer periods of time [68].

ADMINISTRATION ROUTES

The extensive research focused on polymeric carriers with different composition, morphology and sizes, has allowed the formulation of vehicles to be administered through several routes of administration (oral, i.p., i.n., i.d., i.m.), aiming to develop single-dose vaccines or to associate different antigens in one system [66, 68, 73, 91, 129]. On the other hand, vaccination through the mucosa is easier because it does not require the use of needles and syringes, is economic and more effective in the stimulation of mucosal immune responses, when compared with parenteral routes. Consequently, non parenteral immunisation routes are highly desirable [57, 61].

S. equi main port of entry in the host is the nasal mucosa which, associated with the low production costs and easy administration of formulations administered through the nose, justify the choice of nasal route as the one preferred for the administration of a vaccine against a *S. equi* infection. Moreover, previous studies have shown that the i.n. administration of antigen-loaded polymeric particles was capable of inducing not only humoral and cellular immune responses, but also local responses spread at different mucosal membranes, regardless the targeted one, essential to protect the host against a further invasion [2, 71, 72, 124, 134, 135].

Particle size influences their deposition at the nasal cavity and mucosal uptake. It was observed that particles larger than 5 μm are taken up by Peyer patches

and then remained in this area, while carriers with lower mean diameters were identified, not only in these patches, but also in mesenteric lymph nodes and spleen. Therefore, these latter carriers will preferably induce systemic immune responses, while the former will stimulate local immunity associated to the mucosas [125, 139, 140].

The microentrapment of TT [70], DT [141] and hepatitis B surface antigen has been the most studied system for the development of a single dose vaccine [142, 143]. Indeed, the entrapment of many antigens, as TT and DT, in biodegradable polymeric microspheres has been extensively studied for the induction of a systemic but also mucosal immune responses, regardless the administration site [70, 144, 145].

Eyles *et al.* (1998/1999) showed that antigens entrapped in microspheres were able to induce immune responses stronger than those resultant from administration of its soluble form [72, 135]. Almeida *et al.* (1993) found that the i.n. administration of TT adsorbed onto PLA microspheres surface, with mean diameters ranging from 0.1 to 1.6 μm, induced systemic and local immune responses higher than those elicited by their administration through the oral route, which remained even 15 weeks after animal vaccination [124]. Wu and Russell (1997) obtained a similar pattern, which may be the result of the difficult administration of large amounts of antigen to the intestine, as a consequence of the dilution effect and stomach low pH [71].

The administration of nucleic acids by different routes (i.m., oral, i.d., s.c.) induced Th1 type immune responses, along with antibodies production and CTL stimulation. Therefore, some researchers are developing vaccine formulations based on DNA entrapped polymeric particles [75].

Particle surface modification is also one of the most recent approaches to induce phagocytosis [75]. Biodegradable and polymeric particles associated to mucoadhesive compounds, as CS [97, 117, 136, 145] and ALG [146] to increase the particle residence time in the vicinity of cells, and absorption enhancers, such as spermine (SP) and oleic acid (OA), are important approaches in order to

overcome, for example, the nasal barriers and increase antigens uptake through mucosa [145, 147-149].

CS surface modified PLGA microspheres developed by Jaganathan *et al.* (2006) have increased nasal uptake of these vehicles [136]. Their particulate form and high mucoadhesive properties conferred by CS seem to be key factors for the immunostimulating effect of those polymeric carriers [66].

CS is a co-polymer of β-(1-4)-D-glucosamine and β-(1-4)-N-acetyl-D-glucosamine, prepared by the alkaline deacetylation of chitin [150, 151], being widely used in the pharmaceutical field, since it is a biodegradable, nontoxic and positively charged polysaccharide, which safety has already been confirmed [152, 153]. It has the capacity to form colloidal particles and entrap macromolecules by several mechanisms. Encouraging results have been obtained for the tranfection of DNA entrapped in CS nanoparticles [153]. However, low solubility at physiological pH is one of its major drawbacks. Increasing the internal phase viscosity reduces not only the interaction between entrapped proteins and the organic phase, observed at the (o/w) primary emulsion interface during homogenisation process, but also its diffusion through the aqueous phase, resulting in higher loadings [152]. The CS particles are also recognised and captured by cells of the mononuclear phagocytic system, due to the opsonisation process, in which serum proteins and glycoproteins coat these particulate systems [153]. This polymer increases drug bioavailability due to its mucoadhesion property and its ability to open the tight junctions, which promotes the paracelular transport [98, 154-156].

The review of all types of materials used in the formulation of micro and nanoparticles is outside the scope of this introduction. Moreover, the adjuvant properties of many of these polymeric carriers had already been reviewed by several researchers. As a result, PLGA, PLA and PCL polymeric particles will be discussed in more detail, as the present study consists mainly in the development of polymeric systems for mucosal administration of *S. equi* antigens, mostly based on polyesters [56, 75, 91, 134, 157-160]. These are able to

controll release of antigens for prolonged periods of time, avoiding short humoral and cellular immune responses, stimulating CTL and overcoming the antigenic variation of the causative agent. Aspects related to antigen distribution within particles, formulation stability and cost of production will also be evaluated as these are important characteristics that have to be taken into account when chosing the type particulate system.

Poly(lactic acid) and poly(lactic-co-glycolic acid)

The synthetic PLGA is the most studied polymer for vaccine formulation [66, 75, 91]. PLGA is a polyester composed by one or several monomers of D-lactic acid, L-lactic acid and/or glycolic acid. It is a well evaluated polymer, widely used in the preparation of controlled release systems and its degradation properties are already well known [66, 75, 91]. Indeed, there are several publications reporting the use of these polymers in different therapeutic areas. PLGA polymeric carrier systems, with distinct sizes and morphologies, can encapsulate molecules with different physicochemical characteristics. Their chemical composition determines the final structure, which can be highly crystalline (poly(L-lactic acid) or amorphous (poly(D-,L-lactic-co-glycolic acid) [70, 145]. It has been used for decades, in humans and animals, as degradable surgical sutures and in the development of peptide controlled delivery systems. Among these, some have been tested in clinical trials and 12 products are already licensed in 10 different countries by the regulatory agencies [68, 70, 87, 91, 161].

Several studies have shown that PLA and PLGA particles are potential carriers for proteins or antigens [70, 75, 91], entrapped or adsorbed [162], preserving antigen stability and immunogenicity, which is important since their structure has to be maintained in order to stimulate the immune response for extended periods of time, avoiding the administration of repeated doses [70, 73, 91]. These polymeric vehicles can continuously and slowly release peptides and proteins for 1 to 4 months. The development of immune responses and animal

protection for over one year after vaccination with PLGA carriers has been reported [70, 75, 91].

DT entrapped in PLGA microparticles with a mean diameter lower than 5 μm, were found at the injection site, even 4 weeks after their administration, supporting the depot effect commonly associated to their adjuvant properties. This study has revealed the transport of these particles by phagocytic cells from the injection site to distant lymph nodes [163].

In addition, PLGA micro (<10 μm) and nanoparticles have the ability to protect antigens, such as proteins, peptides and nucleic acids against extracellular degradation. These polymeric carriers can be taken up by APCs, such as macrophages and dendritic cells, that will process, transport to the lymph nodes and present the antigens to T lymphocytes [68, 75, 91, 93, 143]. Although biocompatible, a weak inflammatory response also appears to be inherent to their adjuvant effect [91].

After phagocytosis, the intracellular phenomena associated to antigen processing will depend on polymeric composition and surface characteristics of particles. Positively charged particles are rapidly phagocytosed, leading to the formation of phagosomes, resultant from ionic interactions established between particle positive charges and negatively charged phagosome membrane. Pseudopod formation is no evident during the negatively charge particles internalisation, while phagosome maturation and degradation are faster [164].

PLGA monomers and their proportion allow the formulation of different vehicles with characteristics suitable to several types of antigens, routes of administration and type of immune responses required. Since PLA polymer has a degradation rate slower than the poly(glycolic acid) (PLG), the association of both monomers can be used in the controlled release of antigen, being theoretically possible to predict the release rate taking into account their individual degradation pattern. On the other hand, monomer optical properties also play an important role in polymers degradation, which is faster for PLA racemic mixture than for their isolated isomers [66, 73, 143, 165].

Particle surface modification with different functional groups or macromolecules has been explored to assess their influence on phagocytic cells activation and maturation [91]. The i.n. administration of DT-entrapped PLA microspheres containing CS has induced higher specific antibodies levels than those observed for PLA negatively charged particles [70, 145].

PLA and PLGA particles present a negative charge in solution, which is due to their terminal carboxylic groups or to the adsorption of hydrolysed PLGA oligomers [75]. Still, the production of particles with positive surface charge is easily achieved by the addition of cationic molecules as cetyl trimethylammonium bromide (CTAB), stearylmine and polyethyleneimine (PEI) during the particles formulation [87, 166-169].

CS surface modified PLGA microspheres were developed by Jaganathan *et al.* (2006), and used in animal vaccination by i.n. route. Mucus layer is anionic at neutral pH, being therefore able to interact with positively charged particles, leading to a lower particle clearance and hence a prolonged residence time on the mucosal surface [74, 136].

Shen *et al.* (2006) found that ovalbumin (OVA)-entrapped PLGA particles were taken up by dendritic cells, through endocytosis, followed by antigen processing in cytosol proteosomes and presentation by MHC class I molecules [68]. Thus, antigens carried by PLGA particles were found in the cytosol in higher amounts than those obtained when administered in their soluble form or adsorbed onto non-degradable carriers (such as latex), which shows that particle composition is essential for uptake. Moreover, there was a slow and continuous antigen release from PLGA particles, which is important due to the rapid turnover of MHC class I molecules at cells surface [68]. The microentrapment of *Bacillus anthracis* recombinant antigens has stimulated dendritic cells, in contrast to its soluble form [170]. The authors confirmed the observations and conclusions of other studies, showing that the adjuvant mechanism presented by the particulate system was different from that observed when particles and recombinant antigens were administered alone

[171]. Enhanced antigen processing and presentation by dendritic cells were also obtained by Sun *et al*. (2003) [172]. In addition, the ability of particles to stimulate antibody production was reported by O'Hagan *et al.* (1991) and Eldridge *et al.* (1991) [173, 174]. Maloy *et al.* (1994) and Moore *et al.* (1995) observed that systemic and oral administration of PLGA microparticles entrapping OVA or human immunodeficiency virus (HIV) gp-120 to mice, led to the stimulation of CTL and cellular immune responses, which is extremely important as these are weakly induced by new adjuvants [87, 91, 175, 176]. Moreover, PLGA micro and nanoparticles have been proved as potential adjuvants for the delivery of DNA and recombinant antigens, being able to induce humoral, cellular and also mucosal immune responses [66, 70, 75, 89].

Several studies have shown that particle size has an important role in particle uptake by phagocytic cells. In fact, the diameters range presented by PLGA particles entrapping pertussis toxin and filamentous haemagglutinin influenced the type of immune response obtained, being strong Th1 immune responses preferably induced by microspheres, while nanoparticles preferentially stimulated Th2 cells pathway [177]. This physical characteristic is another factor responsible for the extensive investigation towards the development of PLGA-based particulate carriers, as it is possible to formulate antigen controlled release PLGA systems of similar polymeric composition but different diameters [178-181]. Even so, some limitations are still associated to these vehicles, such as their high costs of production, the conditions required for their formulation under aseptic conditions, the common use of organic solvents during preparation method, which can negatively affect antigen structure integrity and immunogenicity, besides others drawbacks related with their *in vitro* and *in vivo* characterisation assays, as well as the complex prediction of particle adjuvant effect in humans based on results obtained in a mouse model [75, 91]. Antigen aggregation, denaturation, and their adsorption onto tubes used for *in vitro* release studies, have hindered the effective characterisation of some of these loaded particulate systems, and their approval by the regulatory authorities [182-184]. For example, Nutropin depot® was the first protein controlled

delivery system, that was introduced in 1999 on the USA market but withdrawn five years later by manufacturer decision based in its high costs of production, mainly due to the sterilisation conditions [185].

The degradation of glycolic and lactic acids homo and copolymers occurs by hydrolysis resulting in the acids above mentioned, which will be eliminated from the body by the Krebs cycle. In fact, one of the major disadvantages associated to these polymers is the extremely acidic (pH 2-3) surrounding media resulting from the accumulation of degradation products, which is not indicated for macromolecules, as several antigens are denatured at these pH values [91, 120]. This can be overcomed by addition of bases (for example, calcium hydroxide and magnesium carbonate) or polyethylene glycol (PEG) to the media, or by reducing the polymer hydrolysis rate (increasing the lactic acid fraction, for example) [91, 119 , 185].

The extensive study of PLGA carriers performed by several groups clarified the influence of parameters such as molecular weight, formulation technique and chemical composition in their adjuvant properties. It was concluded that even polymer low molecular weight and high loadings may provide a continuous antigen release. As a result, some limitations initially associated to these carriers, such as low loading capacity, high burst release and difficult particles dispersion for parentereral administration, have been overcome [91, 186, 187]. Preliminary studies conducted by our group showed that the i.n. and i.m. administration of whole killed *S. equi* cells or bacterial lysate entrapped in PLGA microspheres resulted in a immune response able to protect mice against further infection by the virulent strain [61]. These studies have also shown that polymeric particulate systems are promising *S. equi* antigen carriers and adjuvants showing a high potential for use in strangles prevention.

Poly-ε-caprolactone

PCL is biocompatible polyester that has not yet been fully explored for antigen entrapment. It has a great potential for developing antigen controlled release

matrices by its biocompatibility, low degradation rate, hydrophobicity, good drug permeability, *in vitro* stability and low toxicity [68, 73, 120, 144, 188-190]. A positive correlation between the hydrophobicity and particle uptake by cells was found, whereas cells had greater affinity for PCL particles than for those based on PLA [144, 191].

PCL degradation does not result in media acidification, in contrast to PLA and PLGA polymers, which is an advantage as low pH values can adversely affect protein structure, and therefore be responsible for cellular and humoral immune responses failure [120, 144, 192, 193]. Even so, it is still necessary to assess the antigen structure after being associated to PCL polymeric carriers [91].

PCL particles have a slow degradation rate in an aqueous media due to polymer hydrophobic and crystalline nature, which prevents the incorporation of water in the system, being the antigen release consequence of erosion of polymeric matrix [120, 194]. PCL crystallinity is particularly important in the development of controlled drug delivery systems, as hydrophilic substances will be entrapped only in polymeric amorphous regions. Moreover, high loadings are especially important for expensive antigens or when only a small amount is available [120].

The adjuvant properties of PCL microparticles have been studied by some groups, and their ability to induce immune responses after parenteral and non-invasive administrations has been previously mentioned [72, 117, 124, 135, 136, 144]. However, vaccine controlled release systems, such as nanospheres based on PCL, have not been extensively referred in the literature, probably due to aggregation that often occurs after administration. However, some studies have suggested that PCL is a potential adjuvant, mainly due to its hydrophobic nature responsible for PCL particles higher uptake by APCs [120, 144].

PCL was used for the development of levonorgestel controlled release systems, which were included in clinical trials in several countries [120]. Jameela *et al.* (1996) showed that the i.m. administration of bovine serum

albumin (BSA) entrapped in PCL microspheres, associated to incomplete Freund's adjuvant, were able to stimulate systemic humoral immune responses [195]. Murillo *et al.* (2001) demonstrated in a mouse model that *Brucella ovis* antigen extract loaded in PCL microparticles are a potential vaccine to prevent the infection induced by this microorganism [196]. The oral administration of a single dose of *Schistosoma mansoni* antigen entrapped in PCL microparticles led to a long-lasting immune response, which remained for longer periods than that stimulated after an oral and i.n. administration of PLGA microparticles loaded with the same antigen [120].

The double emulsion (w/o/w) solvent evaporation technique is the most common method used to produce PCL carriers. In general PCL is dissolved in an organic solvent, being after that homogenised in an aqueous phase containing a surfactant, to form a w/o emulsion, which will then be added to an aqueous continuous phase. The microspheres are hardened after solvent evaporation [120].

Particle surface modification by hydrophilic polymers such as CS will increase the w/o/w double emulsion stability, as it will limit the interactions between the organic and external phases. Similarly to PLGA particles, the association of CS and ALG to PCL carriers can increase nasal mucosa residence, as electrostatic interactions will be established with the negatively charged epithellium mucus [136].

Since the production method previously mentioned involves the use of organic solvents, particles interior may be sterile, which along with aseptic conditions and good laboratory practice adopted during particle production, will result in a sterile final product.

Several groups have shown that this method does not compromise the activity and immunogenicity of entrapped antigens, such as ovalbumin [121], BSA [197], TT [124] and CTB [131]. Benoit *et al.* (1999) used double emulsion (w/o/w) solvent evaporation method to formulate PCL microparticles, evaluating parameters such as polymer and BSA protein concentration, external

aqueous phase volume, primary emulsion internal phase viscosity, external phase surfactant type (SDS, PVA and Tween 80) and concentration, time of homogenisation, salt addition to internal phase (NaCl) and pH (pH 3, 7 or 10) [120]. Higher polymer concentrations resulted in larger particles and higher loadings. The size increase may be a consequence of particle agglomeration, even if a monodisperse particle population with 5 μm of mean diameter has been obtained [120, 198]. On the other hand, entrapment efficiency (E.E.) increase could be due to the higher viscosity of the organic phase, which reduces protein migration from the inner phase to the external one [120].

These authors found a significant decrease in E.E. when higher amounts of protein were used, without any change in carrier size or loading, which was explained by the small amount of polymer available for protein entrappment. PVA was selected as the most appropriate surfactant, as emulsions gave particles with satisfactory loadings (1.38±0.11% w/w) and sizes (1-10 μm).

Higher internal phase viscosity led to a small increase in particle size, but did not induce any changes in their loadings and E.E., as reported by Jeffery *et al.* (1993) [121], although these results are opposite to those previously published by Ogawa *et al.* (1988) [199]. The use of higher external phase volumes increased particle mean diameter and loading, probably due to the formation of larger droplets during double emulsion homogenisation. On the other hand, prolonged homogenisation has resulted in particle size reduction probably due to droplet division during the emulsification process. The NaCl addition into the inner phase decreased the amount of protein entrapped in microparticles, but it has not induced any changes in particle size.

Regarding protein release profile, it was obtained a 30% burst release of the associated protein, which was followed by a controlled and sustained period that lasted for 3 months, and appears to be a consequence of PCL low permeability to macromolecules. Moreover, Benoit *et al.* (1997) have demonstrated that protein molecules have affinity to hydrophobic surfaces, which indicates that those 30% are probably to be a result of protein desorption

from particles surface [120, 200]. Neverthless, this release profile appears to be appropriate for vaccine delivery, since particle administration will first mimic the first dose and further simulate boost as they will be able to continuously release the antigen.

ANTIGEN-LOADED BIODEGRADABLE MICRO E NANOPARTICLES

The entrapment of different antigens, such as proteins, peptides and viruses, has been studied by several research groups, which was already discussed in a considerable number of reviews [70, 75, 91, 116, 201, 202].

One of the main aspects related to pre-clinical trials of new vaccines, adjuvants or any drug delivery systems, concerns the evaluation of immune responses in animal models [91]. Some examples of *in vivo* studies performed with antigens loaded in polymeric micro and nanoparticles and the type of immune responses induced by these systems are listed in Table 1.4.

Table 1.4
Antigen-loaded biodegradable micro and nanoparticles

ANTIGENS	POLYMERS	SPECIES	IMMUNE RESPONSES	IMMUNISATION ROUTES	REFERENCES
DT	PLA PLGA	Guinea pigs	IgG	s.c.	[70]
	PLA+ aluminium hydroxide	Rats	IgG ≅ 2 × TT adsorbed onto aluminium hydroxide	i.m. single dose	[73]
	PLGA	Guinea pigs	Systemic IgG	s.c.	[127]
	PLA	Guinea pigs	Systemic IgG	s.c.	[127]
	Poly(DL-lactic acid)+CS	Mice	Systemic specific IgG	i.n.	[145]
	PCL+ alpha-tocopheryol succinate esterified to polyethylene glycol 1000 (TPGS)	Mice	Increased immune response	i.n.	[203]
	CS	Mice	Local (IgA) and systemic (IgG) Systemic IgG	Oral i.n.	[204]
	PCL PLGA PLGA-PCL	Mice	Systemic IgG_{PCL}>$IgG_{PLGA-PCL}$; IgG_{PLGA} Systemic IgG_{PCL}>$IgG_{PLGA-PCL}$; IgG_{PLGA}	i.n. i.m.	[205]
TT	PLA	Rats	IgG	i.m.	[73]
	PLA	Rats Rabbits Guinea pigs	Local (IgA) and systemic (IgG)	i.n.	[124]
	CS	Mice	IgG IgA	i.n.	[206]

Hepatitis B virus core	PLGA PLGA+MPL	Mice	Th1 immune response higher for PLGA+ MPL	s.c.	[207]
Staphylococcus B enterotoxin	PLGA	Mice	IgG	s.c.	[174]
	Alginate	Mice Rabbits Calves	Systemic IgA Local IgA Local and systemic IgA	Oral s.c.+oral	[208]
OVA	PLGA	Mice	Systemic IgG	s.c.	[209]
	PLGA	Mice	IgG	s.c.	[210]
	PLGA	Calves	Local IgA Systemic IgG	i.n.	[211]
OVA+MDP	PLGA	Mice	IgG	i.d.	[212]
Pig serum albumin	ALG	Rams	Systemic and nasal IgG1 Non significant IgG2a and IgA levels Non significant immune responses	i.n. oral	[213]
Salmonella enteric serovar. Abortusovis extract	PCL	Mice	Protection failure	s.c.	[115]
Brucella ovis Extract	PCL	Rams	Protective IgG levels	s.c.	[214]
Salmonella enterica	Polyacrylamide	Mice	Th1/Th2 Th1; IgA local	i.m. Oral	[215]
Helicobater pylori	CS	Mice	Systemic IgG1 and IgG2a Th2/Th1	oral	[216]
Mycobacterium tuberculosis	CS	Mice	Systemic IgG, IgG1 and IgG2a Th1	s.c.	[217]
Plasmodium falciparum	PLGA	Mice	IgG ≅ positive control	s.c.	[218]
DNA (HIV-2 gp140)	CS	Mice	IgG, IgM and IgA systemic	i.d.	[221]
DNA (HPV-16 E7)	PLGA	Humans	Cellular immune responses; IgA	i.m., s.c.	[219]
DNA (HIV-1 p55 gag)	PLGA+CTAB	Mice	IgG;CTL	i.m.	[220]

DNA (E1E2 proteins of Hepatitis C virus)	PLGA+CTAB Boost E1E2+MF59 PLGA+CTAB	Monkeys Mice	IgG >IgG with E1E2/MF59 Systemic IgG	i.m.	[222]
Group C meningococcal conjugated vaccine	CS+LTK63	Mice	Systemic IgG Nasal IgA	i.n.	[97]
HIV-1 p55 gag protein	PLGA+SDS	Mice	CTL stimulation	i.m.	[126]
rPsaA (*Streptococcus pneumoniae* recombinant adhesin A)	ALG ALG+CTB	Mice Mice	Systemic IgM and IgA, local IgA Systemic IgM and IgA , local IgA > ALG	oral	[223]
Yersinia pestis F1 and V subunits	PLA	Mice	i.m. protection ≅ intratracheal (i.t.) protection	i.m. i.t.	[224]
Monovalent influenza A subunit H3N2	*N*-trimethyl CS	Mice	Total IgG and IgA Total IgG	i.n. i.m.	[225]
Live porcine rotavirus VP6 recombinant protein	ALG	Mice	IgG anti-VP6 Local IgA anti-VP6	i.p. oral	[226]

OUTLINES AND AIMS OF THIS THESIS

Horse raising industry generate a considerable number of full time jobs and is vital for the economy of several countries all over the world, such as Australia, New Zealand, England, USA, Brazil, Scotland, Ireland, Canada, Sweden and Japan. Strangles is however a worldwide endemic disease that often leads to large outbreaks, being considered as one of the most important endemic diseases in horses. Treatment with antibiotics is not consensual among the veterinary community, being researchers rather directed to the development of efficient diagnostic tests and vaccines. As mentioned in the previous section, vaccines available in the market have not been able to make major improvements in the effective control of this contagious disease. Therefore, all over the world there have been major efforts in order to develop a new vaccine that have been certainly improved by the *S. equi* and *S. zooepidemicus* genome-sequencing projects, as they have markedly contributed for the identification of bacteria virulence factors.

Our research group has been previously involved in a project (R&D project, SINDEPEDIP, Consórcio Imunopor, P/P/S5) directed to the development of a vaccine against *S. equi* infection. During those experiments, it was observed that SeM identification, production and purification using biotechnology methods is a complex and highly expensive process. Therefore, cheaper antigens, as *S. equi* whole cells or their lysates were microentrapped in PLGA microspheres, being able to induce protection in laboratory animal model. These studies also indicate that PLGA microspheres are a potential carrier system for the delivery of *S. equi* antigens, making possible a vaccine formulation. However, the use of PLGA in the production of a veterinary vaccine can compromise future commercialisation, as it is a truly expensive excipient. Therefore, the major goal of this thesis was to develop and fully characterise stable alternative polymeric carriers for mucosal delivery of *S. equi* antigens, able to induce humoral, cellular and local immune responses, thus protecting animals against strangles. In addition, it is extremely

important that vaccinated animals with *S. equi* antigen-loaded polymeric particles do not show any signs of the disease and adverse reactions, which are the main disadvantages of those vaccines currently available in the market.

In the other host institution (Centre for Drug Delivery Research, University of London School of Pharmacy, UK), where part of the work included in this joint PhD thesis was developed, PCL, PLA and CS were already being tested giving promising results for the controlled release of several clinically relevant antigens, such as DT, TT, hepatitis B surface antigen, as well as plasmid DNA. PCL is cheaper and more hydrophobic compared to PLGA/PLA polymers and therefore may have an extended release profile. For that reason, PCL offers a cost-effective alternative and was used for the production of plain microspheres by double emulsion (w/o/w) solvent evaporation technique (Chapter 3), whose surface was further modified by association with the polysaccharides CS and ALG, in order to evaluate the influence of particle surface characteristics in the immunoadjuvant properties of these polymeric carriers, as it was previously documented that the particles surface charge influences their uptake by APCs. CS and ALG of different molecular weight and concentrations were used to assess their influence in particle physicochemical characteristics. CS is a polycationic polymer produced by chitin deacetylation, which seems to have the ability to reversibly open tight junctions, and therefore increases paracellular transport across cells. CS and ALG, besides natural, biocompatible, biodegradable and mucoadhesive polymers, are able to elicit higher immune responses and somehow have the potential to protect proteins from degradation, due to less harsh formulation conditions. After full particle characterisation, three polymeric systems were chosen for the following *in vivo* studies. Therefore, the total proteins extracted from *S. equi* cell wall were adsorbed onto those selected PCL microspheres, a single dose was administered by s.c. route and the elicited humoral and cellular immune responses were studied for 300

days. This was the first study focused on *S. equi* antigens adsorption onto polymeric carriers, as a potential vaccine system.

After confirming the potential adjuvanticity of PCL microspheres, a second experiment was performed upon particle size reduction. A comparative study was carried out between systemic and local immune responses induced after i.n. administration of *S. equi* enzymatic extract antigens adsorbed and entrapped PCL nanospheres (Chapter 4). Particle size is important as in general smaller particles are more efficiently taken up by APCs than larger ones. Therefore, PCL nanospheres were produced by modification of the w/o/w emulsification solvent evaporation method used in Chapter 1. Besides the CS attractive properties, its main drawback is the low solubility at physiological pH. Consequently, glycol chitosan (GCS) was used in the inner phase of w/o first emulsion, during particle formulation due to its good solubility in broad range pH aqueous systems. Similarly to PCL microspheres developed in Chapter 3, ALG was also used to modify PCL nanospheres in order to extend particle residence time in the nasal mucosa. Particle association with absorption enhancers (SP and OA) was performed to overcome nasal barriers and improve antigen delivery through the mucosal surface. Particle physicochemical characteristics, as volume mean diameter, morphology, surface charge, loadings and antigens structural integrity and antigenicity after either adsorption or entrapment processes, were extensively evaluated.

In Chapter 5 the cellular, humoral and local immune responses induced by PLA nanospheres formulated with those mucoadhesive polymers and absorption enhancers previously associated to PCL carriers (Chapter 4) were evaluated for 12 weeks in an *in vivo* study performed after mice i.n. immunisation, in order to assess polymer influence in the duration and type of immune response elicited.

Besides being a more expensive antigen product, recombinant *S. equi* SeM proteins were entrapped in PLA nanospheres of similar composition than

those previously mentioned (PVA, GCS, ALG, SP and OA) and animals were vaccinated by i.m. route. The IgG and IgG subtype levels, along with cytokine produced after splenocyte co-stimulation with SeM, were assessed and compared with those *S. equi* enzymatic extract antigen-entrapped PLA particles (Chapter 6).

Overall, the main purpose of these extensive *in vivo* studies was to study the possibility of obtaining a fully characterised polymeric particulate adjuvanted system for effective nasal immunisation against strangles in a mice model.

Most of the results described and discussed in this thesis have been submitted or accepted for publication in international journals.

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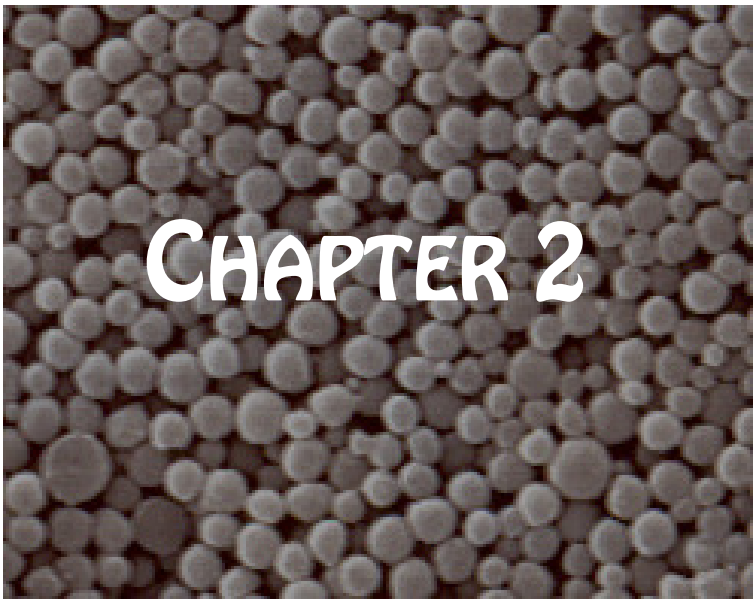

S. equi ANTIGENS ADSORBED ONTO SURFACE MODIFIED POLY-ε-CAPROLACTONE MICROSPHERES INDUCE HUMORAL AND CELLULAR SPECIFIC IMMUNE RESPONSES

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ABSTRACT

Streptococcus equi subsp. *equi* is the causative agent of strangles, which is one of the most costly and widespread infectious diseases, affecting the respiratory tract of *Equidae*. In this work, polyvinyl alcohol, alginate and chitosan were used in formulations of surface modified poly-ε-caprolactone microspheres, which were evaluated after adsorption of *S. equi* enzymatic extract for physicochemical characteristics and *in vivo* immune responses in mice. After subcutaneous immunisation, the formulations induced higher lymphokines levels, in accordance with cellular and humoral immune responses, as compared to the free antigen, successfully activating the paths leading to Th1 and Th2 cells. The obtained results highlight the role of these microspheres as an adjuvant and their use to protect animals against strangles.

Keywords: Poly-ε-caprolactone microspheres, *Streptococcus equi*, vaccine adjuvant.

INTRODUCTION

Streptococcus equi subsp. *equi* (*S. equi*) is a Lancefield group C streptococcus, known as the causative agent of equine strangles, which affects the upper respiratory tract, more specifically, the nasopharynx and draining lymph nodes of horses, donkeys and mules [1, 2]. This disease was first reported by Jordanus Ruffus in 1251 and is characterised by an acute, febrile, suppurative, purulent pharyngitis, lymphadenitis and abscesses in head and neck, which may often burst and exude [2, 3]. *S. equi* is highly virulent and is transmitted through direct contact with the mucopurulent nasal discharges from infected horses or by any equipment and food utensils that are usually near these animals [2, 4]. Animals affected with strangles have a long convalescent period and present a high morbidity level, which can lead to chronic illness or even death [1-5]. Although *S. equi* is sensitive to some antibiotics, most of the treatments are ineffective and prevention is the key [1, 2, 6]. *S. equi* virulence factors are the hyaluronic acid capsule and the antiphagocytic cell wall-associated *S. equi* M-like protein (SeM). SeM is known as the major protective antigen as it stimulates opsonozing antibodies, which are the basis of the so-called bactericidal reaction of immune serum [3, 7, 8]. Indeed, previous infected animals develop a protective immune response against SeM, as they carry immune complexes consisting of IgG and IgA against this protein, both in serum and in nasal secretions. This indicates that to combat and prevent serious complications an efficient vaccine must be achieved [1, 7].

In fact, the strong antibody response has attracted great interest in SeM as a potential vaccine component [5]. However, the efficacy obtained with commercially available and widely used vaccines, that consist of heat-inactivated bacteria or M-protein-rich extracts, is not enough to make major improvements in the control of the disease and thus protection of horses against *S. equi* infection [1-3]. The failure of the current vaccines is presumably due to the vaccines inability to stimulate local nasopharyngeal

antibodies similar to those found in convalescent immune horses, which are considered to be more relevant to protection than serum responses [7].

The therapeutic use of particulate drug carriers for immunisation purposes is currently one of the most promising strategies for fighting infectious diseases and therefore one of the most investigated areas in pharmaceutical technology [9, 10].

Antigens that are responsible for the induction of protective immunity against various pathogens may be immobilised or attached to suitable particulate carriers. The carriers have the advantage to be absorbed with greater efficiency in the mucosal epithelium as compared to soluble molecules, thus providing a long-term depot for the antigen. This is known as the depot theory of adjuvant action, and has been shown to induce effective and long lasting immunity [10-12]. For example, microentrapment of antigens, such as tetanus and diphtheria toxoid, in polymeric and biodegradable microspheres has been extensively studied for its application as antigen carriers in the induction of systemic but also mucosal immune responses regardless of the target site [13-15].

The release properties of microspheres can be tailored for the antigen to produce a sustained immune response [11, 12], providing a release of antigens for weeks to months, a time far exceeding the depot effect of aluminium salts [16] or water/oil emulsions such as Freund's adjuvants [9, 17]. Therefore, these carriers have the potential to reduce the frequency of vaccination required to establish long-term protection [10, 18].

In addition, our preliminary studies showed that the entrapment of whole killed *S. equi* cells or bacterial lysates, respectively in poly(lactide-co-glycolyde) (PLGA) microspheres is a potential carrier system for the delivery of *S. equi* antigens. Furthermore, administration of these microspheres by nasal and intramuscular (i.m.) routes, leads to an enhancement of immune response, causing full protection against the virulent strain upon experimental infection [19]. These studies also indicate that polymeric

microspheres are a potential carrier system for the delivery of *S. equi* antigens, making possible a vaccine formulation.

Researchers have been tempted to expand the use of these carriers, however high loading of large and fragile molecules into microspheres without loss of activity has been difficult to achieve. The adsorption is an important subject in biomedical field and its application as drug delivery systems has been studied for the last decades, as it appears to be an alternative and ideal technique for the loading of proteins onto particles [10, 20, 21].

Poly-ε-caprolactone (PCL) has not been extensively explored for the entrapment of vaccine antigens but its higher hydrophobicity, *in vitro* stability, lack of toxicity and low cost comparatively with PLGA polymeric particles makes it of interest as a matrix for controlled release [14, 22-25]. PCL degradation does not generate an unfavourable low pH micro-environmental for antigens as PLGA, which can be an advantage for the generation of cellular and antibody-mediated responses, crucial for an effective protection against SeM [26, 27]. Some studies have demonstrated the potential of PCL microparticles to be used as a vaccine delivery system [28-30]. For example, Murillo *et al.* 2006 [31] have shown that PCL microparticles entrapping a hot saline antigenic extract of *Brucella ovis* could be used as an anti-*Brucella* vaccine candidate in a mouse model.

In this study, the producing parameters were evaluated for their influence on particles characteristics such as surface morphology, particle size, zeta potential, residual organic solvent concentration, adsorption efficiency and protein integrity. But this paper will mainly focus on the characterisation of humoral and cellular immune responses induced by *S. equi* antigens associated to surface modified PCL microspheres, in order to evaluate the influence of particle surface characteristics on the immunoadjuvant properties of these polymeric carriers.

Materials and Methods

Materials

Polycaprolactone (PCL, average molecular weight (MW) 42.5 kDa), polyvinyl alcohol (PVA, MW 13-23 kDa, 87-89% hydrolysed), polyethyleneimine (PEI), alginate low viscosity (ALG) and sucrose were supplied by Sigma Aldrich Co., UK. Chitosan (CS), low MW (LMW, 150kDa), medium MW (MMW, 400 kDa) and high MW (HMW, 600 kDa) were purchased from Fluka Switzerland. Dichloromethane (DCM) was obtained from BDH Laboratory Supplies, UK. Bicinchoninic acid (BCA) kit for protein determination was provided by Sigma Aldrich Co. U.K, GOSS Scientific Ld. Nuclear Magnetic Resonance (NMR) tubes were obtained from WilMAD/Lab Glass and acetic acid-d4 from Cambridge Isotope Laboratories, Inc., UK.

Streptococcus equi subsp. *equi* (strain LEX) ATCC 53186 was a kind gift from Prof. J.F. Timoney (University of Kentucky, USA).

Other chemicals were used as purchased and reagent grade.

Animals

All procedures carried out in this study were performed in strict accordance with the UK 1986 Animals (Scientific Procedures) Act. Experimentation was carried out using groups of 4 female, 6-8 week old BALB/c mice with food and drink provided *ad libitum*.

Antigen preparation

Antigens were prepared using a modification of a procedure previously developed [32]. Briefly, inactivated *Streptococcus equi* subsp *equi* cells (strain LEX, ATCC 53186) were washed with phosphate buffered saline (PBS, OXOID) 50 mM at pH 6.0 and homogenised by two passages at high pressure (900 PSI, Avestin Inc., Canada). Lysozyme (3 mg/ml), mutanolysin (93.6

Unit/ml) and sucrose (170 mg/ml), all from Sigma Aldrich Co., UK, were added to the homogenised cells (0.4 g /ml; wet weight), and the mixture was incubated at 37°C for 3 h, under constant agitation. Finally, protoplasts were separated by centrifugation at 8,000xg, and supernatants containing the cell wall proteins, including the SeM, were stored at -20ºC for further analysis. Just prior to its utilisation, the enzymatic extract obtained was dialysed at 4ºC, in order to remove sucrose residues. The resultant protein solution was then frozen at -20ºC and freeze-dried (Virtis Advantage freeze dryer, UK) to obtain a dry powder containing ca. 2.7% (w/w) of SeM among many other proteins, including the enzymes used to obtain the extract. The presence of other components of bacterial origin different than proteins may not be discarded.

MICROSPHERE PREPARATION

Polymeric microspheres were aseptically prepared by w/o/w emulsification solvent evaporation method using PVA as a stabiliser (Table 3.1) as described elsewhere [25]. The polymer PCL was completely dissolved in DCM and an internal aqueous phase consisting of 5% (w/v) PVA was emulsified into this organic solution, using an ultra-turrax (T25 Janke & Kunkel, IKA-Labortechnik) at 24000 rpm for 2 min. To prepare negatively charged PCL microspheres, the resulting w/o emulsion was subsequently added dropwise to 2.5 % (w/v) PVA (PCL-PVA microspheres) or into 0.75% (w/v) low viscosity ALG (PCL-ALG microspheres), while positively charged PCL microspheres were prepared using different molecular weights (LMW, MMW or HMW) and concentrations of CS (0.5%, 0.75%, 1% w/v) as the external phase (PCL-CS microspheres). This dispersion was then homogenised (Silverson model L4RT, Chatham Bucks, UK) to create the double w/o/w emulsion. The formulations were magnetically stirred for 4 hours at room temperature to allow organic solvent evaporation and microspheres formation. Sucrose was added to the polymeric particles suspension, which were collected by centrifugation (20,000 rpm, 20 min, 10-15°C ; Beckman J2-21

Hi speed centrifuge), washed with deionised water and freeze-dried. The formulations details are shown in Table 2.1.

Table 2.1
Summary of PCL negatively (-) and positively (+) charged microsphere composition

FORMULATION		ORGANIC PHASE	INTERNAL PHASE	EXTERNAL PHASE	
(-)	PCL-PVA	PCL in DCM	5% (w/v) PVA	2.5% (w/v) PVA	
	PCL-ALG			0.75% (w/v) ALG LV	
(+)	PCL-CS0.5L			0.50% (w/v)	CS LMW
	PCL-CS0.75L			0.75% (w/v)	
	PCL-CS1L			1.0% (w/v)	
	PCL-CS0.5M			0.50% (w/v)	CS MMW
	PCL-CS0.75M			0.75% (w/v)	
	PCL-CS1M			1.0% (w/v)	
	PCL-CS0.5H			0.50% (w/v)	CS HMW
	PCL-CS0.75H			0.75% (w/v)	
	PCL-CS1H			1.0% (w/v)	

PCL-PVA – polycaprolactone – polyvinyl alcohol microspheres; PCL-ALG - polycaprolactone – alginate microspheres; PCL-CS - polycaprolactone – chitosan microspheres; L – low molecular weight; M – medium molecular weight; H – high molecular weight. Chitosan concentrations: 0.5%, 0.75% and 1.0% (w/v).

MICROSPHERES PHYSICOCHEMICAL CHARACTERISATION

PARTICLE SIZE ANALYSIS

The volume mean diameter (VMD) of particles was determined by laser light diffractometry, using a Malvern Mastersizer (Malvern Instruments, UK). The results obtained are represented as VMD (μm).

ZETA POTENTIAL DETERMINATION

The determination of the zeta potential of the particles was performed by anemometry, using the Malvern ZetaSizer (Malvern Instruments, UK) following the dispersion of particles (2-3 mg) in a 10 mM potassium chloride solution to produce a dilute suspension. Zeta potential data (mV) were obtained from the average of three measurements with a standard deviation of ≤ 5%.

SCANNING ELECTRON MICROSCOPY STUDIES

Scanning electron microscopy (SEM, Phillips/FEI XL30 SEM) was used to corroborate the size and to analyse particle surface morphology. Briefly, a thin layer of microspheres was mounted onto aluminium stubs using adhesive carbon pads as a dry powder. The particle surface was then coated with a gold film with a thickness of approximately 20 nm under vacuum in an argon atmosphere using a sputter coater (Emscope SC500) prior to SEM analysis.

ANTIGEN DESORPTION STUDIES

Accurately weighed 10 mg samples of antigen-adsorbed PCL-PVA or PCL-CS0.75L microspheres were placed in LoBind eppendorf tubes (1 tube per time point) and dispersed in 2 ml of a PBS buffer (pH 7.4), containing 5% (w/v) of sodium dodecyl sulphate (SDS, BDH UK) and 0.02% (w/v) of sodium azide, and shaken in a water-bath at 37^{o}C. At predetermined intervals, one eppendorf tube was collected, centrifuged at 7500 rpm for 10 minutes (IEC Micromax eppendorf centrifuge, UK) and the supernatants were analysed by the BCA protein assay (Pierce, USA) and SDS-PAGE.

RESIDUAL SOLVENT QUANTIFICATION

Residual DCM present in the particles (PCL-PVA, PCL-CS0.75L, PCL-ALG) after freeze-drying was quantified using NMR spectroscopy technique. The ^{1}H-NMR spectra were measured using a Bruker spectrometer at a temperature of 300K, using acetic acid-d4 as a solvent and sodium formate (Sigma Aldrich Co., UK) as internal reference.

S. equi ENZYMATIC EXTRACT ADSORPTION

To adsorb the extract, plain PCL-PVA and PCL-0.75CSL microspheres were weighed (10 mg) and dispersed in 900 µl of water. To this suspension, 18.5 mg/ml enzymatic extract solution (100 µl) was added and particles were

incubated at 37°C, for one hour under constant agitation. Particles suspension was then centrifuged (10000 rpm, 5 min; IEC Micromax eppendorf centrifuge, UK), the pellet washed twice and then allowed to dry in a dessiccator, while the supernatant and the washes were kept frozen at -20°C until future analysis.

ANTIGEN LOADING

The amount of protein adsorbed onto the PCL-PVA and surface modified PCL-0.75CSL particles, expressed as total protein (%w/w) per unit weight of microspheres, was determined by a direct and an indirect method. The total amount of protein not adsorbed onto particles was quantified in the supernatants recovered after particle centrifugation, as mentioned above, using the BCA protein assay (Pierce, USA) which can detect 200-1000 μg/ml of protein, by measuring absorbance at 562 nm. To assess the efficiency of protein adsorption onto the particles surface, a known mass of the dried pellet (5 mg) was digested in 2 ml of 5% (w/v) SDS (BDH UK) 0.1 M sodium hydroxide (NaOH, Fisher Co. UK) solution and maintained under magnetic stirring, at 37°C, until a clear solution was obtained. This solution was then neutralised to pH 7.0 with 1N HCl and the total protein amount was determined using the BCA protein assay. Plain microspheres (without antigen) were used as a control. A calibration curve was established using a series of protein standards. Four absorption determinations were made for each standard, blank, control or test sample.

The amount of protein effectively adsorbed onto microparticles surface was then calculated by subtracting the weight of the *S. equi* protein extract recovered from microspheres from the starting amount of antigen initially used during the adsorption process.

STRUCTURAL INTEGRITY OF THE ADSORBED ANTIGEN

Antigen was extracted from freeze-dried microspheres, suspended in PBS (pH 7.4), after incubation at 37ºC. The structural integrity was assessed by SDS-PAGE, compared with native *S. equi* enzymatic extract proteins and a broad range of prestained SDS-PAGE standards (Bio-Rad UK), in order to evaluate the effects of the processing procedures in the structural integrity of the adsorbed proteins. Samples were loaded onto a 10% (w/v) polyacrylamide (Bio-Rad, UK) mini-gel and electrophoresis was performed at a constant voltage of 100 V for 120 minutes using a Bio-Rad 300 power pack (Bio-Rad, Hercules, CA, USA). After migration, the gel was stained with SimplyBlue™ SafeStain solution (Invitrogen, USA), destained, dried and imaged using a UVP gel scanning camera.

IN VITRO CYTOTOXICITY OF PCL MICROSPHERES

Cytotoxicity of PCL microspheres, specifically PCL-PVA, PCL-ALG, PCL-CS0.75L, PCL-CS0.75M and PCL-CS0.75H, to mouse BALB/c monocyte macrophage cells (J774A.1 cells line, American Type Culture Collection; ATCC#TIB-67) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [33], in which the absorbance was measured at 570 nm using a Dynex MRX Microplate Reader (Dynex, UK). All the experiments were carried out in triplicates.

IMMUNISATION STUDIES

Three groups of female BALB/c mice (25g; n=4/group) were immunised subcutaneously on day 1 with a single dose (100 µl) of *S. equi* enzymatic extract equivalent to 10 µg of SeM, either in the free form or adsorbed onto either negatively PCL-PVA or positively PCL-CS0.75L charged microspheres, respectively.

All formulations were prepared freshly and aseptically in a safety cabinet, immediately prior to dosing. For sample preparation, the microspheres were suspended in PBS pH 7.4 and the freeze-dried extract was diluted in water, in order to obtain the desired SeM concentration.

Tail vein blood samples were collected after 7, 45, 90, 180 and 300 days of immunisation. The blood was allowed to clot by overnight incubation at 4^{o}C. The serum was separated by centrifugation at room temperature (15,000 rpm, 10 min) and stored at -20^{o}C until serum humoral immune response (IgG, IgG1 and IgG2a) was assessed by indirect ELISA.

On day 300, mice were ethically sacrificed and their spleens were aseptically removed in order to study the cellular immune response.

Analysis of humoral immune response in serum

The serum was analysed for *S. equi* specific antibodies using a modification of a standard enzyme-linked immunosorbent assay (ELISA) technique [34]. Briefly, 96-well ELISA microtiter plates (Immulon 2, flat bottom plates, Dynatech, UK) were coated overnight at 4^{o}C with 50 µl of 10 µg/ml *S. equi* M proteins in PBS solution (pH 7.4). The plates were washed three times with 0.05% (v/v) solution of Tween 20 (Sigma Aldrich Co., UK) and 1% albumin from bovine serum (BSA, fraction V, Fisher scientific UK) in PBS (PBST) and allowed to dry. Serum samples were added to the wells at a 16-fold dilution in PBS, followed by two-fold serial dilutions and subsequently incubated for 1 hour at 37^{o}C. Plates were washed three times with the PBST solution and antibody binding was detected adding to each well 50 µl of goat anti-mouse antibody-horseradish peroxide conjugate (Sigma, Pool Dorset, UK), diluted in PBS (IgG (Serotec, UK) diluted 1:1000; IgG subclass 1 (IgG1; Serotec, UK) diluted 1:2000; IgG subclass 2a (IgG2a; Serotec, UK) diluted 1:2000). The plates were incubated at 37^{o}C for 1 hour and washed three times with PBST. Fresh substrate solution was prepared by dissolving three tablets of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma Aldrich Co.,

UK) in 50 ml of citrate buffer, plus 5 µl of hydrogen peroxide (H_2O_2; Sigma Aldrich Co., UK), and added to each well. The plates were incubated for 10 min at 37°C for colorimetric reaction development. The citrate buffer was prepared in advance using 44% (m/v) 0.10 M citric acid (Fisher Scientific, UK) and 56% (m/v) 0.2M di-sodium hydrogen orthophosphate (BDH anala VWR international, UK). Absorbance was measured at 405 nm using a Dynex MRX Microplate Reader (Dynex, UK) and compared to sera of naive mice, of the same age housed under the same conditions.

Elicitation of humoral immunity was evaluated by serum *S. equi* proteins-specific IgG, IgG1, IgG2a titres, which were expressed as the average (± S.D.) of the reciprocal of the dilution at which the absorbance 405 nm was 5% higher than the strongest negative control reading, and compared at each time point by analysis of variance (ANOVA) with significance set at $P \leq 0.05$. As an indicator of immune response bias, serum IgG2a/IgG1 ratios were calculated on endpoint titres of group sera obtained at the termination of the experiment.

SPLENOCYTE CULTURE STUDIES

Mice were ethically sacrificed and their spleens were aseptically removed and placed into ice-cold sterile PBS. A crude suspension of spleen cells was prepared in 10 ml of working media RPMI 1640 (Gibco, UK), supplemented to a final concentration of 10 % (v/v) FBS (Gibco, UK), 20mM L-glutamine (Sigma Aldrich Co., UK), 10^5U/l of penicillin and 100 mg/l of streptomycin (Sigma, Poole, Dorset, UK), by gently grinding the spleen on a fine wire screen. The cell suspension was spun at 200 ×g for 10 min, the cell pellet resuspended in 10 ml of fresh working media and the centrifugation procedure was repeated. Following this final centrifugation, the cell pellet was resuspended in 5 ml fresh working media and then diluted out in 15 ml with sterile working media. Using sterile 96-well tissue culture plates (Fisher, UK), 100 µl volumes of cells from individual mice were seeded into 100 µl

volumes of media containing soluble *S. equi* enzymatic extract at the concentration of 2.5 µg/ml and 5.0 µg/ml. Covered plates were incubated at 37°C in a humidified incubator at 5% CO_2 environment. After 48 hours of *in vitro* re-stimulation of splenocytes with soluble antigen, supernatants were obtained and cytokine production was assessed in the culture supernatants using DuoSet® ELISA Development kit (R&D Systems Europe, UK) according to the manufacturer instructions to assay for IL-2, IL-4 and IL-6 and IFN-γ content by sandwich ELISA.

STATISTICAL ANALYSIS

Statistical analysis was preformed using an ANOVA general linear model with SPSS software (Version 13, Microsoft) assuming p-values of $P \leq 0.05$ as significant. In order to specify the difference between the groups, multiple comparisons were also performed following a LSD post hoc test, assuming as well a p-value of ≤ 0.05 as significant.

Results and Discussion

Microsphere characteristics

In the present work, the selection of PCL above other polymers was based especially on its biodegradability, biocompatibility, slow rate of degradation, hydrophobicity, *in vitro* stability, lack of toxicity and low cost [23, 24].

Non-aggregating positively and negatively charged spherical particles were produced by (w/o/w) double emulsion solvent evaporation technique. Particle size ranged from 1.43 to 2.71 μm, depending on the formulation composition, but those made with CS had a narrower size distribution.

Surface modification of PCL microspheres by CS was confirmed by a recorded change in zeta potential. As expected, CS influenced the surface charge of the microspheres resulting in high positive zeta potentials, which increased with raising concentrations and molecular weights (Table 2.2).

Table 2.2
Volume mean diameter (VMD) and zeta potential of PCL polymeric particles, positively (+) and negatively (-) charged (n=3 ± S.D.)

Formulation		VMD (μm)	Zeta potential (mV)
(-)	PCL-PVA	2.71 ± 1.56	-31.2 ± 2.0
	PCL-ALG	2.51 ± 1.50	-37.5 ± 1.5
(+)	PCL-CS0.50L	2.35 ± 0.91	+16.5 ± 1.7
	PCL-CS0.75L	2.02 ± 0.48	+22.4 ± 1.4
	PCL-CS1.0L	1.88 ± 0.98	+28.4 ± 0.8
	PCL-CS0.50M	2.04 ± 0.87	+23.4 ± 1.2
	PCL-CS0.75M	1.75 ± 1.02	+30.2 ± 1.7
	PCL-CS1.0M	1.52 ± 0.88	+34.8 ± 1.7
	PCL-CS0.50H	1.92 ± 0.87	+25.1 ± 1.6
	PCL-CS0.75H	1.65 ± 0.73	+32.2 ± 1.2
	PCL-CS1.0H	1.43 ± 0.89	+39.5 ± 1.3

It is important to mention that the particle size distribution after freeze-drying showed an increase of the number of larger particles when compared with the non freeze-dried particles (results not shown), which may be due to some aggregation, as they were easily dispersible after agitation. Even so, despite its

polymeric composition, all of the prepared PCL particles presented a mean diameter below 5 µm, and consequently should be efficient in targeting antigen presenting cells (APCs), as shown in earlier studies [14, 35, 36].

Scanning electron micrographs show the formation of spherical and reasonably monodisperse microspheres from PCL polymer, all exhibiting a smooth surface morphology (Figure 2.1).

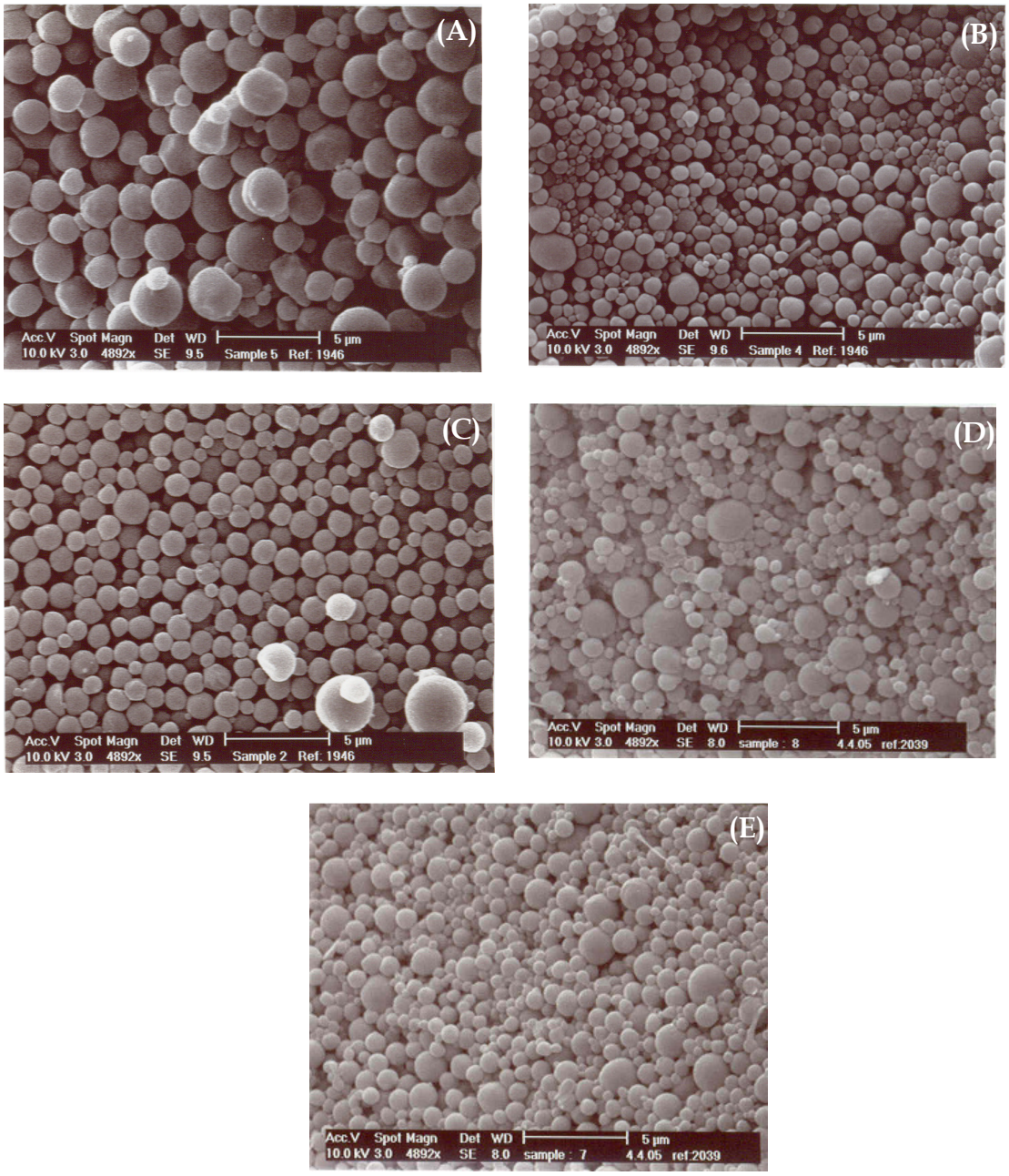


Figure 2.1 - Scanning electron micrographs of the (A) PCL-PVA, (B) PCL-ALG (C) PCL-CS0.75L, (D) PCL-CS0.75M and (E) PCL-CS0.75H microspheres.

Concentration of the internal phase stabilizer (PVA), PCL molecular weight and external phase volume were chosen based on a previous orthogonal design study 3^3 in which the formulations 5% PVA, PCL MMW and 40 ml of external phase seem to result in particles with lower mean diameter but also with minor dispersion of particle sizes (results not shown). The influence of volume ratio dispersion/continuous phase on the microspheres size has been contradictory. Some reports show a mean size reduction with decreasing continuous phase volume [37, 38], probably associated to a decrease in mixing efficiency associated to larger volume, while in other reports no significant difference was obtained [39].

It has been shown that higher surfactant concentration generally increases the stability of the primary emulsion, resulting in a higher loading efficiency. Moreover, addition of a surfactant to the external phase will improve the stability of the second emulsion, as it limits the exchange between both phases. In fact, during solvent evaporation the thin layer of surfactant around the droplets prevents their coalescence. Agglomeration is improved by a gradual decrease of the volume and consequent increase of the viscosity of dispersed phase.

PVA is frequently used as a colloidal polymeric stabiliser by surrounding particles with a hydrophilic polymer layer. Therefore, the increase of PVA concentration has been associated with a decrease in particles size and polydispersity [37, 38]. However, PVA lacking biocompatibility is its disadvantage, since it is not accepted for intravenous administrations (i.v.) and, as a result, is replaced by more biocompatible polymers [40]. For the antigen-adsorbed microspheres prepared with PVA, the PVA concentration did not significantly affect the size and charge of the particles, and therefore for its lacking biocompatibility the smaller concentration tested (5%) was selected.

Adsorption efficiency was almost the same for the positively and negatively charged particles i.e. 53% and 54%, respectively (Table 2.3). Furthermore, the

amount of *S. equi* enzymatic extract adsorbed onto 1 mg of particles was 100 µg for the PCL-PVA microspheres and 98 µg for PCL-CS0.75L, which corresponds to 2.7 and 2.6 µg of SeM, respectively. This supports the theory that the observed differences in size and charge did not to have an influence on the extent of the adsorption of the proteins used in this study [41].

Table 2.3
Total of *S. equi* enzymatic extract protein adsorbed onto PCL microspheres (mean ± S.D.; n=3)

FORMULATION	ADSORPTION EFFICIENCY (%)	*S. equi* PROTEIN (µg) ADSORBED PER mg OF PARTICLES
PCL-PVA	53.0 ± 2.8	98.0 ± 5.18
PCL-CS0.75L	54.0 ± 1.6	100 ± 2.96

In a preliminary adsorption study of PCL microparticles using ovalbumin as a model drug, the adsorption efficiency of ovalbumin was 48% to the negative particles and 100% to the PCL-CS0.75L particles (results not shown). In the case of the *S. equi* enzymatic extract these values were not achieved probably because of the presence of several proteins, especially lysozyme that will certainly compete for adsorption sites. Moreover, protein adsorption is a complex process as several interactions may occur between the proteins and the surfaces. Proteins are macromolecules with inhomogeneous domains, prone to change their conformations after interaction with surfaces. Therefore, the prediction of a protein adsorption from a mixture requires a complete understanding of the competition between charges, depending upon the pH, of electrostatic, van der Waals and steric interactions of the different types of proteins in the mixture and of these proteins with the surface [41].

Figure 2.2 shows the *in vitro* total *S. equi* protein cumulative desorption profiles from PCL-PVA and PCL-CS0.75L microspheres. After a remarkable burst release of 59% for the negatively charged and 48% for the positively charged particles, proteins are slowly desorbed from microspheres throughout time.

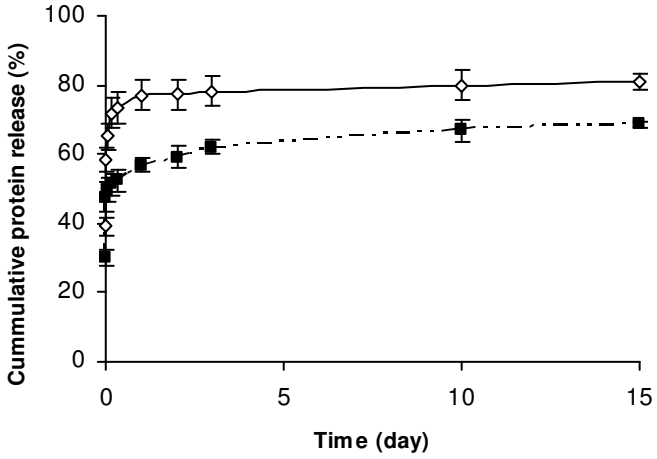


Figure 2.2 - *In vitro* desorption profile of protein antigens from PCL-PVA (◊) and PCL-CS0.5L (■) microspheres (mean ± sd; n = 3).

From Figure 2.3B it can be seen that in both supernatants just the thick lysozyme band and two light bands were seen. In order to confirm the results obtained with the supernatants, particles adsorbed with the *S. equi* extract were also run directly in a SDS-PAGE gel (Figure 2.3A). The difference in concentration between the solutions obtained after extraction of adsorbed proteins and those containing proteins which were not adsorbed to the particles indicates that adsorption really occurred. The native SeM is a 58kDa M-like protein [8, 42], and the corresponding band should appear near that of the 56 kDa molecular weight marker used. However, only 25 μg of SeM are present in 925 μg of *S. equi* extract solution, which can explain the thinner bands seen in lanes 3 and 4, while the extract solution, which was used for adsorption of the particles, presented higher concentration (Figure 2.3A). These results might support the existence of a selective adsorption of some proteins present in the extract solution to the particles. For example, lysozyme is mainly recovered in the supernatant, while a thin band around 56 kDa in both formulation supernatants maybe detected, revealing preferential adsorption. Moreover, no changes in the pattern of protein migration and no additional bands revealing fragmentation or aggregation were detected, suggesting that their integrity was maintained.

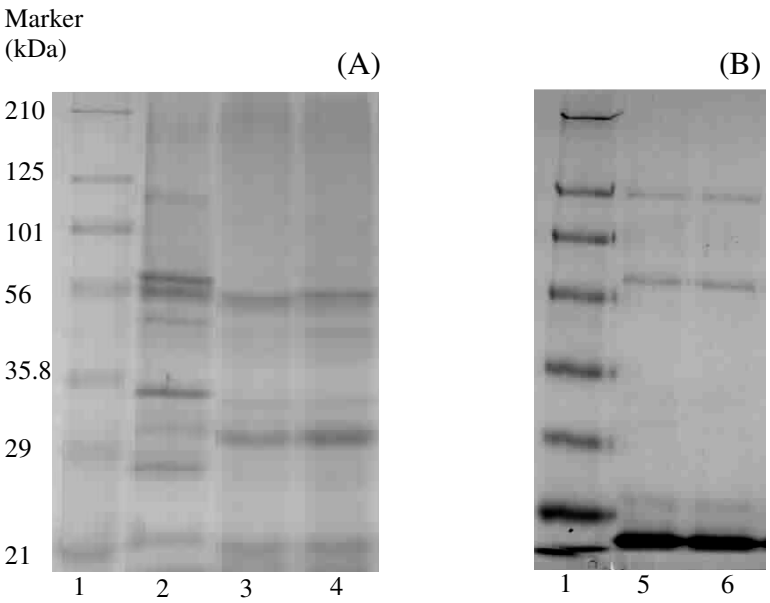


Figure 2.3 - SDS-PAGE (10% gel) of (A) *S. equi* enzymatic extract solutions before and after adsorption onto PCL (MW 40 kDa) microspheres. Lanes: 1) Standard molecular weight markers; 2) *S. equi* enzymatic extract standard solution at 1850 µg/ml; and *S. equi* proteins extracted from adsorbed particles 3) PCL-PVA and 4) PCL-CS0.75L; (B) supernatants obtained after centrifugation of particles 5) PCL-PVA and 6) PCL-CS0.75L.

Based on the results from SDS-PAGE it can be postulated that the formulation by adsorption did not affect the molecular stability and structural integrity of proteins presented in the extract, which is a prerequisite for the antigen to generate a suitable immune response. In fact, using other pharmaceutics formulation methods, processing and storage, proteins could be exposed to conditions that have significant effects on their chemical and physical stability, leading to aggregation and precipitation. For this reason, the adsorption method was proposed in order to avoid protein contact with organic solvents, achieving higher antigen loadings and simultaneously maintaining their structural integrity. However, hydrophobic interactions between proteins and interfaces may lead to structural changes and accelerate aggregation of these macromolecules in solution [21, 43].

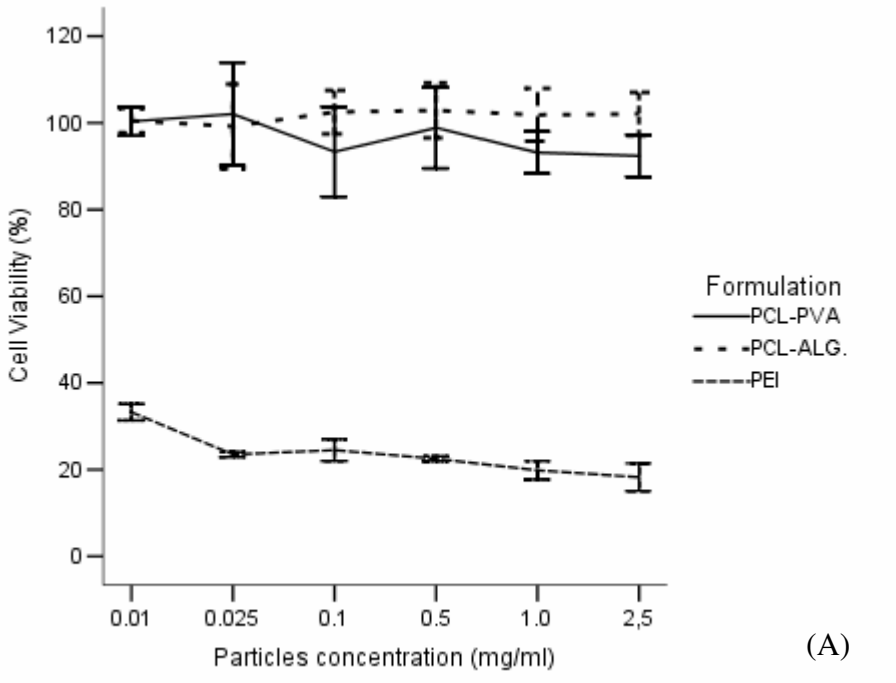
Inclusion of sugars, such as sucrose, while processing the particles is important to maintain the stability and activity of the proteins during storage and desorption from the microspheres surface [44].

RESIDUAL SOLVENT QUANTIFICATION

The use of organic solvents is one of the main disadvantages of using emulsion solvent evaporation methods. Only small amounts of these organics solvents (≤ 600 ppm) are allowed to remain in the particles after the formulation procedure has been ended [45]. In this particular study, DCM was used to dissolve PCL. The DCM residues (% w/w) present in the freeze-dryed microspheres were assessed by NMR and the organic solvent strength showed to be less than 0.04%, thus below the accepted limit.

IN VITRO CYTOTOXICITY OF PCL MICROSPHERES

Cytotoxicity of PCL-PVA, PCL-ALG, PCL-CS0.75L, PCL-CS0.75M and PCL-CS0.75H, to J774A.1 cell line was determined by the MTT assay. For all formulations tested, the MTT test (Figures 2.4A and 2.4B) showed no evidence of toxicity and a significant difference was obtained when the viability was compared with that of the positive control (PEI) (P<0.01 for the CS formulations and P<0.05 for the negatively charged ones).



(A)

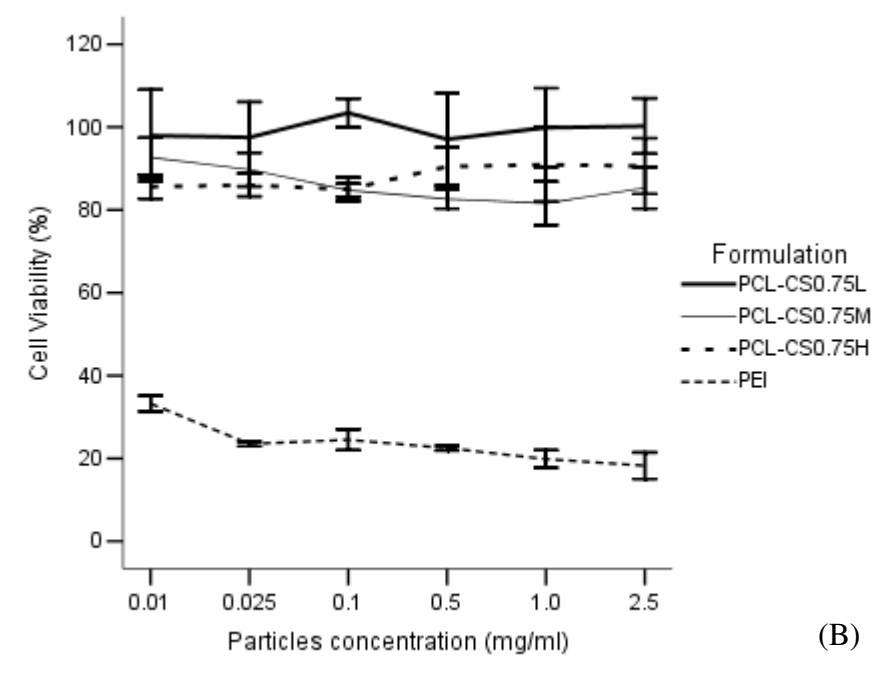


Figure 2.4 - Cytotoxicity of PCL negatively charged (A) and positively charged (B) microspheres to BALB/c mouse monocyte macrophage J774A.1 cell line. PEI-polyethyleneimine; PCL-PVA – polycaprolactone – polyvinyl alcohol microspheres; PCL-ALG - polycaprolactone – alginate microspheres; PCL-CS0.75 - polycaprolactone – chitosan microspheres (0.75%); L – low molecular weight; M – medium molecular weight; H – high molecular weight.

Furthermore, there is a significant difference (P<0.001), between the viability of cells treated with CSLMW and either CSMMW or CSHMW in the particle concentration ranges of 0.1-2.5 mg/ml and 0.01-0.1 mg/ml, respectively (Figure 2.4B). This observation also led to the selection of the PCL-CS0.75L formulation for further studies.

No significant difference was found between the PCL-CS0.75L formulations and both PCL-PVA and PCL-ALG microspheres (P<0.16 and P<0.25) (Figures 2.4A and 2.4B).

SERUM HUMORAL IMMUNE RESPONSE

Biodegradable microspheres have enormous potential as antigen carriers and immunoadjuvants when delivered by parenteral routes, acting as depots for the sustained release of antigens and inducing a significant protection after a single-shot vaccination [37, 46, 47]. Protective immunity following an

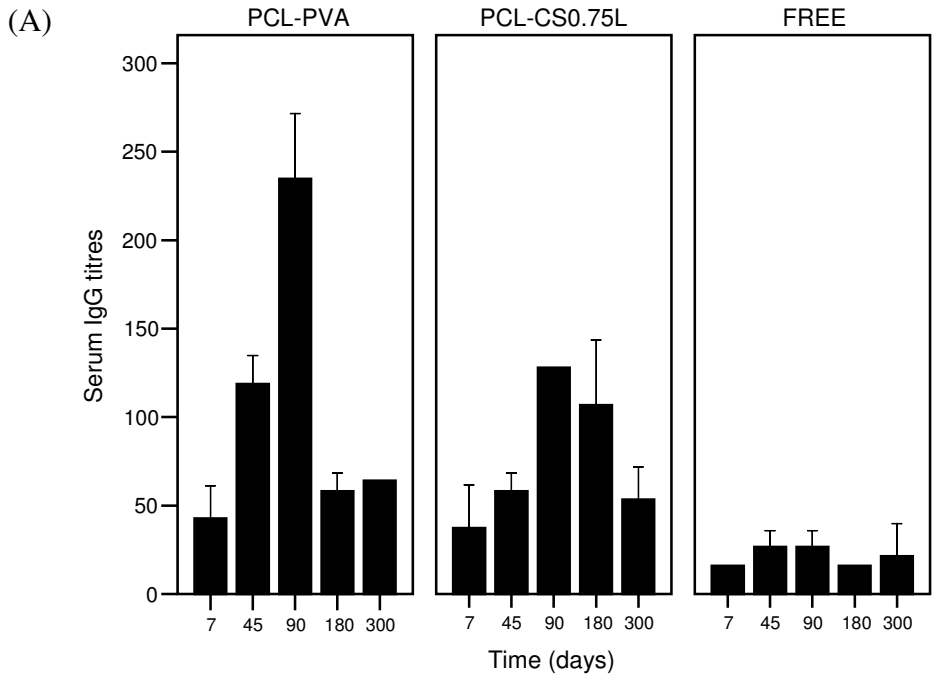
infection with *S. equi* is mediated by serum bactericidal antibody mostly directed against the cell wall SeM [3, 8, 19, 43]. This protein induces a very strong B and T cells response resulting in protective immunity mediated by systemic IgG and secretory IgA [3, 8, 43].

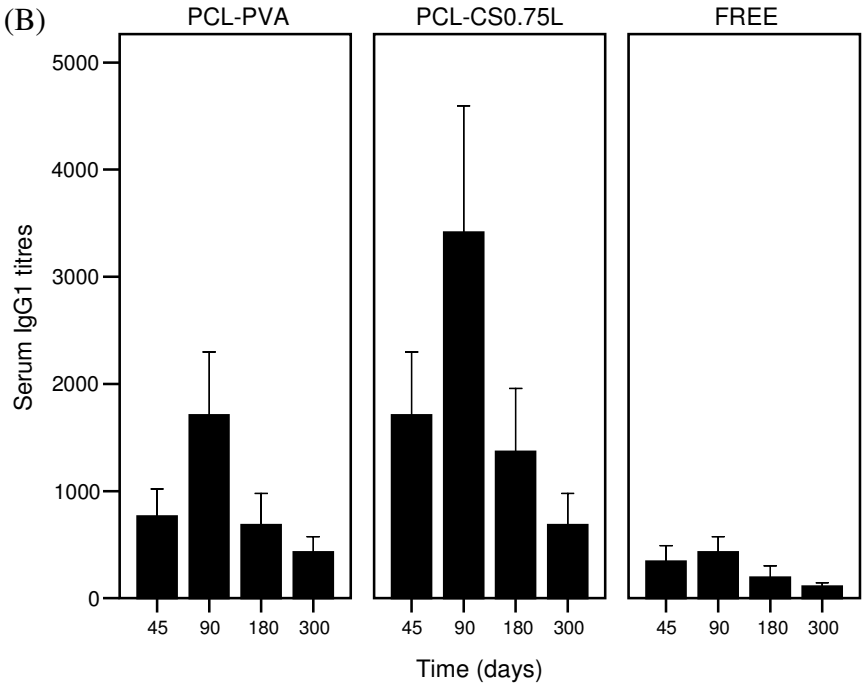
Despite extensive investigation undertaken in this field, the development of an efficient vaccine has still not been achieved. This is due to the fact that not only conventional vaccines, based on live attenuated whole bacteria, but also vaccines that specifically target the SeM have shown little efficacy and have failed to confer protection against *S. equi* in horses [8].

In this study we aimed at developing a single shot vaccine consisting of *S. equi* enzymatic extract antigens adsorbed onto PCL particles. Since no major differences were observed between PCL-PVA and PCL-ALG microspheres, the former was selected as the negatively charged formulation for *in vivo* studies, also because PVA is the most common microsphere stabiliser. Concerning the positively charged formulations, again no major differences were found between formulations. However, the LMW chitosan is the less cytotoxic formulation (section *In vitro cytotoxicity of PCL microspheres*), while give rise to lower viscosity solutions, which are easily handled.

The immune response developed in mice was followed until 300 days after animal vaccination and during the trial period. One week after dosing no significant differences could be observed among the several groups of mice ($P>0.05$) (Figure 2.5A). However, by day 45 all mice had seroconverted and their IgG responses were significantly superior to that of the free antigen immunisation group (Figure 2.5A). At this stage, PCL-PVA microspheres enhanced the serum anti-*S.equi* IgG specific titre three-fold in comparison to free antigen, while the PCL-CS0.75L formulation was only able to double this value. However, the IgG1 responses obtained with CS surface modified PCL microspheres were significantly higher than those observed in the groups immunised with free antigen or with PCL-PVA microspheres ($P<0.005$ and $P<0.03$, respectively) (Figure 2.5B). Moreover, the difference between the

serum IgG2a titres obtained after immunisation with the positively charge particles and the free antigen was statistically significant. But the difference was not as pronounced when compared to the PCL-PVA group (P<0.018 and P<0.17 respectively) (Figure 2.5C).





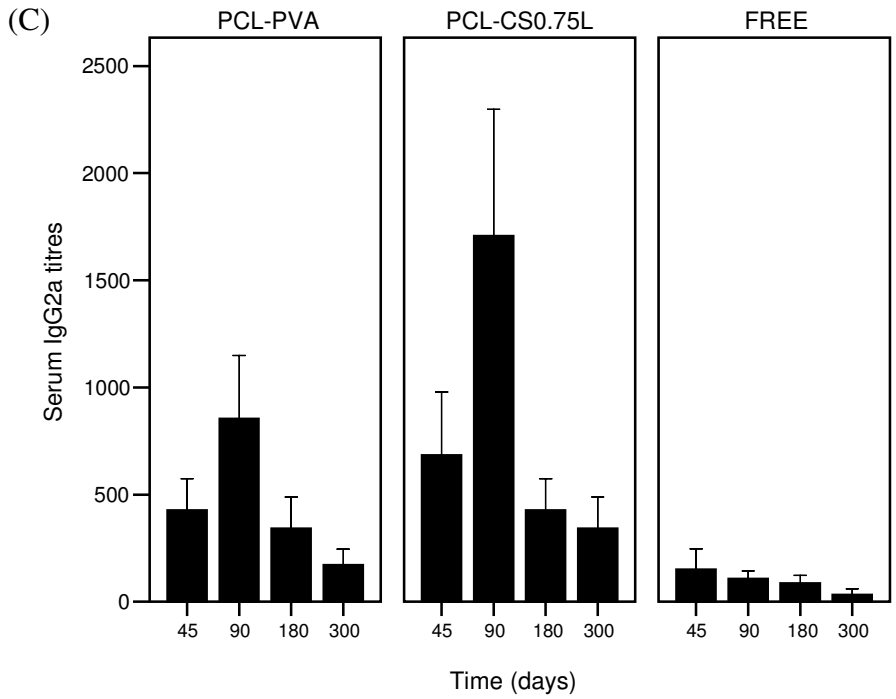


Figure 2.5 - Serum anti-*S. equi* specific IgG (A), IgG1 (B) and IgG2a (C) titres after a single subcutaneous immunisation of female BALB/c mice with PCL-PVA (PVA) or PCL-CS0.75L (CS) microsphere, compared to the free antigen immunisation (FREE).

On day 90 the microsphere-treated groups of mice exhibited higher systemic IgG compared to the free antigen group (P<0.001). Serum IgG titres in the PCL-PVA group were superior to those of the PCL-CS0.75L group (P<0.001). On the other hand, the IgG1 and IgG2a responses of the PCL-CS0.75L group were statistically significant when compared to those obtained with the PCL-PVA (P<0.04) or the free antigen group (P<0.03).

A general decrease in serum anti-*S. equi* IgG and IgG subclass titres was detected after this time-point. However, the decrease in immune response was slower in the mice groups immunised with PCL-CS0.75L as compared to the other groups. The higher level of immune response could be due to impaired desorption from the particle surface, consequence of its positive charge. Therefore, after day 180 the IgG level was still maintained in PCL-PVA group, whereas no significant difference was observed in the free antigen group (P<0.06 and P<0.18, respectively). Furthermore, the PCL-CS0.75L group exhibited the highest IgG antibody titre among all

samples tested (P<0.037) from day 180 and onwards, and a significant difference was only distinguishable when IgG1 and IgG2a titres where compared with the titres induced by the free antigen group (P<0.014).

Even 300 days after a single dose of *S. equi* enzymatic extract adsorbed onto PCL microspheres, both PCL-PVA and PCL-CS0.75L groups showed a serum specific IgG antibody response to *S. equi* proteins slightly higher than that induced by the free antigen.

It is important to mention that from day 180 to 300 the PCL-PVA formulation enhanced the antibody response in terms of peripheral IgG levels, which justifies the considerable difference previously mentioned, opposite to that obtained by day 180. Mice vaccinated with PCL-CS microspheres continued to show the highest IgG1 and IgG2a titres. Nevertheless a significant difference was distinguishable only when compared to the free antigen group (P<0.017 for IgG1 and P<0.08 for IgG2a).

The generation of a dominant Th1 profile as a consequence of an animal vaccination is vital to facilitate the eradication of the microorganism and consequently for a successful prevention against strangles [31, 48]. Upon conclusion of the experiment (day 300) the ratio between IgG titres (IgG2a/IgG1) were suggestive of a more balanced response regarding to the CS surface modified PCL microspheres (IgG2a/IgG1 = 0.464) as opposed to the PCL-PVA microspheres (IgG2a/IgG1 = 0.333), which exhibited a subclass ratio twice as high as that obtained with the free antigen (IgG2a/IgG1 = 0.125). Our results indicate a Th1/Th2 mixed response, after a single dose of *S. equi* enzymatic extract proteins adsorbed onto PCL microspheres, mainly from the positively charged microspheres.

Strangles is a disease that starts at the respiratory tract, therefore local immune response (SIgA) is likely to play an important protective role [19]. For that reason, the evaluation of these mucosal antibodies should help to clarify the differences found between the immune response induced by PCL microspheres. Previous studies have shown that the delivery of antigens

adsorbed onto microparticles up-regulate the gene expression of certain cytokines [49]. In order to further characterise the importance of the vaccine carriers, a study was performed to assess the cytokines produced by splenocytes after their induction by *S. equi* protein antigens.

SPLENOCYTE RESPONSES

Immunisation of animals with PCL microspheres resulted in a general increase of splenocytes responsiveness as observed in terms of lymphokines levels, characterised by high levels of IFN-γ production (Figure 2.6). Differences observed in IL2, IL4, IL6 and IFN-γ induced by microsphere formulations and free antigen were statistically significant (P<0.02).

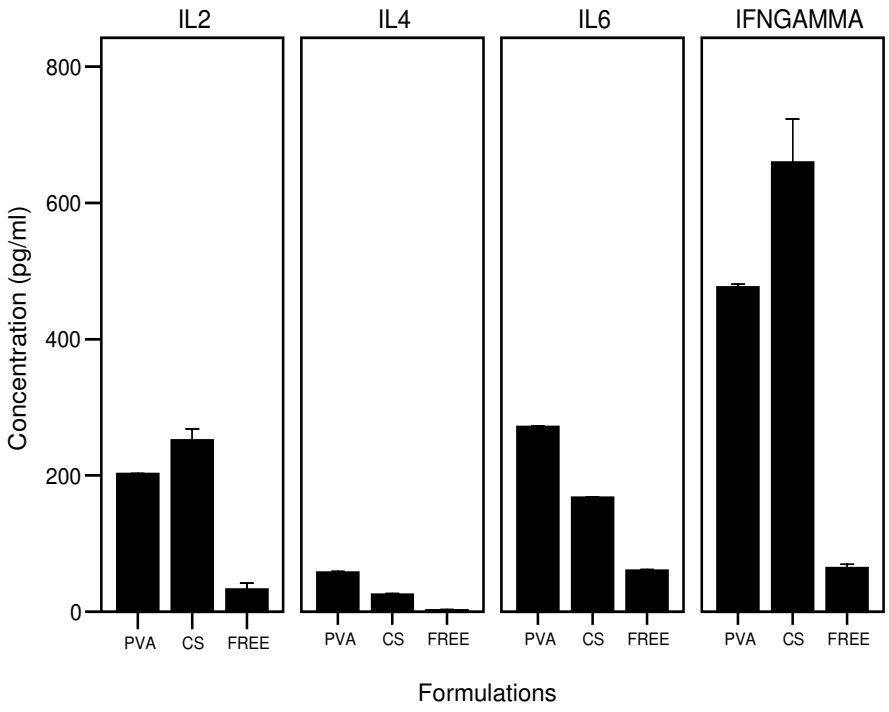


Figure 2.6 - *S. equi*-specific recall responses after stimulation of splenocytes derived from BALB/c mice immunised subcutaneously with *S. equi* enzymatic extract alone (FREE) or adsorbed onto PCL-PVA (PVA) or PCL-CS0.75L (CS) microspheres. Cytokine concentration in culture supernatants was determined in splenocyte cultures stimulated with 5 μg/ml of soluble *S. equi* enzymatic extract.

Interestingly, in contrast to the small differences between the IgG1 and IgG2a antibody titres induced by the particulate formulations of different charge, a significant difference between cytokine titres induced by positively and negatively charged particles was observed: CS surface-modified particles elicited higher IL2 and IFN-γ levels in the vaccinated animals ($P<0.03$), while PCL-PVA microspheres lead to more pronounced amounts of IL4 and IL6 ($P<0.01$). Indeed, PCL-CS0.75L microspheres induced a significantly stronger cellular immune response when compared to the unmodified PCL microspheres ($P<0.03$).

Thus, PCL-CS0.75L microspheres induced a strong cell-mediated immune response according to both IgG2a/IgG1 ratio and interleukin levels, particularly the high levels of Th1-dependent cytokines. The immune response was significantly stronger as compared to the unmodified PCL microspheres ($P<0.03$) and for that reason may be a cost-effective vaccine alternative to the component vaccines as it might facilitate the eradication of *S. equi* infection (Figure 2.6).

CONCLUSIONS

The present study confirmed that adsorption is an alternative method for the production of protein delivery carriers, as it can avoid the possible degradation of proteins caused by organic solvents commonly used in the solvent evaporation technique. At the same time this method was able to achieve higher loadings than those usually reported for the microentrapment process. In addition, non-aggregated PCL microspheres adsorbed with *S. equi* enzymatic extract enhanced serum specific IgG, IgG1 and IgG2a antibody responses, even 300 days after a single dose administration. Both unmodified and CS-surface modified PCL microspheres were capable of eliciting elevated type I immune response cytokines, although it was more pronounced in the latter. In conclusion, PCL-CS0.75L microspheres are a potential vaccine candidate to prevent *S. equi* infection as in this case humoral and cellular responses are needed after host invasion through mucosal infection. Evaluation of mucosal immune response (SIgA) may provide further information regarding the effectiveness of these carriers for the delivery of *S. equi* vaccine.

ACKNOWLEDGMENTS

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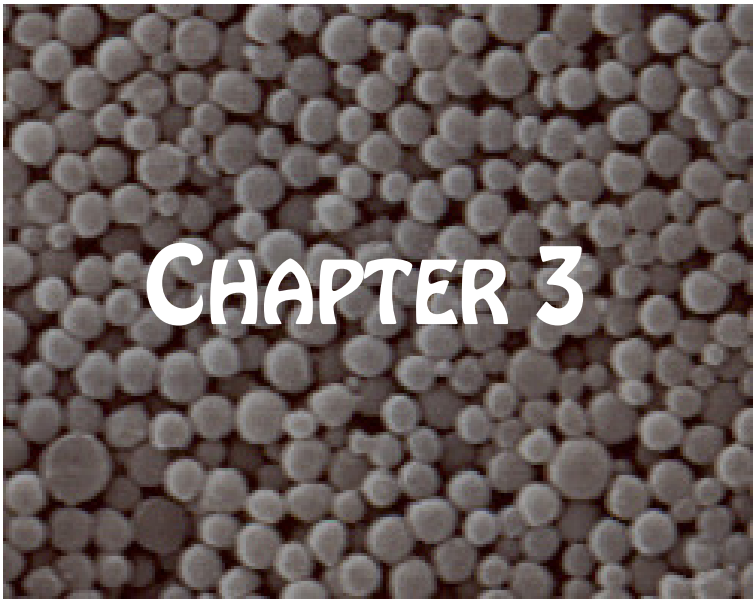

Development of a new mucosal vaccine against equine strangles. Part I: physicochemical and *in vitro* characterisation.

H.F.Florindo, S.Pandit, L. Lacerda, L.M.D. Gonçalves, H.O.Alpar, A.J.Almeida

Adapted from "The enhancement of the immune response against *S. equi* antigens through the intranasal administration of poly-ε-caprolactone-based nanoparticles", paper submitted for publication in Biomaterials.

ABSTRACT

Streptococcus equi subsp. *equi* (*S. equi*) is the causal agent of strangles, a highly contagious disease that affects mainly animals of *Equidae* family. Infected animals present depression, high fever, swallowed lymph nodes and acute pharyngitis and rhinitis. The drainage of exudate occurs after abscess capsule disruption and this mucopurulent liquid contains a high amount of infectious *S. equi*. Therefore, at this phase, there is a high risk of infection of other horses in contact with the infected animal.

Strangles combat seems to be dependent on the development of an effective vaccine, since 75% of animals in the convalescent period have a protective immunity that is maintained for long periods of time, which can go up to 5 years, directed mostly against the protein SeM, although the mechanism of protection is not yet fully known.

The therapeutic use of particulate carriers for the development of an immune response is currently one of the most promising strategies to combat infectious diseases and, hence, one of the areas most investigated in pharmaceutical technology. The goal of the present study was to develop and fully characterise poly-ε-caprolactone (PCL) nanospheres suitable for mucosal immunisation. PCL (42.5kDa) nanospheres were prepared by the double emulsion (w/o/w) solvent evaporation method. Polymer was dissolved in dichloromethane and emulsified by homogenisation, with polyvinyl alcohol (PVA, 13-23kDa), or a glycochitosan (GCS) solution containing the *S. equi* antigens (*S. equi* enzymatic extract). The w/o emulsion was emulsified into a PVA or alginate (ALG) solution. Absorption enhancers, such as spermine (SP) and oleic acid (OA) were also used. Alternatively, *S. equi* antigens were adsorbed onto plain particle surface in order to compare their release profiles with those obtained with the entrapped antigen.

The nanospheres presented a spherical and smooth surface under SEM and particle size ranged from 242.57 nm to 450.2 nm, which means that they may

be suitable for an adjuvant effect as they would be more readily taken up by macrophages and dendritic cells. The entrapment efficiency (E.E.) varied from 49.2% (PCL-SP) to 84.2 % (PCL-GCS), with no compromise of antigen molecular weight by the entrapment process. All PCL nanospheres loaded by adsorption presented higher loading capacities than those resulting from *S. equi* antigen entrapment, although the latter presented better controlled release properties for vaccine delivery. The SDS-PAGE analysis showed no changes in the pattern of protein migration, before and after extraction from the nanospheres, suggesting that their integrity was maintained. MTT studies showed no evidence of toxicity and analysis by confocal microscopy confirmed the uptake of PCL-PVA, PCL-GCS and PCL-SP nanospheres by macrophages, rendering them suitable carriers for mucosal vaccination.

Keywords: Poly-ε-caprolactone, nanospheres, strangles, *Streptococcus equi*, vaccine.

INTRODUCTION

Streptococcus equi subsp. *equi* (*S. equi*) is the causal agent of strangles, a highly contagious disease that affects mainly animals of *Equidae* family [1, 2]. These bacteria cross oral and nasopharynx mucosae, lodge in the lymphatic nodes of pharyngeal and head regions, mainly in retropharyngeal and submandibular lymph nodes, resulting in abscess formation [2-4]. Infected animals present depression, high fever, swollen lymph nodes and acute pharyngitis and rhinitis. The drainage of exudate occurs after abscess capsule disruption and this mucopurulent liquid contains a high amount of infectious *S. equi*. Therefore, at this phase, there is a high risk of infection of other horses in contact with the infected animal [2, 5].

Strangles combat seems to be dependent on the development of an effective vaccine, since 75% of animals in the convalescent period have a protective immunity that is maintained for long periods of time, which can go up to 5 years, directed mostly against the protein SeM, although the mechanism of protection is not yet fully known. As already mentioned, SeM and the capsule hyaluronic acid, are the main factors of virulence of *S. equi*. The IgG and IgA specific anti-SeM present in serum and nasal secretions, will successively recognise, connect to and inactivate the *S. equi* that invade the animal organism. Thus, this protective immune response developed during infection supports the idea that a vaccine will be able to prevent strangles infection [1-4, 6, 7].

The therapeutic use of particulate carriers for the development of an immune response is currently one of the most promising strategies to combat infectious diseases [8, 9]. In fact, these delivery systems can act as a reservoir of the antigen in the APCs, maintaining the presentation of the antigen for prolonged periods of time as shown by Audran *et al.* (2003) [10]. On the other hand, Eyles *et al.* (1998, 1999) demonstrated that antigens entrapped in microspheres have the ability to induce stronger immune response than those elicited by the antigen in the soluble form, which is thought to be due to their

particulate form [11, 12]. As a result, the development of polymeric delivery systems can be used to overcome mucosal barriers and release antigens in the mucosal tissues for prolonged periods of time.

The goal of the present study was to develop and fully characterise poly-ε-caprolactone (PCL) nanospheres suitable for mucosal immunisation, being able to release the antigen into lymphoid tissues. Alginate (ALG) and chitosan (CS) are mucoadhesive polymers and therefore are able to increase particles residence time at the mucosal surface, which can be due to their mucoadhesive hydrophilicity and the electrostatic interactions between the positively charged CS and the negatively charged mucins [13-17]. On the other hand, Schröder and Svenson (1999) have shown that oleic acid (OA) vesicles admixed with diphtheria toxoid were able to induce nasal immune responses in mice as high as those induced by this antigen co-adjuvantised by aluminium [18]. Sugita *et al.* (2007) have demonstrated that spermine (SP) is a polyamine that has the ability to open the epithelium tight junctions and therefore increase the intestinal absorption via the paracellular route [19]. As a consequence, the first objective was to produce PCL nanospheres containing mucoadhesive polymers (glycolchitosan (GCS) and ALG) or absorption enhancers (SP and OA), and associate *S. equi* antigens without compromising their structure integrity and antigenicity.

The adsorbed or entrapped *S. equi* antigens release profiles were assessed to predict the *in vivo* antigen release from these nanospheres. Besides using general agreed biodegradable and biocompatible polymers, cytotoxicity studies in a macrophages cell line (J774A.1) were performed to demonstrate that these delivery systems produced did not present any impurities, resultant either from the production technology or the polymers source. Moreover, the influence of particles composition and surface charge was evaluated in order to assess their potential to be taken up by those macrophages, which is a limiting step for the antigens processing and consequently for the development of an effective immune response.

Materials and Methods

Materials

Polycaprolactone (PCL, average molecular weight (MW) 42.5 kDa), polyvinyl alcohol (PVA, MW 13-23 kDa, 87-89% hydrolyzed), alginate low viscosity (ALG), spermine (SP), oleic acid (OA), glycolchitosan (GCS), sucrose, Bicinchoninic acid (BCA) kit were provided by Sigma Aldrich Co. UK. Micro BCA™ Protein assay kit was supplied by GOSS Scientific Ld and Pierce, UK. Dichloromethane (DCM) was obtained from BDH Laboratory Supplies, UK. *Streptococcus equi* subsp. *equi* (strain LEX) ATCC 53186 were a kind gift from Prof. J.F. Timoney (University of Kentucky, USA).

Preparation of PCL nanospheres

PCL nanospheres were prepared aseptically at room temperature by a modification of a double emulsion (w/o/w) solvent evaporation method previously reported [20]. In brief, polymer was dissolved in 6 ml DCM and emulsified by homogenisation using an Ultra-Turrax T25 (Janke & Kunkel, IKA-Labortechnik) for 3 min at 24,000 rpm, with a 10% (w/v) PVA or a 1% (w/v) GCS solution, which contained *S. equi* antigens (10.0 mg) for the preparation of *S. equi*-entrapped nanospheres. To prepare the *S. equi* antigens, a bacterial culture was harvested and treated with N-acetyl muramidase and lysozyme (Sigma Aldrich Co., UK) to extract the cell wall proteins as previously described [20]. Absorption enhancers (10 mg), such as SP and OA were also used, being the first one dissolved in the PVA solution, while the second was previously molecularly dispersed in 1 ml of ethanol and thus mixed with DCM. The w/o emulsion was then added dropwise into 30 ml of a 1.25% (w/v) PVA or 0.75% (w/v) ALG solution, and homogenised for 7 min at 10,000 rpm using the Silverson homogeniser (Silverson model L4RT, UK). The resultant w/o/w emulsion was magnetically stirred at room temperature for 4 hours to evaporate the organic solvent. The PCL nanospheres were harvested by centrifugation (20,000 rpm, 45 min, 15°C;

Beckman J2-21 High speed centrifuge), washed three times with 0.02% (w/v) sucrose solution and subsequently freeze-dried (Virtis, UK) to obtain a fine, free-flowing dry powder of nanospheres (Table 3.1).

Table 3.1
PCL nanospheres composition

	FORMULATION	ORGANIC PHASE	INTERNAL PHASE (w/v)	EXTERNAL PHASE (w/v)
Ads.	PCL-PVA	PCL (100 mg) in 6 ml DCM	10 % PVA	1.25% PVA
Ads.	PCL-GCS	PCL (100 mg) in 6 ml DCM	1% GCS	1.25% PVA
Ads.	PCL-ALG	PCL (100 mg) in 6 ml DCM	10 % PVA	0.75% ALG
Entrapped	PCL-PVA	PCL (100 mg) in 6 ml DCM	10 % PVA	1.25% PVA
Entrapped	PCL-GCS	PCL (100 mg) in 6 ml DCM	1% GCS	1.25% PVA
Entrapped	PCL-ALG	PCL (100 mg) in 6 ml DCM	10 % PVA	0.75% ALG
Entrapped	PCL-SP	PCL (100 mg) in 6 ml DCM	10 % PVA + 10% SP	1.25% PVA
Entrapped	PCL-OA	PCL (100 mg) 6 ml DCM + 10% OA (1ml EtOH)	10 % PVA	1.25% PVA

To adsorb *S. equi* extract proteins, 20 mg of plain particles (PCL-PVA, PCL-GCS and PCL-ALG) were weighed and dispersed in 2 ml of a 1250 µg/ml protein solution in a LoBind® eppendorf tube (Eppendorf, UK). Particles were then incubated for one hour in a water bath at 37ºC, under agitation (100 rpm). After incubation, particle suspension was centrifuged at 7,500 rpm for 10 minutes (IEC Micromax eppendorf centrifuge, UK), the pellet washed twice and then allowed to dry in a dessiccator, while the supernatant and the washes were kept frozen at -20ºC until future analysis.

PHYSICOCHEMICAL CHARACTERISATION OF PCL NANOSPHERES

The size and surface charge of nanospheres were determined by Malvern ZetaSizer (Malvern Instruments, UK). For zeta potential measurement, a 10 mM potassium chloride solution (Sigma Aldrich Co., UK) solution was used to disperse the particles. Zeta potential data (mV) were obtained from the average of three measurements with a standard deviation ≤5%.

The surface morphology of nanospheres was analysed by scanning electron microscopy (SEM, Phillips/FEI XL30 SEM) as previously described [20].

DETERMINATION OF ANTIGEN LOADING

The total amount (% w/w) of protein entrapped per unit weight of nanospheres (loading capacity, L.C.) was directly measured using the MicroBCA protein assay, after digestion of 10 mg of particles with 2.5 ml of a 5% (w/v) sodium dodecyl sulphate (SDS, BDH UK) in 0.1 M sodium hydroxide (NaOH, Fisher Co. UK) solution, maintained under magnetic stirring at 37°C until a clear solution was obtained. Moreover, the total amount of protein adsorbed onto the surface of particles was determined by an indirect method, assessing the protein concentration in the supernatants using the BCA protein assay. The integrity of protein structure after its adsorption or entrapment was studied by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

IN VITRO RELEASE STUDIES

Antigen-containing nanospheres were accurately weighed (10 mg), placed in three LoBind eppendorf tubes per time point and dispersed in 2 ml of a PBS buffer (pH 7.4), containing 5% (w/v) of sodium dodecyl sulphate (SDS, Sigma Aldrich, Co. UK) and 0.02% (w/v) of sodium azide. Tubes were incubated in a shaking water-bath at 37°C. At predetermined interval, three eppendorf tube were collected, centrifuged at 7,500 rpm for 10 minutes (IEC Micromax eppendorf centrifuge, UK) and the supernatants were analysed for protein content and integrity by BCA, Micro BCA and SDS-PAGE, respectively.

STRUCTURAL INTEGRITY OF *S. equi* ANTIGENS

Dried particles (10 mg) were suspended in 500 µl of a PBS solution, containing 10% (w/v) of SDS, and incubated for 2 hours at 37°C in an orbital shaker. *S. equi* proteins thus extracted from nanospheres, as well as the supernatants

obtained after antigen adsorption were loaded onto 10% (w/v) polyacrylamide (Bio-Rad, UK) mini-gel and run at a constant voltage of 100 V for 120 minutes using a Bio-Rad 300 Power Pack Electrophoresis system (Bio-Rad, Hercules, CA, USA). Proteins bands were observed after staining with SimplyBlue™ SafeStain solution (Invitrogen, USA).

WESTERN BLOTTING

The antigenicity of entrapped and adsorbed *S. equi* antigens was assessed by Western blotting [21]. Samples were transferred from the acrylamide gel onto the PVDF membrane using Bio-Rad mini Trans-Blot Electrophoretic Transfer Cell for 1 hour. The membrane was then washed and blocked by its incubation with 10% (w/v) skim milk powder (Merck KGaA, UK) dissolved in PBS containing 0.02% (v/v) of Tween 20 (PBST; Sigma Aldrich, Co., UK), for one hour under constant agitation in an orbital shaker (100 rpm). The membrane was then incubated at room temperature and for one hour with rabbit monoclonal anti-SeM diluted in the blocking buffer (1:200), under constant agitation. After washing, the blot was incubated with a goat anti-rabbit IgG conjugated to phosphatase alkaline (Sigma Aldrich Co., UK), diluted 1:1000 in blocking buffer for 1 hour at room temperature. The capacity of the anti-serum to recognise *S. equi* antigens was revealed colorimetrically using nitro blue tetrazolinum (NBT) and 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) (Pierce, USA).

IN VITRO CYTOTOXICITY

BALB/c monocyte macrophages (J774A.1 cells line, American Type Culture Collection; ATCC#TIB-67) were used to study the cytotoxicity of PCL nanospheres. Cell viability was evaluated by measuring the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial dehydrogenase of living cells [22]. Cells were treated with samples, in triplicate, at different concentrations and incubated for 4 hours. MTT reagent was added and, after incubation, the complete media was

removed and the absorbance measured at 570 nm at a Dynex MRX Microplate Reader (Dynex, UK) after crystals dissolution with DMSO.

The relative cell viability related to control wells containing non treated cells was determined by the following equation:

$$\%\ Cell\ viability = \frac{A_{Samples}}{A_{Control}} \times 100$$

Where $A_{samples}$ is the absorbance value obtained for cells treated with different particles formulations, while $A_{control}$ was the reading obtained when cells were just incubated with Dulbecco`s modified Eagle's media (DMEM, Sigma Aldrich Co., UK). The % cell viability obtained for treated and untreated cells was compared by one-way ANOVA analysis, followed by LSD post hoc test using SPSS software (Version 13, Microsoft), assuming the differences as significant when $P \leq 0.05$.

CELLULAR UPTAKE STUDIES

Bovine serum albumin fluorescein isothiocyanate (FITC-BSA) was used to assess the binding and uptake of PCL nanospheres by BALB/c monocyte macrophages (J774A.1). To prepare the dye-entrapped nanospheres, FITC-BSA protein was previously dissolved in the internal phase of the first w/o emulsion. The concentration of the FITC-BSA was optimized to 2% (w/w) of polymer weight based on preliminary studies. An efficient loading in PCL nanospheres was achieved with no leaching of protein during incubation in the cell culture medium. ´The cells were previously cultured on a 35 mm glass bottom culture dishes with a cover-slip coated with poly-D-lysine (5×10^4 cell/well; P35GC-0-10C, MatTek® Corporation, USA) in DMEM (Sigma Aldrich Co., UK) supplemented with 4 mM glutamine (Sigma Aldrich Co., UK) plus 10% fetal bovine serum (FBS; Gibco BRL, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, Poole, Dorset, UK). BALB/c

cells were sub-cultured during the experimental period and frequently checked for viability using Typan blue exclusion assay. For qualitative uptake studies, the culture medium was removed and serum free DMEM was added to cells. Freeze-dried *S. equi*-loaded particles (50 µg/ml; 250 µg/ml) suspended in DMEM medium were added to cells and left for 60 minutes at 37°C in 5% CO_2 and 90% relative humidity. The dishes were afterwards observed with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss Microscope Systems, Germany) with Zeiss LSM Image Browser® 4.0 software for the uptake and distribution of particles. Differential interference contrast and fluorescence images were obtained and processed using Adobe Photoshop® software.

STATISTICAL ANALYSIS

Differences of significance between groups of *in vitro* studies were determined by analysis of variance (ANOVA) linear model with SPSS software (Version 13, Microsoft), with significance set at $P \leq 0.05$. This analysis was further complemented by a multicomparison LSD post hoc test in order to state the difference identified between the groups which ANOVA analysis revealed a $P \leq 0.05$.

Results and Discussion

PCL nanospheres physicochemical characteristics

Nanospheres presented a spherical shape, a surface free of any pores or cracks (Figure 3.1) and a narrow particle size distribution particularly PCL-GCS and PCL-OA (Table 3.2).

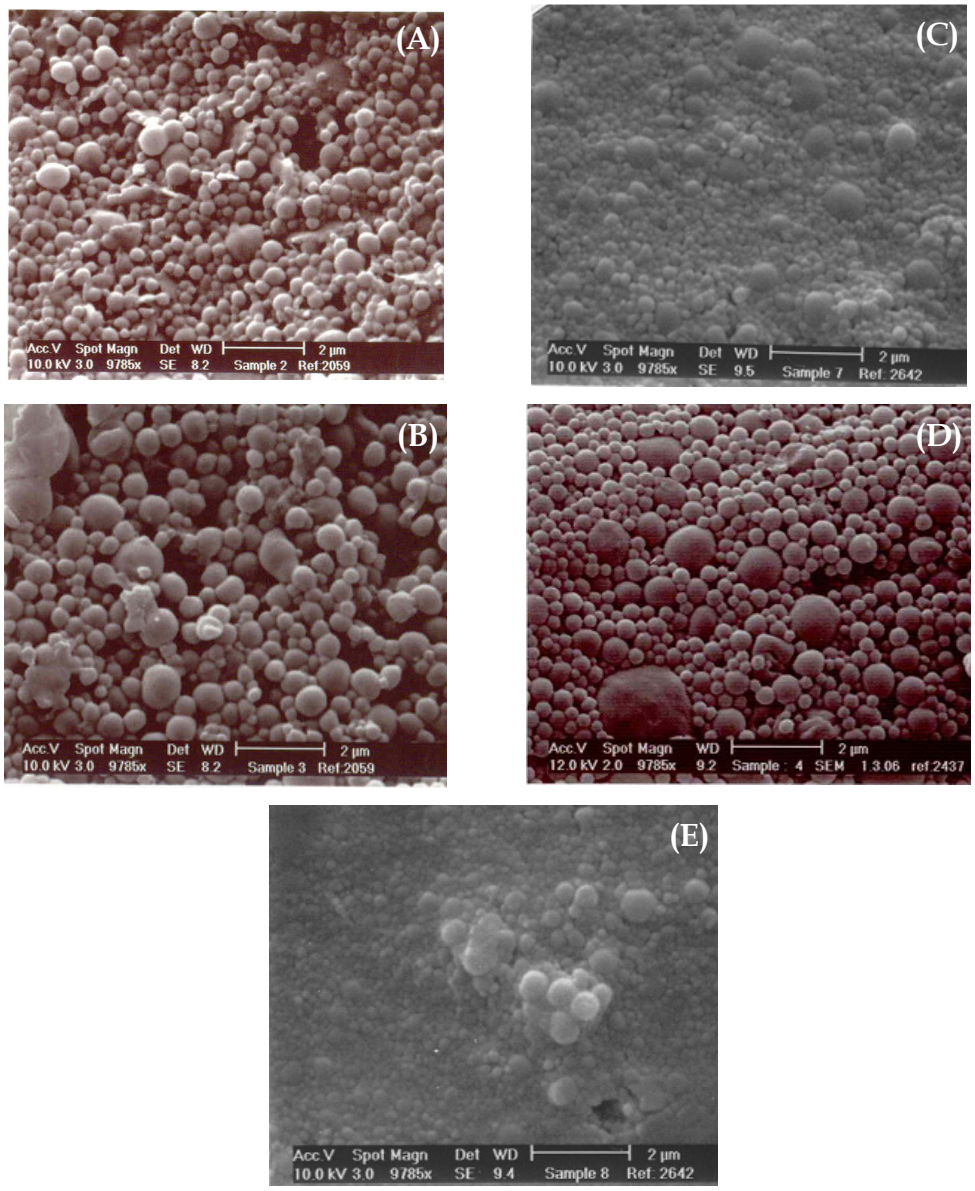

Figure 3.1 – Scanning electron micrographs of the (A) PCL-PVA, (B) PCL-GCS, (C) PCL-ALG, (D) PCL-SP and (E) PCL-OA nanospheres.

PCL particle size ranged from 248.4 to 450.2 nm for antigen-adsorbed particles and 242.57 to 422.83 nm when *S. equi* antigens were entrapped in PCL nanospheres.

Table 3.2
Particles size, zeta potential and loading capacity (L.C.) of PCL nanospheres (mean ± S.D.; n=6)

FORMULATION		VMD[a] (nm)	ZETA POTENTIAL (mV) [c]		L.C. (% w/w)
			BP	AP	
Ads. [b]	PCL-PVA	248.4 ± 55.80	-30.7 ± 6.90	-32.7 ± 7.10	10.75
	PCL-GCS	450.2 ± 32.65	+38.7 ± 7.20	+5.40 ± 7.50	11.38
	PCL-ALG	287.1 ± 21.19	-51.3 ± 6.72	-52.30 ± 7.60	10.13
Entrapped	PCL-PVA	242.57 ± 64.19	-32.8 ± 6.20	-34,30 ± 6.72	5.19
	PCL-GCS	422.83 ± 14.46	+31.7 ± 7.43	+21.10 ± 7.65	8.42
	PCL-ALG	264.67 ± 69.26	-53.1 ± 5. 30	-54.90 ± 5.26	5.04
	PCL-SP	348.37 ± 23.36	-27.8 ± 6.50	-23.10 ± 5.46	4.92
	PCL-OA	243.40 ± 10.60	-31.60 ± 3.70	- 31.73 ± 2.52	6.50

[a]VMD- volume mean diameter;
[b]Ads.-adsorbed;
[c]PCL nanospheres surface charge, before (BP) and after (AP) protein association.

When GCS and SP were added as adjuvants and dissolved in the internal phase of the first (w/o) emulsion, the size of nanospheres increased. The smallest particles were formed when PVA, ALG and OA were used as adjuvants. The 1% (w/v) GCS solution presented a higher viscosity compared to PVA, which can thus explain the increase of nanospheres size. On the other hand, the polyamine SP forms relatively low viscosity solutions after dissolution in the 10% (w/v) PVA solution and therefore an increase of particles size was not predictable. In fact PVA is an excellent colloidal polymeric stabiliser. As it is well accepted, amphiphilies having the property to align at the droplet surface, spontaneously form micelles in an aqueous media, due to intra and/or intermolecular interactions with hydrophobic moieties, so as to promote stability by lowering the free energy at the interface between two phases and resisting coalescence and flocculation of the emulsion droplets, resulting in smaller particle diameters and polydispersity

[23-25]. The resultant carriers are composed by a hydrophobic core that can be used as drugs reservoir. Moreover, the nanospheres surface modification by the use of a hydrophilic polymer, such as alginate, will improve the stability of the second emulsion, as it limits the exchange between the external phase and the organic phase of the first emulsion.

Nanospheres production was previously optimised using OVA as a model drug (results not shown). Therefore, the amounts and concentration of polymers and stabilisers used in the w/o/w double emulsion solvent evaporation technique were chosen in order to produce particles that would fit within the nanometric range, as well as with a low polydispersity index. As a result, despite particles composition, all presented a size lower than 1 μm, which means that they may be suitable for an adjuvant effect as they would be more readily taken up by the macrophages and dendritic cells [26, 27].

For nanospheres formulated with SP and OA, the zeta potential is slightly more negative compared to control, non-modified PCL nanospheres, whereas it became positive when GCS was used due to its cationic polymeric nature, despite its inclusion in the inner phase of the double w/o/w emulsion. PCL-ALG nanospheres presented a high negative charge which was even higher after proteins adsorption (Table 3.2).

CS has been widely used in the pharmaceutical field due to its unique properties as it is a non-toxic, biodegradable and positively charged polysaccharide. However, its low solubility at physiological pH constitutes one of its important limitations. On the other hand, lysozyme is the principal enzyme responsible for CS degradation [28]. This enzyme was present during the extraction of *S. equi* cell wall proteins and remained in the mixture of *S. equi* extract antigens used for PCL particle loading. As a result, a biocompatible CS derivative (GCS) was chosen for particles production, as its good solubility in a broad range pH aqueous systems makes possible its inclusion in the internal phase of w/o first emulsion, being less accessible for lysozyme when the PCL nanospheres are suspended in *S. equi* antigens

solution [29, 30]. Surprisingly, no destabilisation of internal aqueous solution was observed when *S. equi* antigens were dissolved in 1% (w/v) GCS solution, contrary to that observed immediately after dissolution of *S. equi* enzymatic extract in a 0.75% (w/v) CS solution. Actually, as already reported in the literature, GCS has indeed the ability to protect proteins not only during particles formation, but also throughout release in physiological media [29]. The increase in viscosity of the inner phase not only reduces the interaction between the entrapped proteins and organic phase, in the interface, during the homogenisation of the first w/o emulsion, but also reduces leakage from the nanospheres, resulting in a higher L.C. [29]. In fact, the entrapment efficiency (E.E.) varied from 49.2% (PCL-SP) to 84.2 % (PCL-GCS), with no compromise of antigen molecular weight by the formulation process (Figure 3.2). It was observed that GCS substantially increased the L.C. of *S. equi* antigens in the nanospheres (Table 3.2). Besides PCL-SP lower L.C., particles were very easy to disperse in water evidently due to the high hydrophilicity of the molecule. As a result, sample preparation for animal dose will not be compromised by the amount of particles needed to be administered in order to keep the SeM dose.

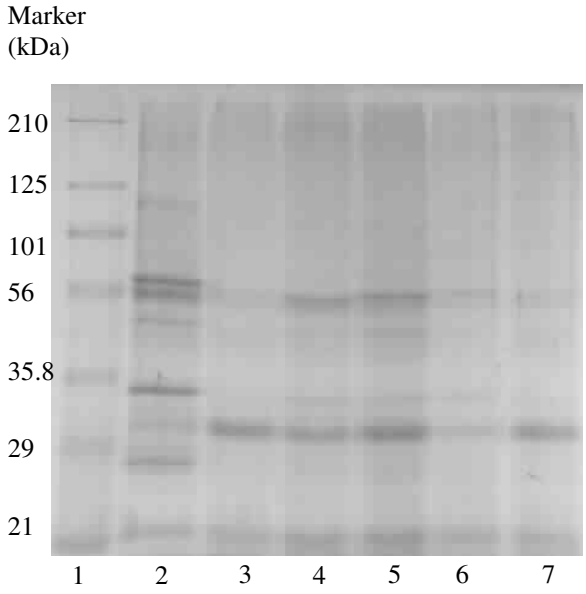

Figure 3.2 – SDS-PAGE (10% gel) analysis of *S. equi* enzymatic extract solution before and after entrapment in PCL (MWT 42.5 kDa) nanospheres. Lanes: 1) Standard molecular weight marker; 2) *S. equi* enzymatic extract standard solution at 1250 µg/ml; and *S. equi* proteins extracted from 3) PCL-PVA, 4) PCL-GCS, 5) PCL-OA, 6) PCL-SP and 7) PCL-ALG.

In the present work, kinetic studies were undertaken in order to evaluate the adsorption capacity of different PCL particles and to estimate the equilibrium

time of the adsorption reaction. Protein adsorption seems to be a time-dependent phenomena, but one hour was enough to reach the maximum amount of protein adsorbed onto the solid particles (results not shown) [3].

All PCL nanospheres loaded by adsorption presented higher L.C. than those resulting from *S. equi* antigen entrapment. The total protein amount adsorbed was 86% (w/w) onto non-modified PCL-PVA nanoparticles, 91% (w/w) onto PCL-GCS nanospheres and 81% (w/w) onto PCL-ALG, which supports the idea that, for the mixture of proteins studied, both the differences on particle size and charge seemed not to have an influence in the adsorption phenomena.

For the antigen-loaded nanospheres to function as controlled release delivery systems for the generation of long lasting immune responses, they should not only contain suitable high levels of antigen, but also maintain its antigenicity [31]. In *S. equi* antigens entrapped in PCL nanospheres, proteins were exposed to potentially harsh conditions, such as shear force, contact with surfactants and organic solvent DCM, which can potentate degradation. Denaturation or inactivation of proteins during processing causes alteration of their native structure, indicated by a shift in band formation higher or lower than the standard. Therefore, western blotting (Figure 3.4) was used to evaluate antigenicity, and polyacrylamide gel electrophoresis (SDS-PAGE) (Figures 3.2 and 3.3) was undertaken in order to assess protein integrity after entrapment and adsorption in PCL nanospheres.

SeM is a 58kDa M-like protein therefore, in Figures 3.2 and 3.3, its band may be seen near the 56kDa marker [32, 33]. As it can be observed, protein bands pattern of migration obtained for native *S. equi* antigens and those extracted from loaded nanospheres are identical, which suggests that protein molecular weights were not affected by the entrapment or adsorption techniques used for protein association to PCL carriers. In addition, *S. equi* epitopes necessary for immune responses were as well preserved as an antiserum raised against

protein SeM still recognise entrapped and adsorbed *S. equi* antigens (Figure 3.4).

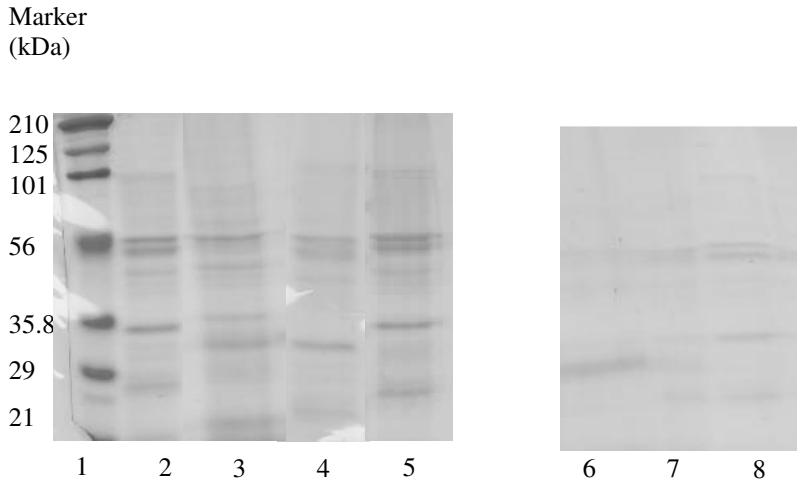

Figure 3.3 - SDS- PAGE (10% gel) of *S. equi* enzymatic extract solutions before and after adsorption onto PCL (MWT 40 kDa) nanospheres. Lanes: 1) Standard molecular weight markers; *S. equi* proteins extracted from adsorbed particles 2) PCL-PVA, 3) PCL-GCS and 4) PCL-ALG; 5) *S. equi* enzymatic extract standard solution at 1250 μg/ml; and supernatants obtained after particles centrifugation 6) PCL-PVA, 7) PCL-GCS and 8) PCL-ALG.

The total amount of protein adsorbed was similar in particles with different composition, size and charge (Table 3.2), but SeM band is stronger in the solution obtained after protein extraction from PCL-GCS nanospheres surface, which is in accordance with loading results (Table 3.2). Even so, it was assessed that 25 μg of SeM are present in 925 μg of *S. equi* extract solution, which can explain the thin band generally obtained. Therefore, adsorption can be successfully used for the development of *S. equi* enzymatic extract vaccine formulations using nanospheres, resulting in higher loadings than those obtained for the antigens entrapment, with no compromise of its molecular structure.

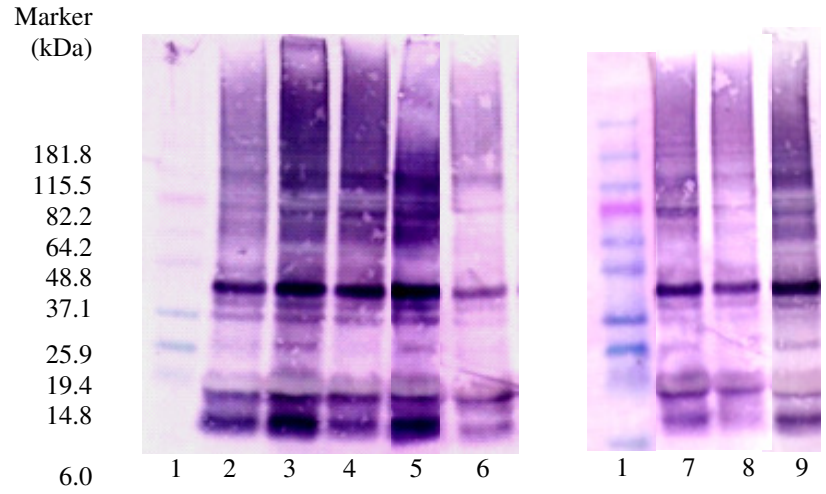

Figure 3.4 – Western blot analysis *S. equi* antigens extracted from PCL (MWT 42.5 kDa) nanospheres blotted against anti-SeM antibody. Lanes: 1) Standard molecular weight marker; *S. equi* proteins extracted from 2) PCL-PVA and 3) PCL-GCS, 4) PCL-OA, 5) PCL-SP and 6) PCL-ALG; *S. equi* proteins removed from the surface of 7) PCL-PVA and 8) PCL-GCS; and 9) *S. equi* enzymatic extract standard solution at 1250 µg/ml;

S. equi antigens remained largely linked to the PCL nanospheres surface for 4 hours, as until this time point 45% (w/w), 55% (w/w) and 42% (w/w) of the adsorbed antigen remained in the surface respectively of PCL-PVA, PCL-GCS and PCL-ALG, under physiological conditions (Figure 3.5). Therefore, this system is suitable for nasal delivery as the antigen associated to particles will not be released to a large extend before antigen-loaded PCL nanospheres are taken up by the nasal epithelium and release the antigen in the nasal cavity. CS and ALG are bioadhesive polymers, therefore PCL-GCS and PCL-ALG nanospheres may resist to the mucociliary clearance in the nose more efficiently than PCL-PVA. Besides the mucoadhesive properties, the positive charge of PCL-GCS nanospheres may also contribute to its prolonged residence time in the nasal mucosa, as it will establish electrostatic interactions with the negatively charged mucus that covers the epithelium. It is important to mention that even 30 days after particle incubation in PBS (pH 7.4) buffer, 22% (w/w) of loaded *S. equi* protein remained adsorbed onto PCL-GCS particles surface, while only 10,7% (w/w) and 12,2% (w/w) were still adsorbed in PCL-ALG and PCL-PVA particles, respectively (Figure 3.5) .

For this reason, in spite of adsorbing a mixture of proteins, it seems that the positive charge presented by PCL-GCS particles contributed for a stronger interaction between *S. equi* proteins and PCL particle surface, as it significantly released a lower amount of adsorbed antigen.

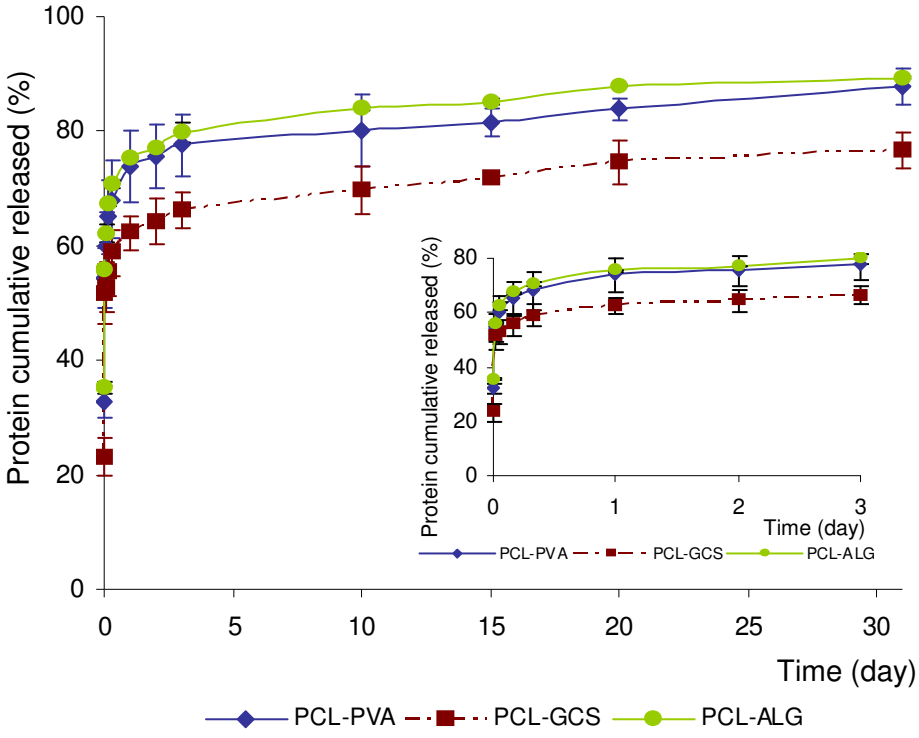

Figure 3.5 – *In vitro* cumulative release of *S. equi* enzymatic extract proteins adsorbed onto the surface of different PCL-based nanospheres, for a period of 30 days (mean ± S.D.; n=3).

All the particulate systems containing *S. equi* antigens presented a burst release that varied from 26% (w/w) for PCL-GCS, to 33% (w/w) for PCL-OA. After this time point, all particles presented a sustained release up to 30 days. At this time, 40% (w/w) for PCL-OA to 55% (w/w) for PCL-GCS remained entrapped, which is in accordance with the degradability of PCL polymer (Figure 3.6). For PCL nanoparticles produced with a 14.8 kDa PCL polymer, it was not observed a significant change of polymer molecular weight even 140 days after particles incubation in PBS at 37ºC. PCL nanospheres degrade slowly in aqueous media due to their hydrophobic and crystalline nature. The cristallinity increases with the decrease of polymer molecular weight [34]. As

a result, it is possible to state that, in the present study the optimisation of the process variables and the degradation characteristics of the polymer chosen resulted in nanospheres with reasonable L.C. which demonstrated to be able to release the entrapped *S. equi* antigens over an extended period of time, especially the PCL-GCS particles.

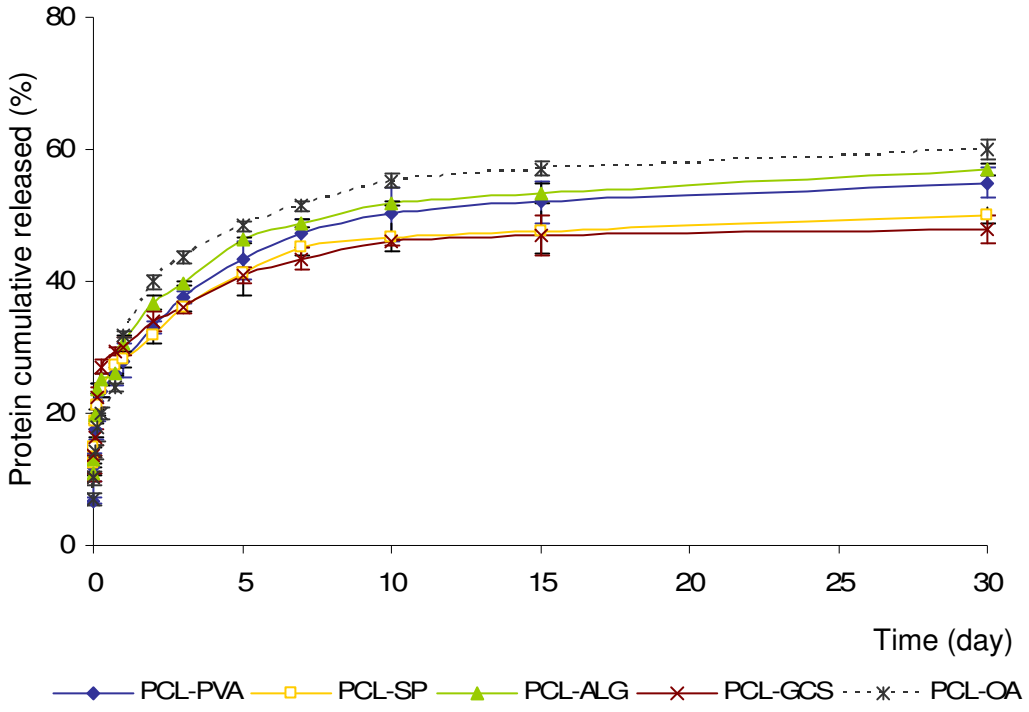

Figure 3.6 – *In vitro* cumulative release of *S. equi* enzymatic extract proteins entrapped in different PCL-based nanospheres, for a period of 30 days (n=3, mean ± S.D.).

PCL NANOSPHERES *IN VITRO* CELL VIABILITY ASSAY

The cytotoxicity of PCL nanospheres was assessed by determining the viability of cells using a MTT test on microtitre plates [22]. PCL nanospheres showed no cytotoxicity effect on BALB/c cells (J774A.1 cell line) in a concentration up to 2.5 mg/ml. Incubation with PCL nanospheres lead to viability as high as 117% compared to control cells, which was significantly higher (P<0.001) than that obtained with the positive control PEI. The viability curve is similar for all PCL nanospheres studied, but PCL-OA led to the

highest reduction in cell viability, being in a range of 93.72% to 99.24% of the control cells (Figure 3.7).

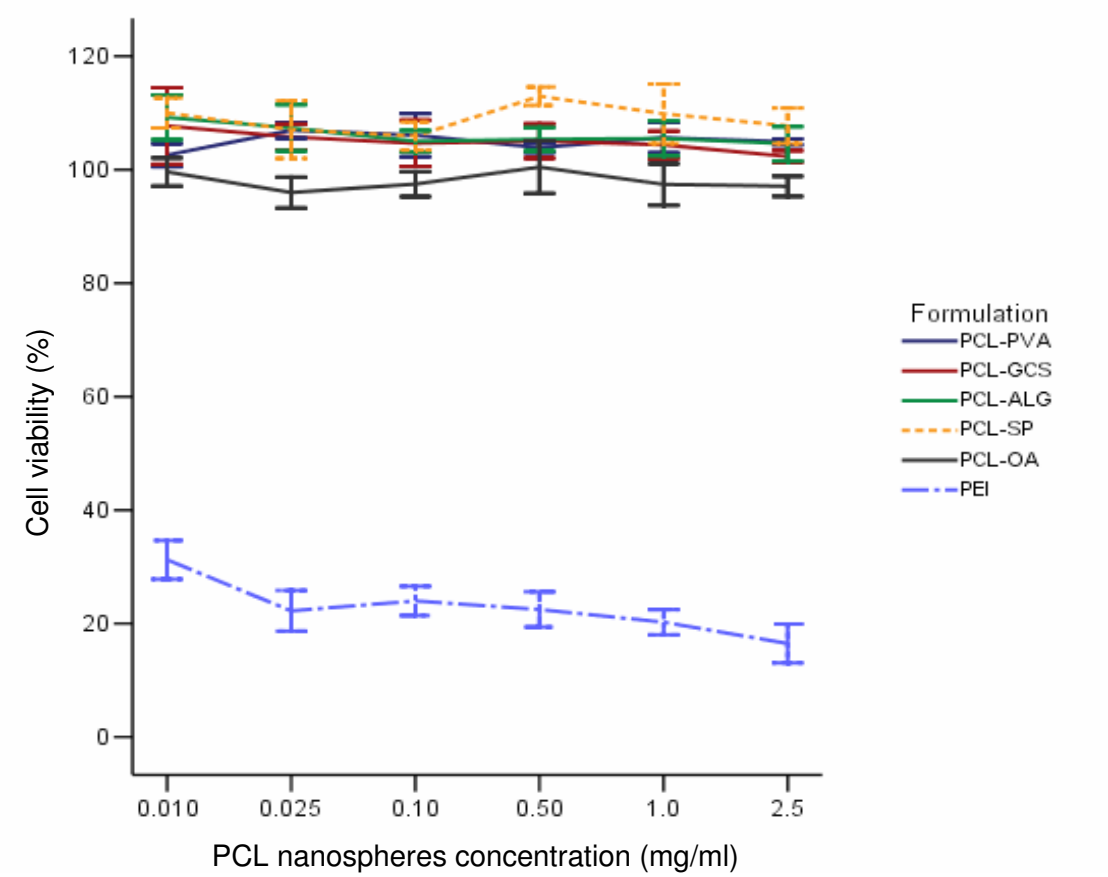

Figure 3.7 – Viability of mouse BALB/c monocyte macrophage cells (J774A.1 cell line) after incubation with increasing concentration of PCL nanospheres (mean ± S.D.; n=3).

IN VITRO CELLULAR UPTAKE STUDIES

Particle uptake is determined by several factors, including particle size, charge, hydrophobicity, but also by adjuvants associated such as GCS, ALG, SP and OA [13, 14, 19, 35]. Particles with hydrophobic surfaces were observed generally to be more readily phagocytosed than those with hydrophilic surfaces [36, 37]. Despite PLA and PLGA being the most frequently used biomaterials for antigen entrapment, PCL is one of the widely used biodegradable polymers due to its biodegradability, biocompatibility, slow rate of degradation, hydrophobicity, good drug permeability, *in vitro* stability,

lack of toxicity and low cost [38-40]. A positive correlation was found for the hydrophobicity and the uptake of particles by cells, and these cells had higher affinity for PCL particles when compared with PLA delivery systems [37, 41].

PCL microparticles have been investigated in the literature as a vaccine delivery system, but PCL nanospheres used in immunisation studies have not been extensively reported, which can be due to the difficulty of producing non-aggregating PCL nanospheres. However, the existing studies have suggested promising results of this polymer as a matrix of a vaccine adjuvant, which is thought to be due to an increase of particles uptake by M cells [37, 40].

The adjuvant effect of PCL and CS microspheres has been studied and their ability to induce an immune response following parenteral and non-parenteral administration has previously been reported [15, 16, 42].

In fact, the cellular uptake of polymeric carriers is a size dependent phenomenon, as particles with a mean diameter less than 10 μm are able to be taken up by M cells in Peyer's patches of mice and pigs as previously shown by several investigators. In addition, particle size may determine the type of immune response induced in animals, as particles with a diameter > 5 μm are taken up by Peyer's patches and remain in their area, and carriers with lower diameters were identified not only in Peyer's patches, but also in mesenteric lymph nodes and spleen, and as a result would preferably induce a systemic immune response, while the first ones will preferentially elicite a mucosal immunity as they remain in IgA inductive environment [43-45].

As it can be seen in Figure 3.8, cells tested do not have background in the fluorescence emission range of FTIC. Presence of green fluorescent nanospheres surrounding the nucleus of several cells could be found in PCL-PVA, PCL-GCS and PCL-SP nanospheres. It is important to reinforce that differential interference contrast (A, B and D) and fluorescence images (C and E) were obtained at Z of 4 μm, in which the nucleus of cells was focused.

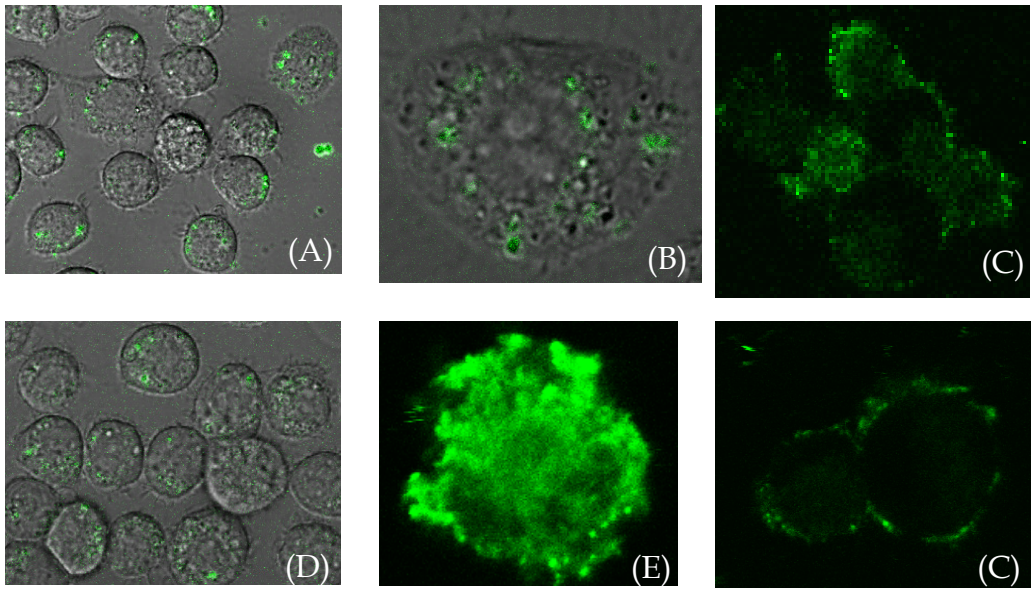

Figure 3.8 – Cellular uptake of PCL nanospheres entrapping BSA-FITC, by mouse BALB/c monocyte macrophage cells (J774A.1) : (A) PCL-PVA; (B) PCL-GCS; (C) PCL-ALG; (D) PCL-SP; (E) PCL-OA (mean ± S.D.; n=3).

These observations suggest that one hour was enough to verify that PCL-GCS nanospheres were successfully taken up by cells and therefore can function as an effective antigen delivery system. In fact, PCL-ALG nanospheres were only able to attach to the membrane of cells during the time of experiment, which can be due to its high negative charge, as its size is as small as those presented by PCL-PVA and PCL-OA. Probably, a prolonged time of observation would have permitted to see the particle inclusion in the cytoplasm, as the cellular uptake efficiency was previously found to be dependent on incubation time [46].

CONCLUSIONS

The purpose of this study was to characterise a delivery system based on PCL nanospheres associated to *S. equi* antigens by adsorption or entrapment method.

Particles fitted the nanosize range and *S. equi* extracted proteins were successfully associated without any damage to their structure, although adsorption, a very mild process, resulted in a higher L.C.. Macrophages (J774A.1 cell line) viability seemed not to be affected by any of the PCL particulate systems prepared, and therefore the preparation method did not introduce any toxic compounds on particles. It was shown that PCL particles modification by the mucoadhesive polymers (ALG and CS) and the absorption enhancers (OA and SP) influenced particles uptake. In fact, one hour after particles incubation with cells, it was possible to see that the positively charged PCL-GCS nanospheres were taken up by macrophages, while the negatively charged PCL-ALG were only able to surround cells surface.

Overall, particles size, L.C., controlled release properties and uptake studies may predict that these systems are promising carriers for mucosal immunisation. As a result, *in vivo* studies including the assessment of systemic, cellular and mucosal immune responses induced in a mouse model will be presented in the second part of this report (Chapter 4) [47].

ACKNOWLEDGMENTS

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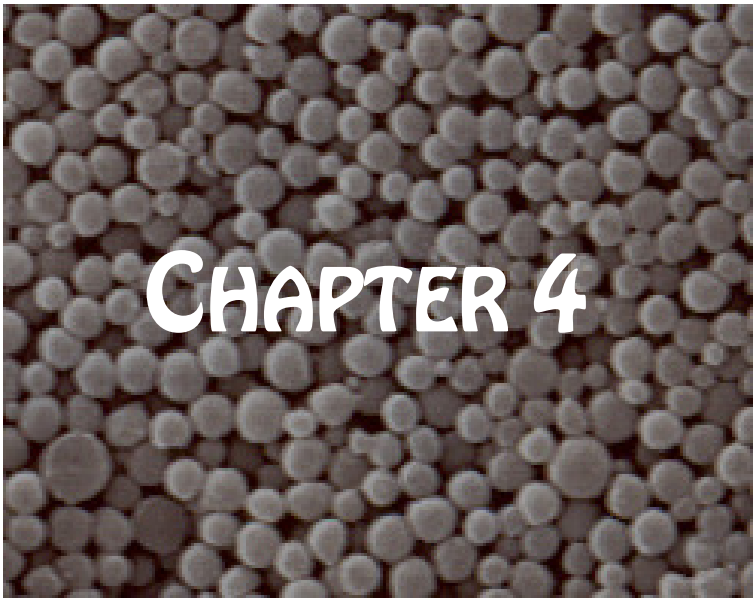

DEVELOPMENT OF A NEW MUCOSAL VACCINE AGAINST EQUINE STRANGLES. PART II: SYSTEMIC, CELLULAR AND MUCOSAL IMMUNE RESPONSES

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Adapted from "The enhancement of the immune response against *S. equi* antigens through the intranasal administration of poly-ε-caprolactone-based nanoparticles", paper submitted for publication in Biomaterials.

ABSTRACT

Strangles is a bacterial infection of *Equidae* family that affects the nasopharynx and draining lymph nodes, caused by *Streptococcus equi* subspecies *equi*. This agent has been reported as responsible for 30% of all worldwide equine infectious and is quite sensitive to penicillin and other antibiotics. However, prevention is still the best option because the current antibiotic therapy and vaccination are often ineffective. As *S. equi* induces a very strong serum and mucosal responses in convalescent horses, an effective and economic strangles vaccine is still a priority. In this study, the humoral, cellular and mucosal immune responses to *S. equi* antigens entrapped or adsorbed onto poly-ε-caprolactone nanospheres were evaluated in mice. Particles were produced by a double (w/o/w) emulsion solvent evaporation technique and contained mucoadhesive polymers (alginate or chitosan) and absorption enhancers (spermine, oleic acid). The intranasal administration of *S. equi* antigen-loaded nanospheres, particularly those constituted by the mucoadhesive polymers, increased the immunogenicity and mucosal immune responses (SIgA) to the antigen. The inclusion of cholera toxin B subunit in formulations successfully further activated the paths leading to Th1 and Th2 cells. Therefore, these PCL nanospheres are potential carriers for the delivery of *S. equi* antigens to protect animals against strangles.

Keywords: Poly-ε-caprolactone, nanospheres, adjuvants, *Streptococcus equi*, mucosal immunisation.

INTRODUCTION

Streptococcus equi subsp. *equi* (*S. equi*) is the etiological agent of strangles, one of the most costly, commonly diagnosed and widespread infectious disease of *Equidae* worldwide, that has lead to devastating epidemics in stables where horses are housed [1, 2]. This is an acute, contagious and deadly respiratory tract disease, which typical signs of infection include pyrexia, suppurative, mucopurulent nasal discharge, lymphadenitis and abscessation, often in the lymph nodes of the head and neck [1-3]. It occasionally affects other lymph nodes and organs, resulting in a severe stage of the disease called bastard strangles. Because *S. equi* persists in the environment for only a few weeks, the most important factor for the maintenance of infection is the infected horse [3, 4]. Even if generally after 4-6 weeks the infected animals recover from disease eliminating *S. equi* from their organism, 10% will constitute long-term *S. equi* carriers, harbouring the microorganism for months. The presence of the pathogen is not detectable in *S. equi* long-term carriers and the animals do not show any clinical signs of disease [1-6]. On the other hand, although *S. equi* is sensitive *in vitro* to some antibiotics, its use is not consensual as most of the treatments are ineffective when external signs of disease are already detectable [3, 7].

Animals that recover from a *S. equi* natural infection show a protective immunity, mostly against one of the most virulence factor of these bacteria, the antiphagocytic cell wall-associated *S. equi* M-like protein (SeM), that seems to remain in 75% of the animals for five years, although the protection mechanism is not yet fully understood [3, 8]. In fact, convalescent horses have antibodies IgG and IgA against SeM, both in serum and nasal secretions, which encourages the development of efficient vaccines. As a result, the inclusion of animals in regular vaccination programmes simultaneously with an effective diagnostic test constitutes the key for the combat of large strangles outbreaks [1, 2]. Bacterins and adjuvant extracts have been widely used in the field since the 1980s, although the efficacy of most available

vaccines is not sufficient to confer protection against *S. equi* infection in horses [1, 3, 9, 10]. Secretory IgA (SIgA) antibodies locally produced in the respiratory tract are extremely important for the prevention and control of infection at mucosal sites [11]. However, those vaccines, which specifically target the SeM protein, did not show useful protection in horses. Therefore, it appears that an effective strangles vaccine must be able to stimulate a nasopharyngeal immune response [12, 13].

Biodegradable particles are of particular interest as technology platforms, for the generation of both protective local and systemic immunities. These carriers increase the level concentration of antigen over an extended time and its uptake by lymph nodes, promoting the interaction between the antigen and specific antigen presenting cells (APCs), protect the antigen from proteolytic enzymes and have a great ability to gain access to the mucosal-associated lymphoid tissue (MALT), when compared to free antigens, thus providing a means of target vaccine delivery [14-16]. These delivery systems have the capacity to be modified in order to achieve a prolonged and pulsatile release of the entrapped antigen, mimicking the primary dose and boosters commonly used in the conventional vaccination programs, reducing therefore the number of doses needed to be administered in order to maintain an eventual immune response for a long period [17-20, 21, 22]. The efficacy of polymeric carriers in inducing effective immune response was reviewed by Lemoine *et al.* (1999) [23].

Polymeric particles induce a mucosal response, after intranasal (i.n.) administration, in lymphoid tissues regardless of target site and the response is paralleled by the appearance of antibodies in secretions of glands distant from the site of immunisation, due to the role of the common mucosal immune system (CMIS), which highlights the immunoadjuvant properties of these carriers [24]. The modification of polymeric and biodegradable particles with mucoadhesive compounds as chitosan (CS) [20, 25-28] and alginate (ALG) [29] to extend residence time, and absorption enhancers as spermine

(SP) [30, 31] and oleic acid (OA) [25, 32], constitute an interesting approach in order to overcome the nasal barriers and improve the delivery of antigens administered by mucosal routes [32]. Accordingly, in the present study SP was used as a potential adjuvant to improve *S. equi* absorption through the nasal epithelium. In order to overcome CS poor solubility in physiological pH, a glycolchitosan (GCS) was used in this work as it is soluble and active as an absorption enhancer at pH 7.4 [33].

Antigens adsorbed and entrapped in particulate systems have been able to induce strong immune responses.

In a previous work, our group have showed that *S. equi* enzymatic extract proteins adsorbed onto non-aggregated PCL microspheres enhanced serum specific IgG, IgG1 and IgG2a antibody responses and elicited an elevated type I immune response (Th1) cytokines, even 300 days after a single-dose subcutaneous administration [34].

The purpose of this study is to use PCL nanospheres, modified by different adjuvants (GCS, ALG, SP and OA), as potential carriers for *S. equi* surface proteins, and compare their ability to induce both systemic and local protective immunities after mucosal administration in a mouse model. As cholera toxin B subunit (CTB) is one of the most potent mucosal adjuvant, both *S. equi*-loaded PCL nanospheres and free *S. equi* antigens were admixed with CTB and the resultant immunogenicity was evaluated and compared with the aforementioned formulations [14, 17, 35].

Materials and Methods

Materials

Polycaprolactone (PCL, average molecular weight (MW) 42.5 kDa), polyvinyl alcohol (PVA, MW 13-23 kDa, 87-89% hydrolysed), alginate low viscosity (ALG), spermine (SP), oleic acid (OA), glycolchitosan (GCS), sucrose, cholera toxin B subunit (CTB), trypsin-chymotrypsin inhibitor, EDTA sodium salt, iodoacetic acid and phenylmethanosulfonyl fluoride solution (PMSF) were supplied by Sigma Aldrich Co., UK. Dichloromethane (DCM) was obtained from BDH Laboratory Supplies, UK. *Streptococcus equi* subsp. *equi* (strain LEX) ATCC 53186 were a kind gift from Prof. J.F. Timoney (University of Kentucky, USA).

Animals

Each group of experimentation used in the *in vivo* studies was composed by 4 female, 6-8 weeks old BALB/c mice with food and drink provided *ad libitum*. Experiments were performed in strict accordance with the UK 1986 Animals (Scientific Procedures) Act.

Preparation of PCL nanospheres

PCL nanospheres were prepared by double emulsion (w/o/w) solvent evaporation technique, as described previously [34]. Briefly, polymer was dissolved in DCM and emulsified with a 10% (w/v) PVA or a 1% (w/v) GCS solution, which contained the *S. equi* antigens (10.0 mg) for the entrapment of these antigens in nanospheres. Particles containing absorption enhancers (10.0 mg), such as SP and OA were also prepared. The w/o emulsion was then homogenised with a 1.25% (w/v) PVA or 0.75% (w/v) ALG solution and the resultant w/o/w emulsion was magnetically stirred for 4 hours to evaporate the organic solvent. The PCL nanospheres were harvested by centrifugation

(20,000 rpm, 45 min, 15°C; Beckman J2-21 High speed centrifuge), washed and freeze-dried (Virtis, UK).

Alternatively, *S. equi* extract proteins were adsorbed onto plain particles surface (PCL-PVA, PCL-GCS and PCL-ALG) following the protocol described in a previous study [34].

For i.n. immunisations, 50 µl of a particles suspension in PBS (pH 7.4) containing adsorbed or entrapped *S. equi* antigens equivalent to 10 µg of SeM were administered per mice. Plain particles and free antigen were used as controls (Table 4.1).

Table 4.1
Design of intranasal immunisation study

GROUPS		FORMULATION
1	Adsorbed	PCL-PVAads.
2		PCL-GCSads.
3		PCL-ALGads.
4		PCL-PVAads.+CTB
5	Entrapped	PCL-PVA
6		PCL-GCS
7		PCL-ALG
8		PCL-SP
9		PCL-OA
10		PCL-PVA+CTB
11	FREE antigens	
12	FREE antigens+ CTB	

IMMUNISATION SCHEDULE

Twelve groups of female BALB/c mice (25g; n=4/group) were immunised by i.n. route on day 1 and boosted on day 21, by instillation using a micropipette tip to administer 50 µl of sample (25 µl in each nostril) containing *S. equi* antigen equivalent to 10µg of SeM, either free, entrapped or adsorbed in nanospheres (Table 4.1). Before each administration, mice were lightly anesthetized with 3% of isoflorane in oxygen (300 cm^3 min^{-1}). The

formulations were delivered slowly onto the nares, so that the mice could inhale it in. Controls consisted of plain particles and 10 μg of CTB (Sigma Aldrich co., UK) associated to the free antigen or *S. equi*-loaded nanospheres. All the formulations were freshly and aseptically prepared by particles dispersion in PBS pH 7.4 in a biological safety cabinet, immediately prior to dosing.

COLLECTION OF SAMPLES

Blood samples were collected in heparinised capillary tubes (Vitrex, Denmark) by tail vein bleeding. The blood was incubated overnight at 4°C and the serum was separated by centrifugation at 15,000 rpm for 20 min at 4°C, and stored at -20°C until further analysis.

In order to assess the mucosal immune response, lung and gut secretions were collected from the ethically sacrificed animal following a method previously reported [36]. Following the culling of animals, the small intestines were aseptically isolated, longitudinally sectioned and transferred into a Petri-dish which contained 4 ml of ice-cold enzyme inhibitor mix (1mM iodoacetic acid, 0.1% (v/v) trypsin inhibitor soybean type 1 and 10 mM EDTA sodium salt). These were scraped to expose the mucus and the liquid was transferred into a centrifuge tube and stored in acid-bath. Next, the tube was sonicated for 30 seconds to release protein from mucin, and centrifuged at 20,000×g for 30 min at 4°C. The supernatants were removed and centrifugation was repeated in order to obtain a clear sample, which were then stored at -70°C overnight and lyophilised (Virtis Advantage freeze dryer, UK) to obtain a dry powder. These samples were then reconstituted with 500 μl of PBS immediately before the SIgA quantification by ELISA. To collect the lung secretions, the trachea and lungs of the sacrificed animal were exposed and 5 ml of lavages solution (1mM PMSF, 0.9% (w/v) sodium chloride, 0.5% (v/v) tween 20 and 0.1% (w/v) sodium azide) were added into the trachea to

inflate the lungs. The liquid was immediately removed and treated as aforementioned for the gut washes.

QUANTIFICATION OF SYSTEMIC (SERUM) AND MUCOSAL ANTIBODY RESPONSES

The level of anti- *S. equi* specific IgG, IgG1, IgG2a antibodies in serum and IgA *S. equi* specific antibodies in lung and gut washes were assessed by indirect ELISA using Immulon 2, flat bottom plates (Dynatech, UK) [37]. Each well was coated overnight at 4^{o}C with 1.5 µg/ml *S. equi* M proteins in PBS solution (pH 7.4). After washing, non-specific protein-binding sites were blocked with 2% (w/v) bovine serum albumin solution (BSA, Sigma Aldrich Co., UK) in PBS and, after 1h incubation at 37^{o}C, the plates were washed with PBS containing 0.05% of tween 20 and 0.1% of BSA, fraction V (washing buffer). Serum samples, controls, lung and gut washes were added to the ELISA plates at a 32-fold dilution in PBS (100 µl), and serially diluted two-fold with the same buffer. After incubating the plates at 37^{o}C for 2h, these were washed before being incubated for 90 min at 37^{o}C with horseradish peroxide conjugate goat antibody (Sigma, Pool Dorset, UK) against mouse IgG (Serotec,UK) diluted to 1:1000 in PBS, IgG subclass 1 (IgG1; Serotec, UK) and IgG subclass 2a (IgG2a; Serotec, UK) or IgA (Serotec, UK) all diluted to 1:2000. After washing, bound antibodies were detected by a colorimetric reaction developed by 2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) [ABTS] (Sigma Aldrich Co., UK) in citrate buffer and optical densities were read at 405 nm.

The serum antibody titres were expressed as the average (mean ± S.D., n=4.) of the reciprocal of the dilution at which the optical density (OD) at 405 nm was 5% higher than the strongest negative control reading. The IgA titres were presented as the absorbance value directly obtained from the optical densities resultant from IgA quantification in lung washes.

SPLENOCYTE CULTURE STUDIES

The spleens were aseptically removed from sacrificed animals and a spleen cells suspension was prepared as mentioned elsewhere [34]. These cells homogenisates (100µl) obtained from the four mice of each group were plated individually in 96-well tissue culture plates (Fisher, UK) in triplicate, along with 100µl of RPMI 1640 (Gibco, UK) containing soluble *S. equi* enzymatic extract at the concentration of 2.5 µg/ml. DuoSet® ELISA Development kit (R&D Systems Europe, UK) was used to quantify, according to the manufacturer's instructions, the interleukin concentrations (IL-2, IL-4, IL-6 and IFN-γ) in cell supernatants obtained after 48h of splenocytes stimulation with the soluble antigen at 37°C, in a humidified incubator at 5% CO_2 environment.

STATISTICAL ANALYSIS

Differences of significance between groups of *in vitro* and *in vivo* studies were determined by analysis of variance (ANOVA) linear model with SPSS software (Version 13, Microsoft), with significance set at $P \leq 0.05$. This analysis was further complemented by a multicomparison LSD post hoc test in order to state the difference identified between the groups which ANOVA analysis revealed a $P \leq 0.05$.

RESULTS AND DISCUSSION

SYSTEMIC IgG ANTIBODY IMMUNE RESPONSES

The development of new protein carrier systems is highly dependent on the reduction or prevention of chemical degradation, denaturation and aggregation, in order to preserve their antigenicity [20].

Our previous results suggested that PCL microspheres adsorbed with *S. equi* antigens may work as an antigen depot that will release the antigen over a long period of time, as the immune response was still maintained 300 days after a single subcutaneous (s.c) administration to BALB/c mice [34].

A previous manuscript described the preparation and fully characterisation of these *S. equi*-loaded PCL nanospheres (Chapter 3) [38]. Non-toxic nanospheres presented a spherical shape and a narrow particle size distribution, particularly PCL-GCS and PCL-OA. The mean diameter of various batches of PCL nanospheres used in these studies ranged from 242.57 to 450.2 nm and it was observed that the production method did not affect protein structure integrity, which is vital for the development of an efficient immune response. The *in vitro* studies previously performed have showed their controlled release properties for vaccine delivery and confirmed their *in vitro* uptake by macrophages [38]. As a result, the purpose of this *in vivo* study was to evaluate whether a mucosal immunisation of BALB/c mice with those *S. equi* antigens adsorbed onto PCL nanospheres were able to induce systemic IgG and IgG subtype antibodies and mucosal SIgA, and compare these immune responses with those elicited by the *S. equi* antigens entrapped in the same polymeric system. Two i.n. administrations were conducted to confirm the immunogenicity of *S. equi* antigen-loaded PCL nanospheres.

In addition to the entrapment technologies believed to enhance the immune response by promoting the antigen uptake by APCs or M-cells, immunoadjuvants as CTB, can be used in order to up-regulate mucosal responses [35, 39]. Therefore, the association of CTB to non modified

polymeric nanospheres (PCL-PVA) and their administration by a mucosal route was expected to improve both local (SIgA) and systemic (IgG) immune responses.

S. equi-specific IgG responses were detected in sera of mice after 2, 4, 7 and 12 weeks of animal inoculation. No *S. equi* specific-IgG antibodies was detected in serum of animals vaccinated with plain particles (data not shown), which emphasised the utilisation of these PCL polymeric systems as an adjuvant for *S. equi* antigens, as they have the ability to change *S. equi* antigens immunogenicity (Figures 4.1 and 4.2).

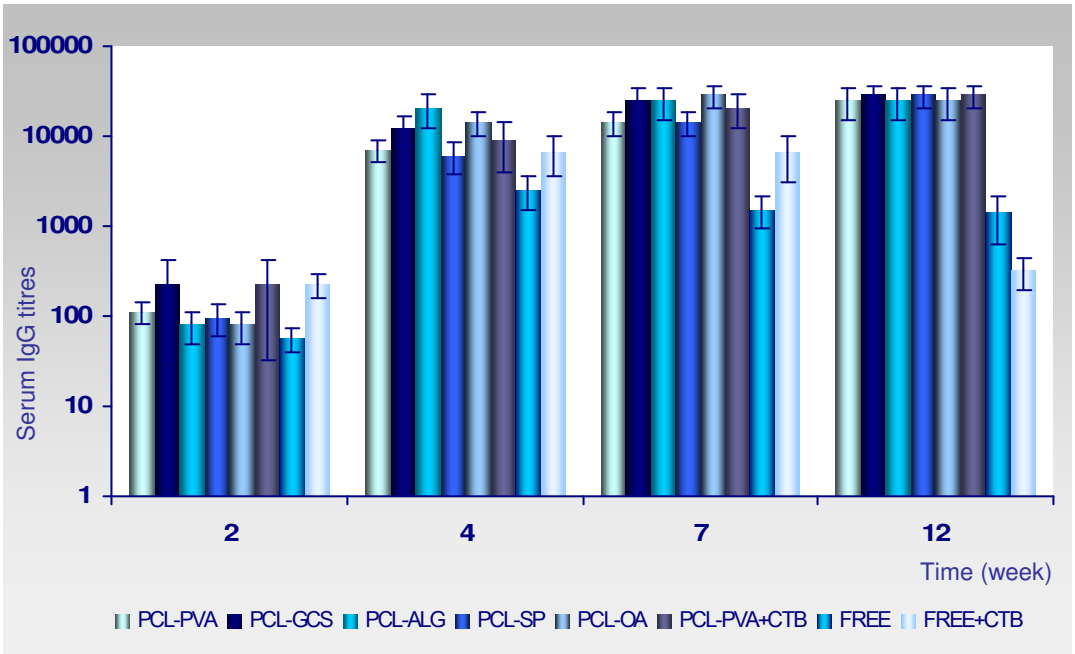


Figure 4.1 – Serum anti-*S. equi* specific IgG profile of mice immunised by i.n. route with *S. equi* antigens in solution (FREE), co-administered with CTB (FREE+CTB) and entrapped in different formulations of PCL nanospheres (mean ± S.D., n=4).

Animals vaccinated with *S. equi* antigen-loaded PCL nanospheres did not show at any period any sign of the disease or other severe adverse reactions, which is of vital importance as it constitutes one of the major disadvantages of *S. equi* extract-based vaccines [1].

Two weeks after animal's vaccination, only *S. equi* antigen-adsorbed PCL nanospheres groups produced serum anti-*S. equi* IgG specific titre significantly higher than those elicited by free antigen, free antigen associated with CTB and *S. equi* antigen-entrapped PCL-PVA nanospheres alone and co-administered with CTB (Figure 4.2).

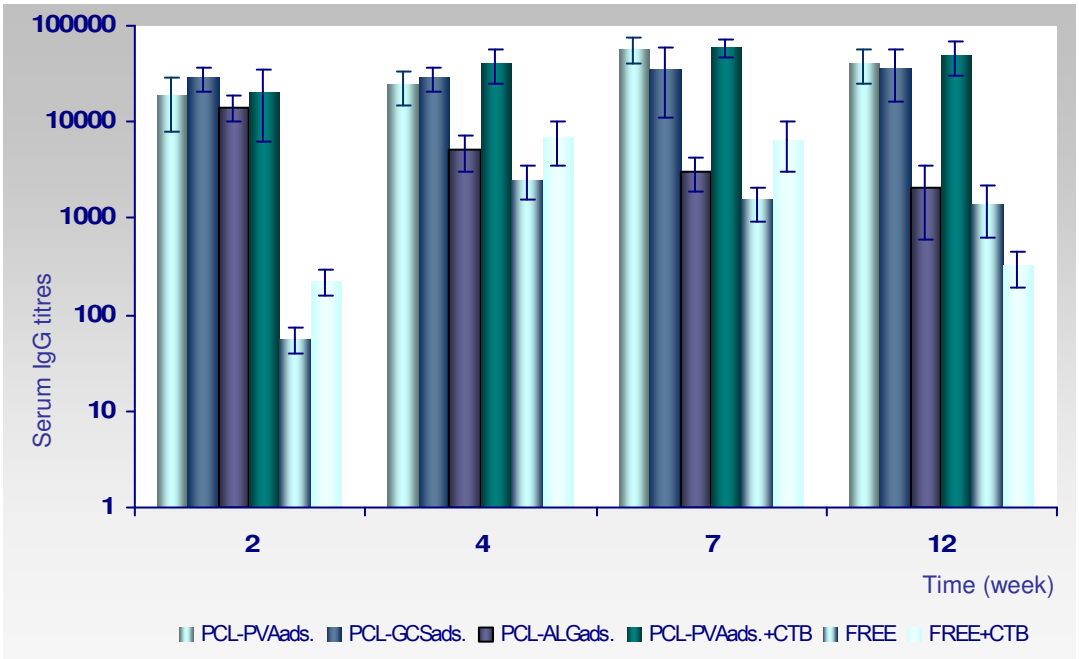


Figure 4.2 - Serum anti-*S. equi* specific IgG profile of mice immunised by i.n. route with *S. equi* antigens in solution (FREE), co-administered with CTB (FREE+CTB) and adsorbed onto different PCL nanospheres (mean ± S.D.; n=4).

PCL-GCS lead to a noticeable higher and more prolonged antibody IgG levels of vaccinated animals, and the difference between these titres and those induced and quantified in all other animals vaccinated with loaded particulate systems was significant ($P<0.001$).

By day 28, not only PCL-PVAads and PCL-GCSads induced significantly higher IgG systemic levels ($P<0.001$), but also *S. equi* antigens entrapped in PCL-ALG and PCL-OA had already seroconverted and their IgG responses were significantly superior ($P<0.02$) than those induced by FREE and FREE+CTB. *S. equi* antigen-adsorbed PCL-PVA nanospheres co-administered with CTB markedly enhanced the immunogenicity compared to FREE and

FREE+CTB groups (P<0.002), opposite to the specific anti-*S. equi* IgG titres that have resulted from the i.n. immunisation of mice with PCL-PVA nanospheres with entrapped *S. equi* antigens and associated to CTB (P>0.176) (Figures 4.1 and 4.2).

PCL had a MW of 42.5kDa which may justify the slower increase of antibody titres in animals vaccinated with *S. equi*-entrapped PCL nanospheres, as it presents a slow degradation rate.

The second i.n. immunisation enhanced the immune response induced by PCL-GCS, as after 7 weeks, the IgG titres of animals vaccinated with these nanospheres, as well as by PCL-ALG and PCL-OA, were significantly higher than those obtained from the free antigen and FREE+CTB (P<0.02 for PCL-GCS and PCL-ALG, and P<0.008 for PCL-OA). PCL-PVA and PCL-SP induced IgG antibody levels statistically higher than those elicited by the free antigen (P<0.05). On the contrary, those IgG levels were different from those obtained with the FREE+CTB-treated group (P=0.131) (Figure 4.1). Moreover, in PCL-PVAads and PCL-GCSads-treated groups it was still possible to distinguish IgG levels that were statistically different from those assessed in sera of animals treated with FREE and FREE+CTB. However, PCL-PVAads elicited a systemic IgG immune response that, at this period, was statistically higher than that induced by whatever formulations tested, with exception only when this formulation was associated to the potent adjuvant CTB (Figures 4.2).

Twelve weeks after the beginning of the *in vivo* study, all particulate formulations enhanced the antibody response in terms of peripheral IgG levels, and the difference between these responses and those induced by the FREE and FREE+CTB were statistically higher (Figures 4.1 and 4.2). CTB admixed with free antigen increased the serum IgG titres by a factor of at least 4 compared with the antibody levels induced by i.n. administration of non-adjuvantised free antigens. It is important to mention that co-administration of CTB with *S. equi* entrapped-PCL-PVA nanospheres did

not resulted in a considerable increase of serum IgG antibody levels when compared with PCL-PVA group, and therefore it was not identified a synergic adjuvant effect from the association of both adjuvants (P=0.107) (Figure 4.1). This finding indicates that CTB seems not to have a substantial additional adjuvant effect when associated to these carriers with entrapped *S. equi*. On the contrary, i.n. administration of CTB admixed with *S. equi* antigen-adsorbed PCL-PVA nanospheres, significantly increased the IgG titres (P=0.018) to one and half times the level induced by nanospheres in the absence of this adjuvant (Figure 4.2). Among animals vaccinated with PCL nanospheres, the difference between systemic anti-*S. equi* IgG specific titre induced by polymeric carriers was only significant when compared with PCL-ALGads-treated group (P<0.004). In fact, this polymeric system induced the lowest IgG levels when compared with those quantified in the serum of animals vaccinated with other PCL nanospheres.

Over the trial period, serum IgG antibody levels of animals vaccinated with *S. equi* antigens entrapped or adsorbed onto PCL particles, despite the type of antigen association, was significantly higher (P<0.005) than those elicited by the control (plain particles), free antigens, or even free antigen adjuvantised with CTB, with exception for *S. equi*-adsorbed PCL-ALG nanospheres, which systemic IgG immune response was not different from that elicited by FREE or even FREE+CTB solutions (Figures 4.1 and 4.2). The exact reason for this effect remains uncertain but antigens immobilised in particles are taken up and processed in a different way, presenting altered properties in terms of uptake and trafficking, as compared with soluble proteins [40]. For example, Eyles *et al*. (1998, 1999) [41, 42] and Baras *et al*. (1999) [43] obtained an immune response higher after an i.n. administration of antigen that was entrapped in microspheres, when compared with its soluble form. Almeida *et al*. (1993) used tetanus toxoid-adsorbed PLA microspheres ranging from 0.1 to 1.6 μm and obtained humoral immune response stronger, even 15 weeks after the priming dose, than that obtained after the i.n. immunisation with the soluble antigen, which indicates that the presentation of antigen by microspheres

enhance its uptake by NALT and eventually by other lymphatic tissues, as the spleen [44].

Despite the lower serum *S. equi*-specific IgG responses obtained after animal i.n. immunisation with PCL-ALG particles, this natural polymer is known by its bioadhesive properties and consequently might enhance a local immune response to strangles [45].

Even if the second i.n. immunisation generally raises the systemic immune response, in this case the antibody levels were not significantly higher, which indicates that one single immunisation of PCL nanospheres may be enough to induce elevated immune response in a mouse model.

Moreover, the antibody titre generated by i.n. administration of these different PCL nanospheres was higher ($P<0.04$) than those induced after a single dose of PCL microspheres previously studied, which highlights the importance of the route of administration and the particle size in the uptake and subsequent presentation of the antigen [34]. This makes PCL nanospheres especially attractive as a nasal vaccine delivery system, as it elicited strong immune responses after a single dose, while most of available nasal vaccines need booster vaccination to reach antibody levels as high as those obtained with a parenteral administration [11]. The adjuvant activity of these PCL nanospheres of different composition was found to be comparable, with the exception of PCL-ALGads, as all *S. equi* antigen-loaded PCL nanospheres tested were equally effective, highlighting the significant adjuvant effect of these polymeric carriers and the versatility of this approach.

Similar results were more recently obtained in mice with whole killed *S. equi* cells or bacterial lysates entrapped in PLGA microspheres, which induced protective immunity to a strangles murine model, after their i.n. administration [40]. In addition, PLGA microspheres modified with CS were successfully developed to improve nasal vaccine delivery by Jaganathan *et al*. (2006) [26]. Indeed, as mucus layer is anionic at a neutral pH value, positively charged groups on particles surface can promote interaction between mucus

and particles, leading to a lower clearance rate of nanospheres and consequently to an increase of their contact time with the nasal mucosa [17, 26, 46].

IgG SUBTYPE PROFILING

As observed for serum specific anti-*S. equi* IgG immune response, analysis of IgG subclasses in serum collected from mice vaccinated with *S. equi* antigen-adsorbed PCL-PVA or PCL-GCS, two weeks after the prime dose, revealed that IgG1 serum titres were significantly higher than those quantified in all other groups ($P< 0.013$ for PCL-PVAads and $P< 0.001$ for PCL-GCSads). In fact the antibody levels induced by PCL-GCS were markedly higher than those induced by PCL-PVAads ($P<0.001$) (Figures 4.3 and 4.5).

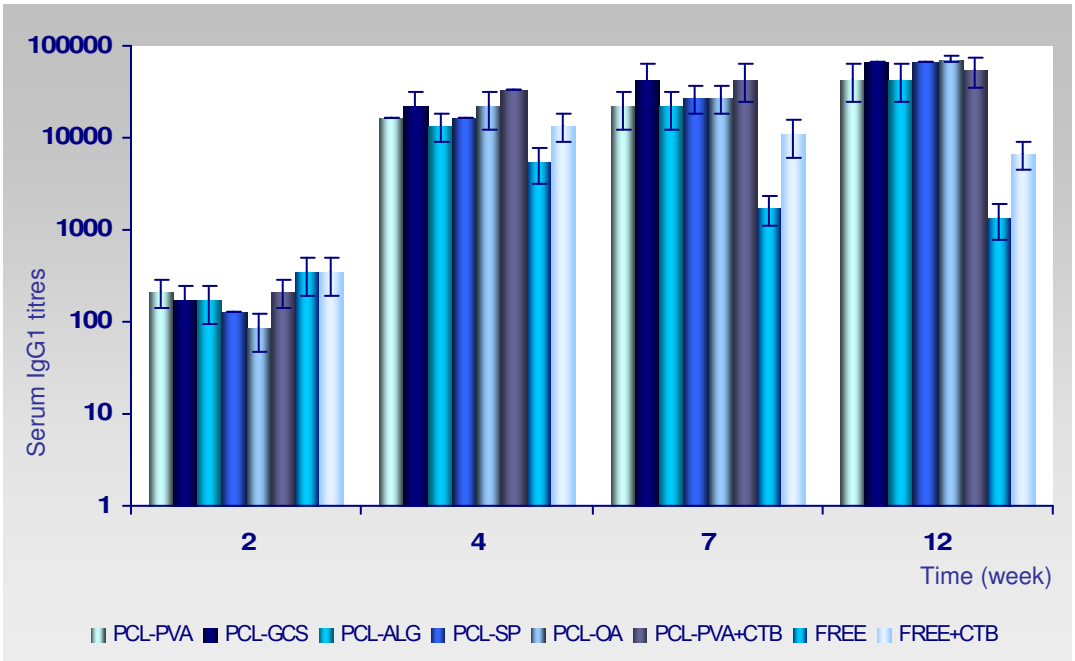


Figure 4.3 – Serum anti-*S. equi* specific IgG1 titres induced after i.n. administration of *S. equi* antigens entrapped in PCL nanospheres to female BALB/c mice, compared to *S. equi* antigens in solution (FREE) and co-administered with CTB (FREE+CTB) immunisations (mean ± S.D.; n=4).

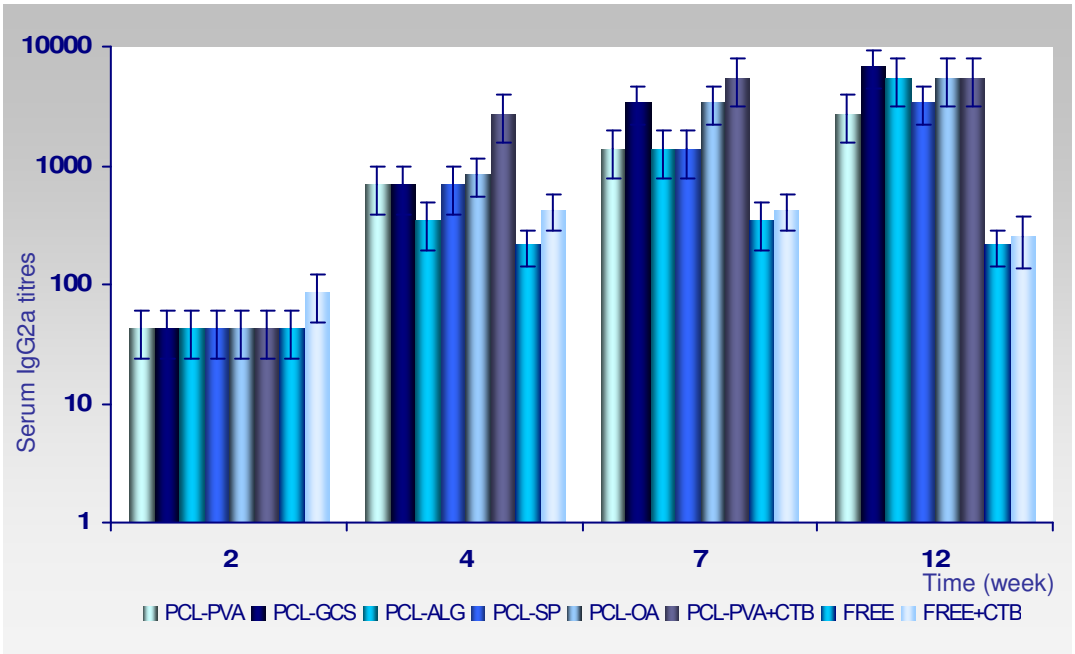


Figure 4.4 - Serum anti-*S. equi* specific IgG2a titres induced after i.n. administration of *S. equi* antigens entrapped in PCL nanospheres to female BALB/c mice, compared to *S. equi* antigens in solution (FREE) and co-administered with CTB (FREE+CTB) immunisations (mean ± S.D.; n=4).

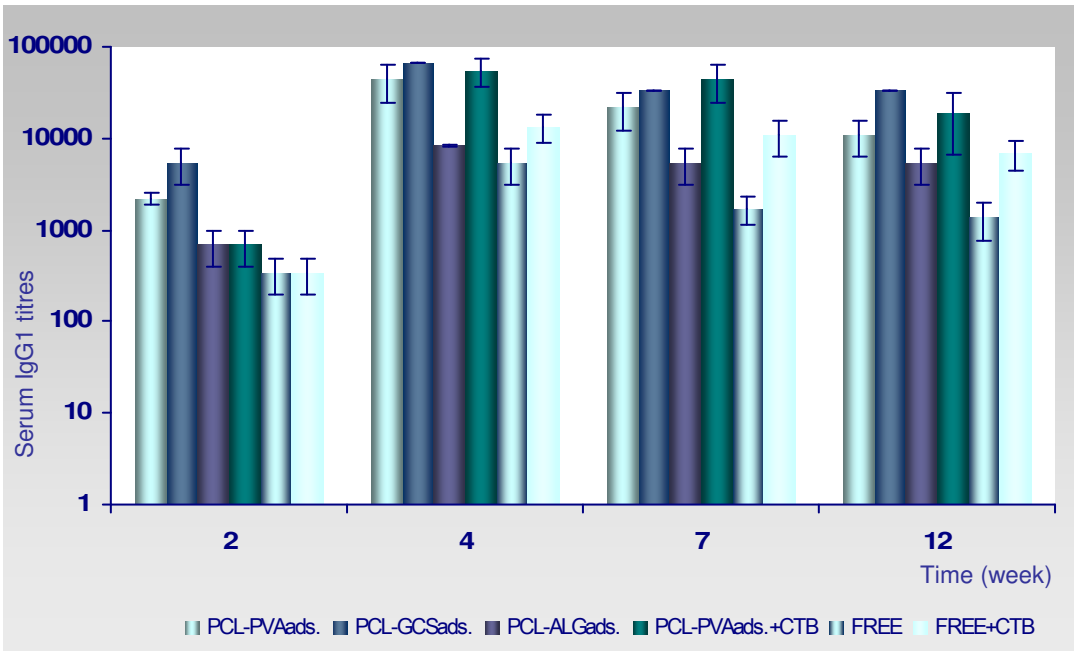


Figure 4.5 - Serum anti-*S. equi* specific IgG1 titres induced after i.n. administration of *S. equi* antigen-adsorbed PCL nanospheres to female BALB/c mice, compared to *S. equi* antigens in solution (FREE) and co-administered with CTB (FREE+CTB) immunisations (mean ± S.D.; n=4).

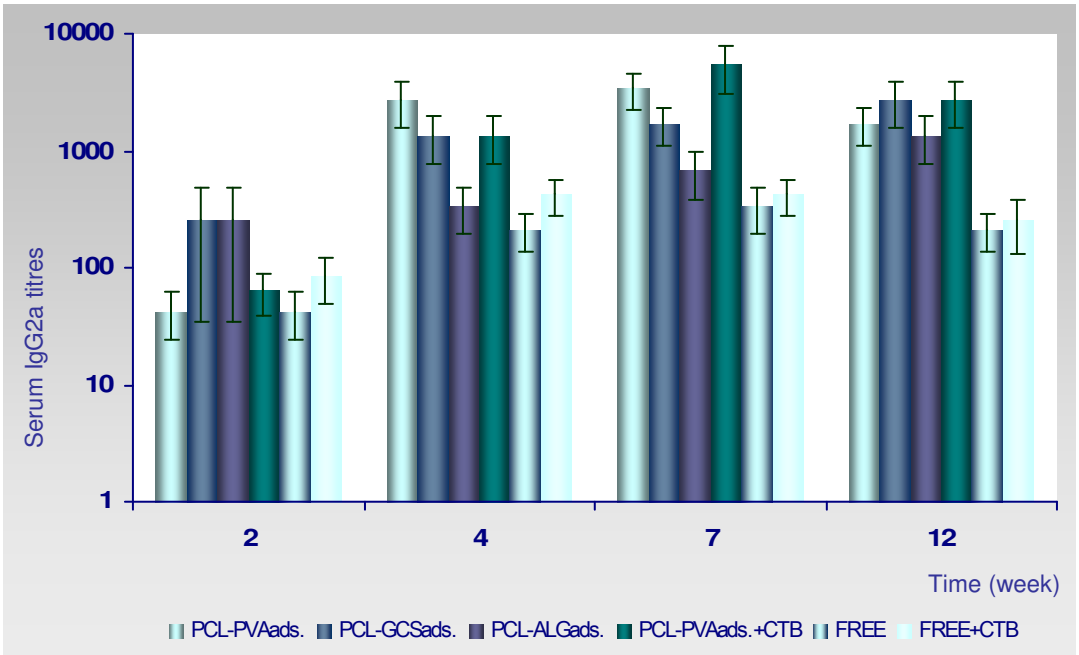


Figure 4.6 - Serum anti-*S. equi* specific IgG2a titres induced after i.n. administration of *S. equi* antigen-adsorbed PCL nanospheres to female BALB/c mice, compared to *S. equi* antigens in solution (FREE) and co-administered with CTB (FREE+CTB) immunisations (mean ± S.D.; n=4).

In addition, IgG2a immune responses induced by PCL-GCSads (P<0.010) and PCL-ALGads (P<0.035) nanospheres were statistically different from those induced by any other vaccinated group in this study (Figures 4.4 and 4.6). Moreover, the systemic responses to *S. equi* were dominated by the IgG1 subclass.

At this point, no efficient specific IgG1 and IgG2a response against *S. equi* antigens could be detected in mice vaccinated with *S. equi* antigens entrapped in any of PCL nanospheres.

CTB enhanced the development of IgG1 and IgG2a to *S. equi* only marginally in mice treated with free or entrapped antigens (Figures 4.3 and 4.4). Conversely, inclusion of CTB in the suspension of PCL-PVAads nanospheres significantly enhanced anti-*S. equi* IgG1 titres, 4 weeks after i.n. immunisation, compared with *S. equi*-entrapped nanospheres, PCL-PVAads and PCL-ALGads experimental groups (P<0.01) (Figure 4.5). Therefore, only antigen adsorbed onto PCL-GCS and PCL-PVA nanospheres surface

markedly enhance the immunogenicity in animals, eliciting an anti-*S. equi* IgG1 titres statistically higher when compared with all experimental groups (P<0.006), excepting those induced by CTB associated to PCL-PVAads nanospheres (Figure 4.5). Serum from mice treated with antigens entrapped in PCL-GCS and PCL-OA nanospheres contained higher concentrations of anti-*S. equi* IgG1 and IgG2a than serum collected from animals vaccinated with antigen in the free form (P<0.033) (Figures 4.3 and 4.4). PCL-PVAads particles induced IgG2a levels statistically higher than those dosed in the serum of all treated mice (P<0.01), with exception for specific anti-*S. equi* IgG2a assessed in animals vaccinated with CTB admixed to the *S. equi* adsorbed onto PCL-PVA surface nanospheres (P=0.283). Conversely, at this time point the immunisation of mice with PCL-GCSads induced superior IgG2a antibody titres compared with *S. equi*-loaded ALG nanospheres (adsorbed and entrapped) (P<0.038) and the free antigen, either alone or combined with the adjuvant CTB (P<0.05) (Figures 4.4 and 4.6).

After boosting, IgG subtypes quantification indicated a decrease of IgG1 antibodies levels in animals vaccinated with the *S. equi*-adsorbed polymeric particles, while general elevated levels of IgG2a were obtained, and no change in these antibody titres was obtained in mice treated with FREE or FREE+CTB (Figures 4.5 and 4.6). As a result, PCL-PVAads and PCL-GCSads at this time point induced IgG1 and IgG2a titres higher than those elicited by the antigen, free or associated with CTB (P<0.04 and P<0.06, respectively). These observations were maintained even 12 weeks after the beginning of the study (Figures 4.5 and 4.6). After 7 weeks, particulate systems co-administered with CTB still induced higher IgG2a levels than all the other experimental groups (P<0.03), while IgG1 antibodies were only extremely higher than those induced by PVA and ALG formulations (P<0.027), as well as FREE and FREE+CTB groups (P<0.001) (Figures 4.3 to 4.6). At the end of the experiment, it is important to mention that PCL-PVAads coupled to CTB induced IgG1 (P<0.008) and IgG2a (P<0.04) levels significantly higher than those elicited by the entrapped antigens, except for IgG2a obtained with PCL-PVA and PCL-SP

nanospheres. In contrast, CTB co-administered to *S. equi* antigens entrapped in PCL-PVA has markedly elevated the IgG subclass titres relative to the antigen-adsorbed particulate systems (P<0.041 for IgG2a and P<0.033 for IgG1), including PCL-PVAads+CTB.

After boosting, entrapped antigens induced a continuous increase of both IgG subclasse levels (Figures 4.3 to 4.6). Despite of the serum of all animals treated with this *S. equi*-entrapped systems containing IgG1 and IgG2a levels significantly elevated in comparison with those assessed in animals vaccinated with the free form (P<0.011 and P<0.06), PCL-GCS nanospheres induced IgG1 antibody levels different than those obtained in mice immunised with antigen adsorbed or entrapped in PCL-PVA (P<0.027) and PCL-ALG (P<0.001) nanospheres. Anti-*S. equi* IgG1 assessed in the serum of animals vaccinated with *S. equi*-entrapped PCL nanospheres continued to increase even 12 weeks after animal immunisation (Figures 4.3). Therefore, these antibody titres were still statistically elevated when compared with the antigen, either FREE (P<0.001) or co-adjuvantised by CTB (P<0.001). On the other hand, IgG2a titres to *S. equi* in the experimental groups treated with antigen entrapped in GCS, ALG and OA nanospheres had raisen to a level that was statistically elevated relative to groups PCL-PVA (P<0.045), all groups of mice immunised with *S. equi*-adsorbed PCL carriers (P<0.007), including PCL-PVAads+CTB, and PCL-SP (P<0.045) (Figures 4.4 and 4.6). Therefore, despite the immune responses induced by antigen adsorbed onto PCL nanospheres being higher during the first seven weeks of the study, the antibody titres induced by the antigen entrapped in nanospheres continuously increased even 12 weeks after the immunisation, which supports the hypothesis of the delayed immune responses induced by these particulate carriers being due to the slow release and biodegradation of the PCL polymer. As a result, it seems that the best system may result from the design of a PCL polymeric carrier containing the antigen adsorbed but also entrapped, in order to not only induce a high immune response just after the

prime dose, but also to maintain the antibody levels for a prolonged period of time.

Yanagitu *et al*. (1999) have shown that a nasal immunisation was capable of inducing both Th1 and Th2 cells, which can inhibit the bacterial attachment to the epithelium and the resultant inflammatory cytokine production [47].

Overall, mice vaccination with *S. equi* antigen-loaded PCL nanospheres, in the absence of CTB, maintained statistically elevated IgG subtype titres in comparison with animals treated with free vaccine (P<0.04) throughout the 12-weeks schedule, indicating the generation of Th1/Th2 mixed response. In fact, by the end point of the experiment the ratio of different anti-*S. equi* IgG subclasses (IgG2a/IgG1) assessed in the serum of treated animals appeared not to be markedly influenced by the presence or absence of CTB in the vaccine formulation. Serum of mice immunised intranasally with *S. equi* antigens associated (entrapped or adsorbed) with nanospheres, formulated with ALG and GCS, contained elevated levels of anti-*S. equi* IgG1 and IgG2a suggestive of a more balanced immune response (IgG2a/IgG1 = 0.35 and IgG2a/IgG1 = 0.49, respectively), opposite to those obtained with the remaining particulate systems. Mice treated with vaccine formulations co-adjuvantised with CTB presented Th1/Th2 mixed immune responses slightly more balanced than those induced by the mucoadhesive polymeric particles previously mentioned.

In addition, until this point, having in mind the particles physicochemical characteristics, the loading capacity and these humoral *in vivo* results, GCS seems to be an attractive adjuvant for nasal vaccination of mice against strangles.

LOCAL IgA IMMUNE RESPONSES

The mucosal immunisation used to vaccinate animals in this study is known to be able to induce, with lower amounts of antigen, not only the production of SIgA, but also a systemic antibody and cell-mediated immunity [48]. The

systemic and local immune responses induced after an i.n. vaccination depends on several factors, namely the antigen nature, the correct selection of an adjuvant, the dose volume, the frequency of administration and the delivery system [49]. Wu and Russel (1997) have showed that an appropriated systemic and local immune responses can not only prevent infection but also induce a mucosal immunisation, as a means of inducing SIgA antibodies directed against specific pathogens of mucosal surfaces, which is of vital importance especially when mucosa constitutes the portal of bacteria entry (eg *S. equi*), as they may block the attachment of this bacteria to the mucosal surfaces and stimulates a cross protective immunity more efficiently than serum IgG antibody [41, 49]. Larger doses administered into the nose may be swallowed, leading to oral delivery. Eyles *et al*. (1998) have seen that 40% of the dose was found in the lung after an administration of 50 µl of labelled microspheres, while most of the particles were retained in the nose when 10 µl was dosed [41]. Moreover, the efficacy of vaccines to prevent strangles seems to be dependent on the induction of a mucosal immune response [13].

Soluble antigens need to be associated or delivered by adjuvants as, after animal i.n. vaccination, they will cross the nasal epithelium, interact with dendritic cells, macrophages and lymphocytes, and then be transported to posterior lymph nodes. On the other hand, particulate delivery systems are taken up by M-cells in the NALT, which transport the antigen preferentially to the cervical lymph nodes. As a result, local and distant mucosal immune responses can be elicited as a result of the maturation of the precursors of IgA mucosal cells in regional lymph nodes or, after being transported, in the *lamina propria* of distant mucosal sites of the CMIS [48]. Accordingly, the induction of an immune response at one mucosal surface will induce the production of SIgA specific for the antigen at mucosal surfaces far from the target, as it has been strengthened more recently by Mestecky *et al*. (1997) [50].

In the present work, the two i.n. immunisations induced elevated *S. equi*-specific IgA antibodies in lung washes of all groups vaccinated with *S.*

equi-loaded PCL nanospheres compared to controls (plain particles, $P<0.002$) or antigen, free ($P<0.01$) or admixed with CTB ($P<0.02$) (Figure 4.7). In fact, modest SIgA levels were detected in lung washes of animals vaccinated with antigen in solution. In addition, i.n. administrations did not induced detectable SIgA levels in intestinal contents of all animals (data not shown).

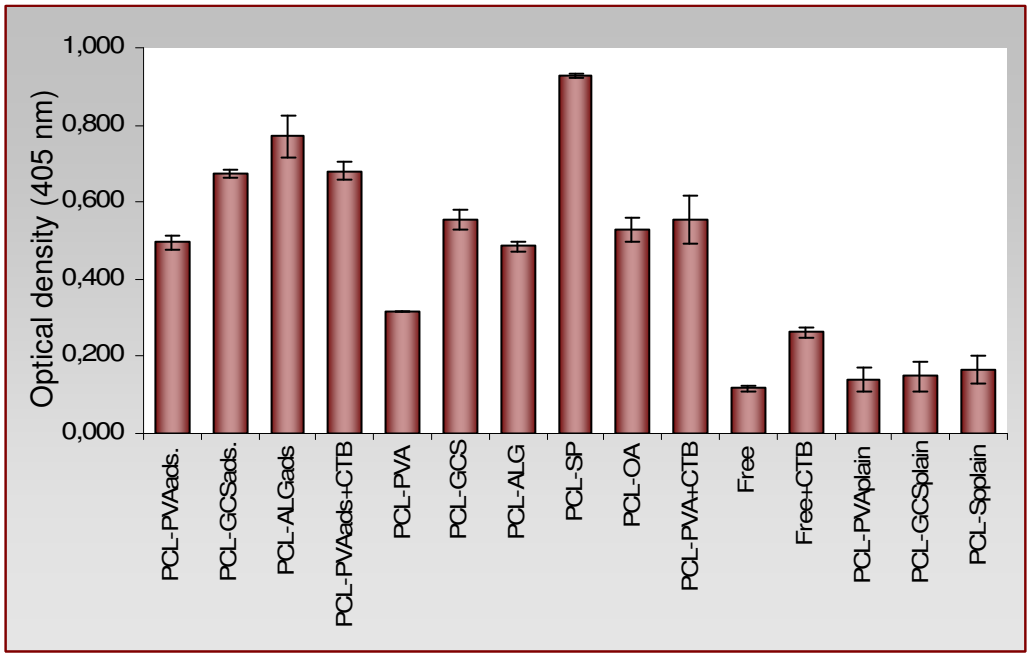


Figure 4.7 – Secretory IgA (SIgA) level in lung washes of mice immunised intranasally with different formulations, at the end of the study (mean ± S.D.; n=4).

The PCL-SP group had the highest SIgA antibody response, besides not being statistically different from that assessed in animals immunised with PCL-PVA+CTB ($P=0.157$) (Figure 4.7).

Among the adsorbed nanospheres, PCL-ALGads group induced the highest SIgA antibody response, which was significantly different from those induced by all other groups ($P<0.01$), although PCL-GCSads had also highly enhanced this antibody level. Among all the particulate systems tested, only non-modified PCL nanospheres (PCL-PVA) formulation did not induce an extremely elevated mucosal response after their i.n. administration (Figure 4.7). These results were expectable, as these natural polymers (GCS and ALG)

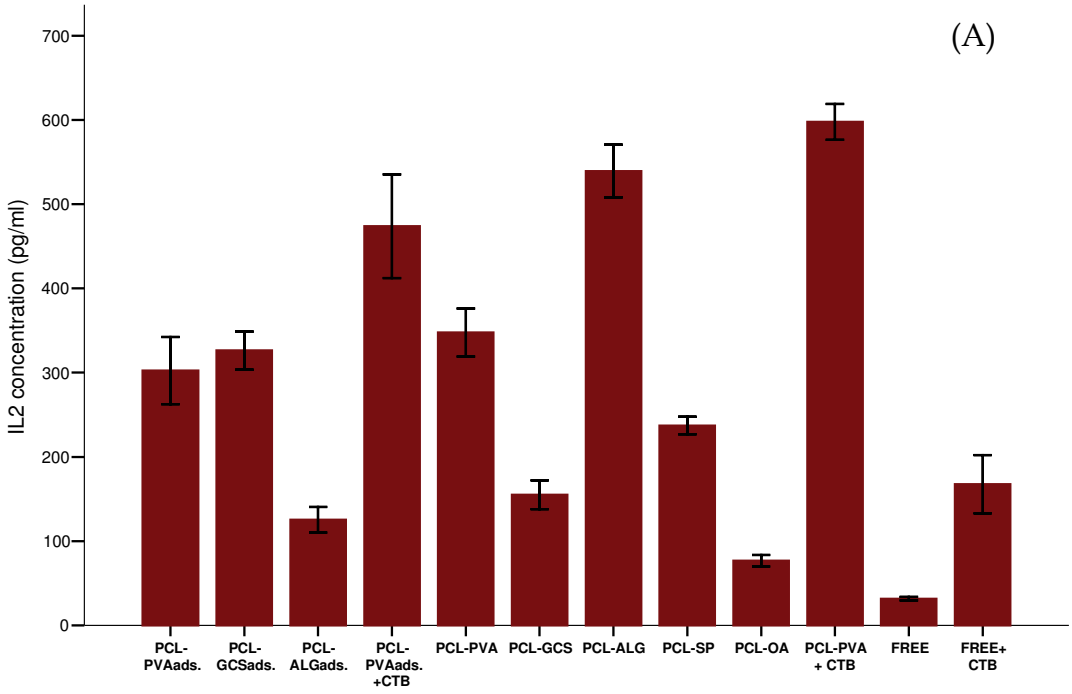
are known by their bioadhesive properties and consequently would probably enhance a local immune response to strangles after animal mucosal vaccination. In addition, PLGA microspheres modified with CS were successfully developed to improve nasal vaccine delivery by Jaganathan *et al*. (2006) [26]. The strong mucoadhesiveness and the particulate nature of these carriers are important properties for its immunostimulatory effect. PCL-GCS nanospheres are positively charged and therefore are likely to establish electrostatic interactions with the negatively charged mucus, and thereby prolong the residence time of the antigen in the nasal cavity [17, 26, 46]. The factors that most probably contributed for the immunostimulating effect of GCS and ALG-based systems, after their i.n. administration, were the improved uptake by M-cells at the nasal mucosa and efficient delivery to the mucosal lymph nodes, consequence of the prolonged exposure of the antigen. Therefore, it is clear from these results that the *S. equi* antigen-loaded nanospheres successfully induced not only a systemic but also a local immune response, after i.n. antigen exposure.

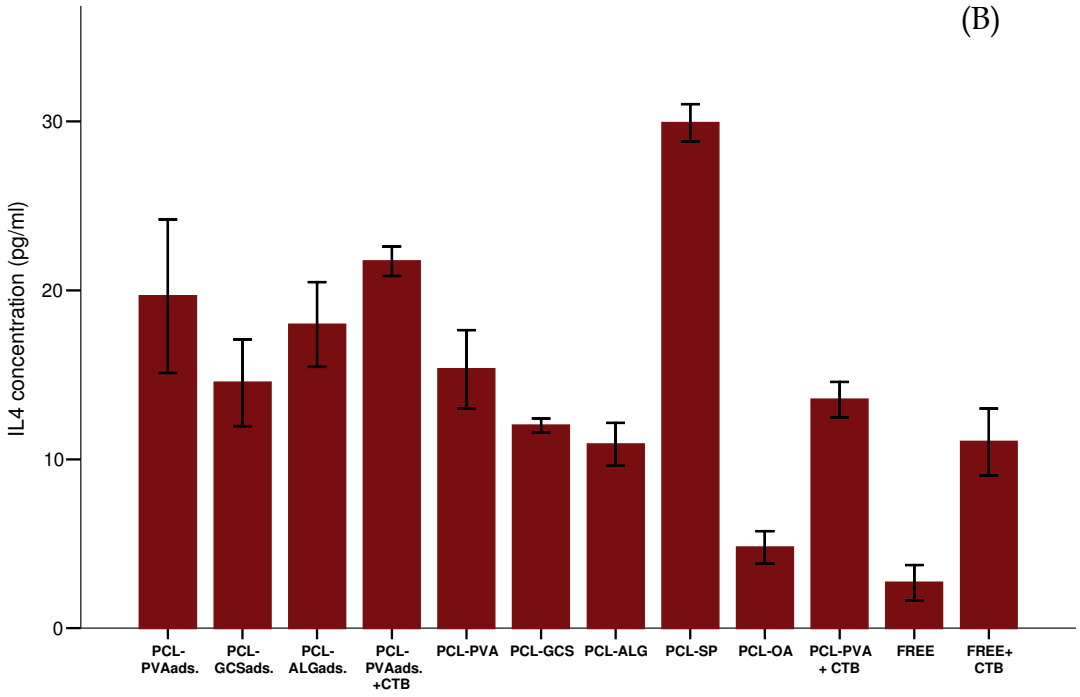
SPLENOCYTE CULTURE STUDIES

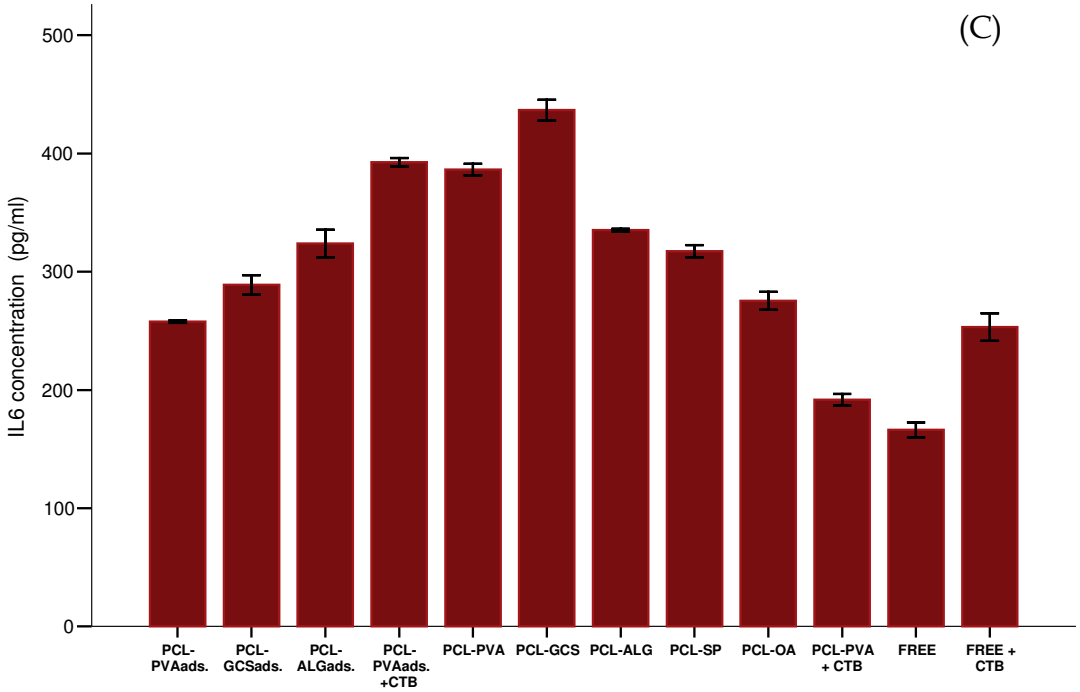
It is generally accepted that the way that the antigen is delivered to APCs is one key factor for eliciting Th1/Th2 relation [17, 51, 52]. Cytokines released from cells play an important role in the activation, growth and differentiation of B and T lymphocytes, which interact with each other and secret factors that will stimulate other cell types and eventually themselves. T-helper cells can be divided in two groups depending on the lymphokines secreted upon their activation. Th1 cells secrete interleukin 2 (IL2), interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β), that will increase gene expression in APCs and activate B cells to differentiate and secrete IgG2a, while Th2 produces IL4, 5, 6, 10 and 13, being the IgG1 the major isotype elicited from B cells following Th2 cells activation. Therefore, Th1 cells are involved in the cell mediated immunity, while Th2 cells are related with humoral immunological response [53].

Previous studies have shown that the delivery of antigens adsorbed onto microparticles up-regulated the cytokine gene transcriptions [54]. More recently, whole killed *S. equi* cells or bacterial lysates entrapped in PLGA microspheres, after i.n. administration in mice, induced protective immunity to a strangles murine model [40]. As a result, the nasal administration of *S. equi* antigens-loaded particles (adsorbed or entrapped) were undertaken in order to assess if PCL, a polymer generally with higher hydrophobicity than PLGA, associated with several adjuvants can induce an immune response after i.n. administration.

From figure 4.8 it can be seen that, in general, the particulate systems induced an increase of splenocyte response as the differences observed in IL2, IL4, IL6 and IFN-γ stimulated by nanosphere formulations and free antigen were significant (P<0.021).




(B)
IL4 concentration (pg/ml)
30
20
10
0
PCL-PVAads.
PCL-GCSads.
PCL-ALGads.
PCL-PVAads. +CTB
PCL-PVA
PCL-GCS
PCL-ALG
PCL-SP
PCL-OA
PCL-PVA + CTB
FREE
FREE+ CTB


(C)
IL6 concentration (pg/ml)
500
400
300
200
100
0
PCL-PVAads.
PCL-GCSads.
PCL-ALGads.
PCL-PVAads. +CTB
PCL-PVA
PCL-GCS
PCL-ALG
PCL-SP
PCL-OA
PCL-PVA + CTB
FREE
FREE + CTB

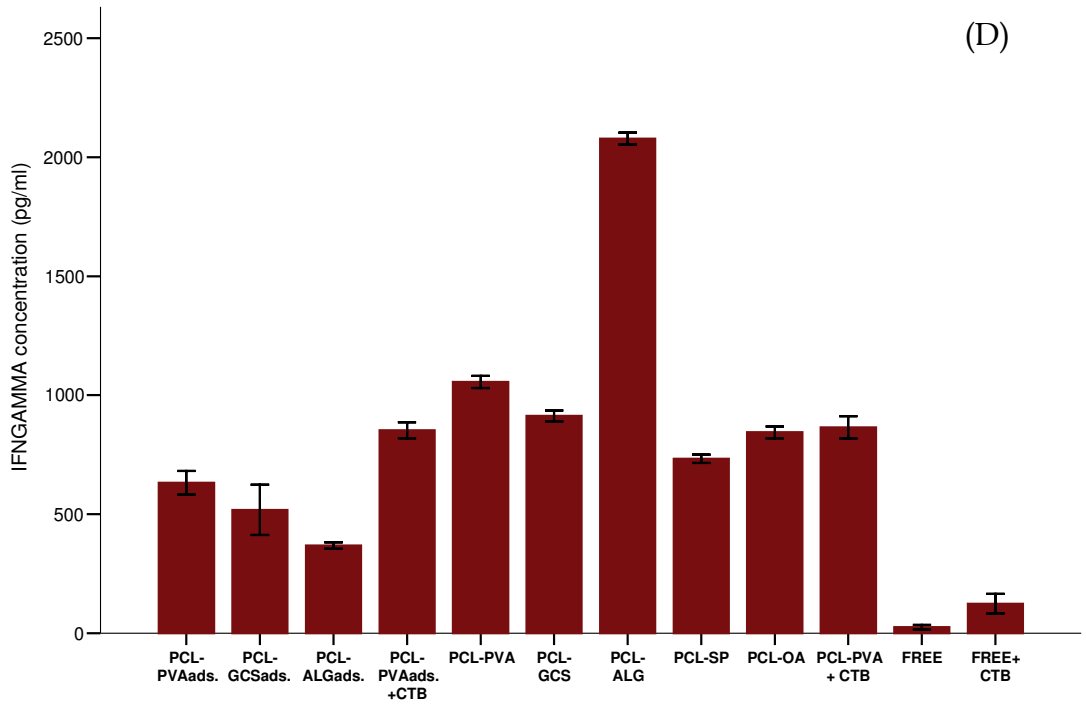


Figure 4.8- *S. equi*-specific recall responses after stimulation of splenocytes derived from BALB/c mice immunised subcutaneously with *S. equi* enzymatic extract in solution (FREE), co-administered with CTB (FREE+CTB) and associated (adsorbed or entrapped) to PCL nanospheres. Cytokine concentration in culture supernatants was determined in splenocyte cultures stimulated with 5 µg/ml of soluble *S. equi* enzymatic extract: (A) IL2; (B) IL4; (C) IL6 and (D) IFN-γ (mean ± S.D.; n=4).

Among the *S. equi* antigen-adsorbed particles, PCL-PVAads and PCL-GCSads induced an elevated IL2 production after spleen cells stimulation with soluble antigen (5 µg/ml), which concentration was statistically different from those elicited by the remaining experimental formulations (P<0.011), except when compared with IL2 levels consequence of antigens-entrapped PCL-PVA nanospheres dosing (P=0.67) (Figure 4.8A). In addition, the differences found in IL2 amounts are statistically significant for all tested formulations, with the exception of those induced by PCL-ALGads when compared with the ones elicited in animals vaccinated with PCL-GCS (P=0.061). Even so, PCL-ALG not only elicited the highest IL2 concentration, but also noticeable IFN-γ levels, being the latter statistical higher than those induced by all other groups (P<0.001) (Figure 4.8B), which is in accordance with the SIgA antibody levels (Figure 4.7). Nevertheless, all the differences between IFN-γ levels observed

in Figure 4.8B are significant (P<0.04), except for PCL-GCS nanospheres when compared to PCL-OA (P=0.059) and CTB associated to nanospheres treated groups (P=0.094).

Indeed, both Th1-dependent cytokines and their high levels indicate that a strong cell-mediated immune response was elicited by the i.n. administration of *S. equi* antigens entrapped in ALG modified PCL-based formulations. The generation of Th1 cytokine profile is extremely important to permit the eradication of *S. equi* pathogen, being therefore a useful vaccine for therapeutic immunisation of *S. equi*-long term carriers.

IL4 production was significantly induced by PCL-SP among the particulate systems tested (P<0.001) (Figure 4.8C). Also CTB admixed with *S. equi*-adsorbed PCL-PVA particles elicited IL4 amounts significantly higher than all formulations, except those elicited by PCL-SP and PCL-PVAads nanospheres (P=0.23) (Figure 4.8C).

All antigen-loaded nanospheres induced high levels of IL6, but those elicited by PCL-GCS were statistically higher than the IL6 concentration assessed in sera of all the remaining groups (P<0.001) (Figure 4.8D).

Despite the differences above mentioned, in accordance with cellular and humoral immune responses, *S. equi* antigens-loaded modified PCL carriers gave higher lymphokines levels when compared to the immunological responses induced by non-modified particles (PCL-PVA and PCL-PVAads) and free antigens, successfully activating the paths leading to Th2 and Th1 cells, which highlights the role of these nanospheres as an adjuvant and their use to protect animals against strangles.

Altogether, the data suggest that the quality of immune response to *S. equi* antigens is increased by its association to the PCL polymeric carriers formulated, mainly to those modified by the mucoadhesive polymers GCS and ALG, as not only a pronounced systemic but also local and mixed cellular immune responses have been detected after animals i.n. vaccination.

CONCLUSIONS

The purpose of this study was to compare the systemic and mucosal immune responses induced after i.n. vaccination of mice with *S. equi* antigens associated (adsorbed or entrapped) to PCL nanospheres with different compositions. These antigens were successfully associated to PCL nanospheres without causing any damage to the protein structure, although adsorption resulted in higher loadings. Even so, both *S. equi*-adsorbed and entrapped nanospheres induced significantly higher specific immune responses to *S. equi* antigens, which were confirmed by cytokine levels. Therefore, in a future work, it would be interesting to combine the adsorption and entrapment of *S. equi* antigens in a single PCL polymeric system, in order to obtain a combined immune response profile. Moreover, modified PCL nanospheres are potential nasal delivery systems to vaccinate animals against strangles, as humoral, cellular and mucosal immune responses were noticeable induced.

ACKNOWLEDGMENTS

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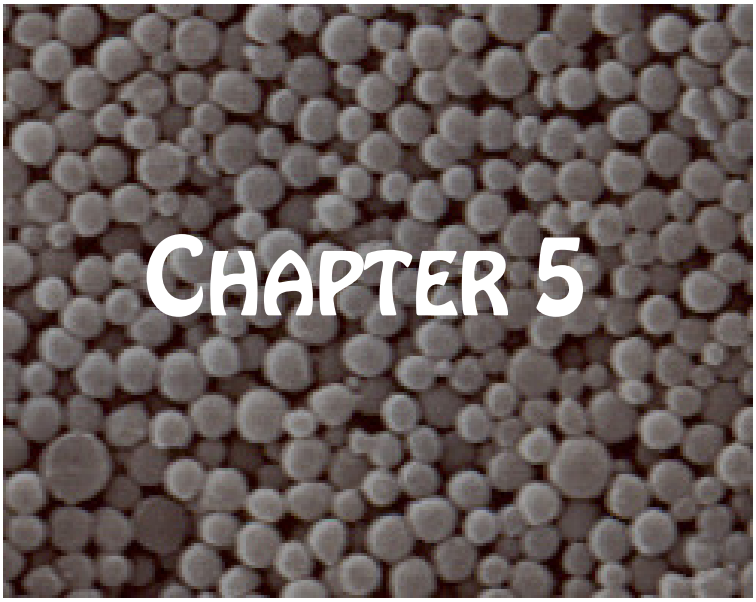

NEW APPROACH ON THE DEVELOPMENT OF A MUCOSAL VACCINE AGAINST STRANGLES: SYSTEMIC AND MUCOSAL IMMUNE RESPONSES IN A MOUSE MODEL

H.F.Florindo, S.Pandit, L.M.D. Gonçalves, H.O.Alpar, A.J.Almeida

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ABSTRACT

Streptococcus equi subspecies *equi* (*S. equi*) infects animals of the *Equidae* family and is the causative agent of strangles, an acute, extremely contagious and deadly disease that affects the upper respiratory tract, leading to devastating epidemics in stables. This agent is transmitted through a variety of mechanisms, most of them without involving direct contact of horses-to-horses, which makes it difficult to identify and isolate the infected animals and to prevent the spreading of this disease, which in addition can last for 3-4 weeks. Despite efforts directed to the formulation of an effective vaccine, prolonged periods of protection associated to absence of serious adverse reactions were not yet achieved. Thus, this experimental work is focused on the study of mucosal, humoral and cellular immune responses developed in a mouse model, after the intranasal administration of *S. equi* antigens associated by adsorption or entrapment to poly(lactic acid) nanospheres, modified by mucoadhesive polymers and absorption enhancers. Particles fitted the nanometric range and proteins integrity and antigenicity were not affected. PLA nanospheres induced a mixed Th1 and Th2 response, being therefore potential carriers for the delivery of *S. equi* antigens.

Keywords: Poly(lactic acid) nanospheres, strangles, mucosal vaccine

INTRODUCTION

Streptococcus equi subspecies *equi* (*S. equi*) is the causative agent of strangles, which is an acute, highly contagious and deadly respiratory disease of horses, donkeys and mules, although its presence in humans has been previously reported [1,2]. This is a prolonged disease, quickly and easily transmitted, leading to large outbreaks and consequently to an important economic burden. In some cases, animals present abscesses in other regions of their body, mainly in the abdomen and thorax resulting in a stage of the disease called 'bastard strangles', which often results in animal's death. Its long convalescent period, associated with the long-term *S. equi* carriers with no sign of the disease, constitutes the main reason for the limitations attributed to the development of diagnostic tests capable of detecting this agent in infected animals, despite the recent and commercial available new diagnostic techniques based in enzyme-linked immunosorbent assays (ELISA) methods. The infected horse, which can harbour the bacteria for several months, constitutes the most important factor for the maintenance of the disease for prolonged periods of time.

S. equi is in fact sensitive to some antibiotics as penicillin, but in most cases treatment is not effective in eradicating the microorganism. As a result, in order to combat this disease, the development of an efficient vaccine is the aim of most of the research related with this subject, as animals that recover from a *S. equi* infection present a prolonged protective immunity, mostly directed against the antiphagocytic cell wall associated *S. equi* M-like protein (SeM) which, associated to the hyaluronic acid capsule and the maturase lipoprotein, are believed to be the basis of S. *equi* virulence [3-7]. The protection mechanism is not yet fully understood but it seems that the most important antibodies for the prevention and combat of this disease are mainly those locally produced in the nasopharynx, although it was previously demonstrated that serum antibodies are the first to be produced [3, 4]. Specific anti-*S. equi* antibodies found in serum and produced by B cells will

recognise, subsequently bind and inactivate these bacteria. However, horse vaccination with bacterins, adjuvanted *S. equi* extracts and, more recently, SeM associated to different adjuvants, did not protect animals against infection with *S. equi* [4, 8-10]. Therefore, several researchers are still developing strategies to prevent infection of animals and spreading of the disease.

The main entrance for *S. equi* is the nasal mucosa which, associated to its easy administration and reduced costs of production, justifies the choice of *S. equi* nasal vaccine administration. In addition, previous studies have shown that the intranasal (i.n.) delivery of polymeric particles associated to antigens was able to induce not only humoral and cellular immune responses, but also desirable mucosal immunity, important for the defence after *S. equi* invades the host via the mucosal surface [11-13].

The purpose of the present research work was to study the immune response developed after mouse vaccination with PLA carriers containing entrapped or adsorbed *S. equi* proteins, previously extracted from bacterial wall. PLA particles were produced by a double (w/o/w) emulsion solvent evaporation technique and the effect of the mucoadhesive polymers (glycolchitosan (GCS) [14-16] and alginate (ALG) [17]) and absorption enhancers (spermine (SP) [18] and oleic acid (OA) [19]) associated to the PLA particles in the immune response following an i.n. administration was evaluated. In fact, we hypothesised that these carriers are able to target and be taken up by antigen presenting cells (APCs) making it possible to prime the animals prior its infection by *S. equi*, resulting in the stimulation of a cell mediated immunity and the formation of memory B cells, which would prevent and combat subsequently infections.

A similar system was previously developed by our group, using instead poly-ε-caprolactone (PCL) for particles matrix constitution [20, 21]. That study has shown that after i.n. vaccination of mice with *S. equi* antigens associated to PCL nanospheres, either by adsorption or entrapment method, it was induced

not only a systemic humoral and cellular immune response, but also secretory IgA (SIgA) locally produced antibodies [20]. Therefore, a comparison will be established between the mucosal SIgA and serum specific IgG, IgG1, IgG2a antibody responses induced by these PLA carriers and those previously obtained for the aforementioned PCL nanospheres.

Materials and Methods

Materials

Poly(L-lactic acid) (PLA, average molecular weight (MW) 2.0 kDa) was acquired to PolySciences, Inc., UK. Polyvinyl alcohol (PVA, MW 13-23 kDa, 87-89% hydrolysed), alginate low viscosity (ALG), spermine (SP), oleic acid (OA), glycolchitosan (GCS), sucrose, cholera toxin B subunit (CTB), trypsin-chymotrypsin inhibitor, EDTA sodium salt, iodoacetic acid and phenylmethanosulfonyl fluoride solution (PMSF) were from Sigma Aldrich Co., UK. Dichloromethane (DCM) was obtained from BDH Laboratory Supplies, UK.

Bicinchoninic acid (BCA) kit and Micro BCA™ Protein assay kit, both for protein determination, were supplied by Sigma Aldrich Co. UK, GOSS Scientific Ltd and Pierce UK, respectively.

Streptococcus equi subsp. *equi* (strain LEX) ATCC 53186 were a kind gift from Prof. J.F. Timoney (University of Kentucky, USA).

Animals

Female BALB/c mice (n=4/ group), 6-8 weeks old with food and drink provided *ad libitum*, were used in the *in vivo* studies, which were performed in strict accordance with the UK 1986 Animals (Scientific Procedures) Act.

Preparation of PLA nanospheres

The double emulsion (w/o/w) solvent evaporation method, previously described elsewhere, was used to prepare PLA nanospheres at room temperature, under aseptic conditions with minor modifications [21]. Briefly, the organic phase was prepared by the polymer dissolution in DCM, while 10% (w/v) PVA or 1% (w/v) GCS solution constituted the aqueous phase (Table 5.1). The w/o emulsion was prepared by homogenisation using an ultra-turrax (T25 Janke & Kunkel, IKA-Labortechnik) for 3 min at 24,000 rpm.

For *S. equi* antigen-entrapped particles (10.0 mg), a previous dissolution of these proteins in the aqueous phase was performed. The proteins were extracted from the bacteria cell wall using N-acetyl muramidase, lysozyme and mutanolysin (Sigma Aldrich Co., UK), as previously reported [21]. In order to study the effect of the inclusion of absorption enhancers (10 mg) in particles composition, SP was dissolved in the PVA solution, while OA, previously dissolved in 1 ml of ethanol, was diluted in the organic phase. The w/o emulsion was then added dropwise into 30 ml of a 1.25% (w/v) PVA or 0.75% (w/v) ALG solution, and homogenised for 7 min at 10,000 rpm with the Silverson homogeniser (Silverson model L4RT, UK), and then maintained at room temperature, under constant magnetic stirring for 4 hours, to remove the organic solvent and, therefore, allow particles formation (Table 5.1). These carriers were separated from the preparation media by centrifugation (15,000 rpm, 45 min, 15°C; Beckman J2-21 High speed centrifuge) and freeze-dried (Virtis, UK) after being washed and dispersed in a cryoprotectant 0.02% (w/v) sucrose solution.

Table 5.1
PLA nanospheres composition

	FORMULATION	ORGANIC PHASE	INTERNAL PHASE (w/v)	EXTERNAL PHASE (w/v)
Ads.	PLA-PVA	PLA (100 mg) in 6 ml DCM	10 % PVA	1.25% PVA
	PLA-GCS		1% GCS	1.25% PVA
	PLA-ALG		10 % PVA	0.75% ALG
Entrapped	PLA-PVA		10 % PVA	1.25% PVA
	PLA-GCS		1% GCS	1.25% PVA
	PLA-ALG		10 % PVA	0.75% ALG
	PLA-SP		10 % PVA + 10% SP	1.25% PVA
	PLA-OA	6 ml DCM + 10% OA (1ml EtOH)	10 % PVA	1.25% PVA

PLA-PVA – poly(lactic acid) – polyvinyl alcohol nanospheres; PLA-GCS – poly(lactic acid) – glycolchitosan nanospheres; PLA-ALG - poly(lactic acid) – alginate nanospheres; PLA-SP - poly(lactic acid) – spermine nanospheres; PLA-OA - poly(lactic acid) – oleic acid nanospheres;

A *S. equi* antigen solution (1250 µg/ml; 2 ml) was added to twenty milligrams of PLA-PVA, PLA-GCS and PLA-ALG plain particles, in a LoBind® eppendorf tube (Eppendorf, UK), and incubated for one hour in a water bath at 37ºC, under agitation. This suspension was centrifuged at 7,500 rpm for 10 minutes (IEC Micromax eppendorf centrifuge, UK) and the pellet, after being washed two times, was dried in a dessiccator, while supernatant and washes were kept frozen at -20ºC until future analysis.

S. equi antigen-adsorbed or -entrapped particles were administered to animals as described elsewhere [20].

Characterisation of PLA nanospheres

The volume mean diameters (VMD) of the *S. equi* antigens-adsorbed or -entrapped PLA nanospheres were determined in double distilled and filtered water (0.2 µm Whatman filters) by photon correlation spectroscopy (PCS; Malvern ZetaSizer, Malvern Instruments, UK). The zeta potential (mV) of particles dispersed in a 10 mM potassium chloride solution (Sigma Aldrich Co., UK) was assessed using the same equipment.

The morphology of the nanospheres was examined and photographed using a scanning electron microscope (SEM, Phillips/FEI XL30 SEM), after samples have been sputter coated (Emscope SC500) with gold as described before [21].

Determination of antigen loading

To evaluate the amount of *S. equi* antigens entrapped or adsorbed on PLA nanospheres, a known amount of antigen loaded particles (10 mg) was accurately weighed and dissolved in 0.1 M sodium hydroxide (NaOH, Fisher Co. UK) solution, containing 5% (w/v) sodium dodecyl sulphate (SDS, BDH UK), under magnetic stirring at room temperature. A bicinchoninic acid protein assay (BCA™ or Micro BCA™ Protein assay kit, Pierce, USA) was used to determine the *S. equi* extract proteins concentration. From these results, the percentage (w/w) of total protein entrapped or adsorbed per dry

weight of nanospheres (*S. equi* extract proteins loading capacity (L.C.)) was determined as described elsewhere [20].

IN VITRO RELEASE STUDIES OF *S. equi* EXTRACT PROTEINS FROM PLA NANOSPHERES

The *in vitro* release study of *S. equi* antigens from loaded PLA nanospheres was carried out, in triplicate, by placing accurately weighed amounts (10 mg) of these particles into LoBind eppendorf tubes (three per time point, Eppendorf, UK) containing 2 ml of PBS buffer (pH 7.4), 5% (w/v) of SDS (Sigma Aldrich, Co. UK) and 0.02% (w/v) of sodium azide. The tubes were incubated in a water bath at 37°C, under continuous shaking. At selected time intervals, three eppendorf tubes were collected and centrifuged at 7,500 rpm for 10 minutes (IEC Micromax eppendorf centrifuge, UK). The amount of protein released was dosed by BCA™ and Micro BCA™ protein assays.

STRUCTURAL INTEGRITY AND ANTIGENICITY OF *S. equi* ANTIGENS

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The molecular weight integrity of adsorbed and entrapped *S. equi* antigens extracted as mentioned in a previous report [21], native *S. equi* enzymatic extract proteins and molecular weight (MW) reference marker (MW 6.0-181.8 kDa, Invitrogen UK) were determined by polyacrylamide gel electrophoresis (PAGE) under denaturating conditions. These samples were loaded onto 12% (w/v) polyacrylamide (Invitrogen, UK) mini-gel and run using a Bio-Rad 300 Power Pack Electrophoresis system (Bio-Rad, Hercules, CA, USA). Proteins were visualized by Coomassie blue staining (SimplyBlue™ SafeStain solution, Invitrogen, USA).

WESTERN-BLOT

Western blotting [22] was used to evaluate the antigenicity of both entrapped and adsorbed *S. equi* enzymatic extract antigens in PLA nanospheres. Antigen

samples were transferred from the SDS-PAGE gel and treated as described earlier [20].

PLA NANOSPHERES *IN VITRO* CELL VIABILITY ASSAY

BALB/c monocyte macrophages (J774A.1 cells line, American Type Culture Collection; ATCC#TIB-67) were plated in 96-well tissue culture plates (Fisher, UK) and cytotoxicity of freeze-dried PLA nanospheres was evaluated, in triplicate, by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; [23]) method as previously performed, using polyethyleneimine (PEI) as a positive control [21]. Samples were prepared by particles suspension, at different concentrations (0.01-2.5 mg/ml), in Dulbecco`s modified Eagle`s media (DMEM, Sigma Aldrich Co., UK), supplemented with 4 mM glutamine (Sigma Aldrich Co., UK) plus 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich Co., UK).

IMMUNE RESPONSE

Four female BALB/c mice per group were vaccinated intranasally, into the two nostrils, with *S. equi* antigens equivalent to 10 µg of SeM per dose, in 50 µl of nanospheres suspensions. One group received unadjuvanted antigens, while other mice were vaccinated with *S. equi* antigens associated to the mucosal adjuvant CTB (10 µg), by the same route of administration. The immune response elicited after i.n. administration of plain particles was as well evaluated. Details of immunogens are given in Table 5.2 and all mice were anesthetised (3% of isoflorane) prior immunisation. Regardless samples composition, all doses were freshly prepared in sterile PBS (pH 7.4), under aseptic conditions, just before animal dosing and boosting, respectively, on day one and 21.

Table 5.2
Immunisation study design

GROUPS	FORMULATION	
1	Adsorbed	PLA-PVAads.
2		PLA-GCSads.
3		PLA-ALGads.
4		PLA-PVAads.+CTB
5	Entrapped	PLA-PVA
6		PLA-GCS
7		PLA-ALG
8		PLA-SP
9		PLA-OA
10		PLA-PVA+CTB
11	FREE antigens	
12	FREE antigens + CTB	

BALB/c female mice 6-8 weeks old were vaccinated intranasally at weeks 0 and 3. Tail veins blood samples were collected at weeks 2, 4, 7 and 12 for antibody titres analysis by enzyme-linked immunoassay. After 12 weeks of study, animals were ethically sacrificed and lung and gut washes were performed at the end of the study to assess the local antibodies (SIgA) levels, by ELISA. Spleens were aseptically removed and splenocytes were co-stimulated with soluble antigen in order to estimate cytokine concentrations.

Blood samples were collected from the tail vein after 2, 4, 7 and 12 weeks of immunisation and sera separated by centrifugation (15,000 rpm, 20 min at 4^{o}C) and stored at -20^{o}C until tested by antigen specific ELISA for IgG, IgG subclass 1 (IgG1) and IgG subclass 2 (IgG2a).

Lung and gut washes were collected according to the method previously reported [20]. Briefly, a gut fraction was removed, longitudinally sectioned and scraped into an ice-cold solution (1mM iodoacetic acid, 0.1% (v/v) trypsin inhibitor soybean type 1 and 10 mM EDTA sodium salt). Samples were vortexed to disrupt all solid material, centrifuged at 20,000×g for 30 min at 4^{o}C, and clear supernatants were freeze-dyed (Virtis Advantage freeze dryer, UK). On the other hand, lung washes were collected by trachea cannulation of sacrificed mice. Trachea and lungs were flushed three times with 5 ml of lavages solution (1mM PMSF, 0.9% (w/v) sodium chloride, 0.5% (v/v) tween 20 and 0.1% (w/v) sodium azide), which were immediately removed and similarly treated until tested by ELISA for secretory antibody

(SIgA) levels. Before lyophilised, washes were stored at -70°C with PMSF as a protease inhibitor. Lung and gut lavages were reconstituted with PBS (pH 7.4) just before ELISA analysis.

QUANTIFICATION OF HUMORAL (IgG AND IgG SUBTYPES) AND MUCOSAL (IgA) ANTIBODY IMMUNE RESPONSES

S. equi specific IgG, IgG1, IgG2a and IgA antibodies, respectively in serum samples and in lung/gut washings were detected by ELISA following a protocol previously referred [21]. In brief, 96-well plates (Immulon 2, flat bottom plates, Dynatech, UK) were coated overnight with 1.5 µg/ml *S. equi* M proteins in PBS solution (pH 7.4) at 4°C, washed with PBS (pH 7.4) containing 0.05% of tween 20 and 0.1% of BSA fraction V, and then blocked with 2% (w/v) BSA solution (Sigma Aldrich Co., UK) in PBS (pH 7.4).

The serum samples were 1:32 diluted in PBS (pH 7.4), and 100 µl was added in quadruplicate to the wells of coated plates and serially diluted two fold in the same buffer. Detection was performed using horseradish peroxide conjugate goat anti-mouse IgG (1:1000), IgG1 and IgG2 (1:2000; Serotec, UK) diluted in PBS (pH 7.4), and 2, 2'- azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [ABTS] (Sigma Aldrich Co., UK) in citrate buffer was used as enzyme substrate. The colour development was stopped with H_2SO_4 after 5 minutes and optical density (OD) values were read at 405 nm. Negative control sera obtained from mice prior the beginning of this study were included in each plate and the reciprocal of the dilution, at which mean OD was 5% higher than the negative control, was used to compare titres.

For analysis of lung and gut washes, the ELISA method was essentially the same, with some exceptions. After freeze-drying, proteins were reconstituted with 500 µl of PBS (pH 7.4) and directly added to the plate wells. The horseradish peroxide conjugate goat anti-mouse IgA (1:2000; Serotec, UK) was used to detect this antibody, and ABTS substrate was then added as

mentioned above. The mean OD was determined for each treatment group and directly used to compare mucosal response.

Splenocyte culture studies

After 12 weeks of study, animals were ethically sacrificed and their spleens aseptically removed as mentioned elsewhere [21]. Briefly, spleens were homogenised in working media RPMI 1640 (Gibco, UK), supplemented to a final concentration of 10 % (v/v) fetal bovine serum (FBS, Gibco, UK), 20mM L-glutamine (Sigma Aldrich Co., UK), 105U/l of penicillin and 100 mg/l of streptomycin (Sigma, Poole, Dorset, UK). Homogenates were centrifuged at 200 ×g for 10 min, cells were resuspended in the above mentioned working media, plated in 96-well tissue culture plates (Fisher, UK), along with 2.5 µg/ml soluble *S. equi* enzymatic extract proteins, and incubated for 48 hours at 37^{o}C in a humidified incubator at 5% CO_2. Cell supernatants were used for estimation of interferon-γ (IFN-γ) interleukine-2 (IL2), interleukine-4 (IL4) and interleukine-6 (IL6), by using separate ELISA Kits (DuoSet® ELISA Development kit, R&D Systems Europe, UK).

Statistical analysis

Statistical analysis was performed on data obtained in the *in vitro* and *in vivo* studies by one-way analysis of variance (ANOVA), using SPSS software (Version 13, Microsoft), followed by LSD post hoc test if ANOVA results were found significant (P<0.05).

Results and Discussion

PLA nanopsheres physicochemical characteristics

PLA particles were spherical, with no cavities and presented a smooth surface, as it can be seen in Figure 5.1.

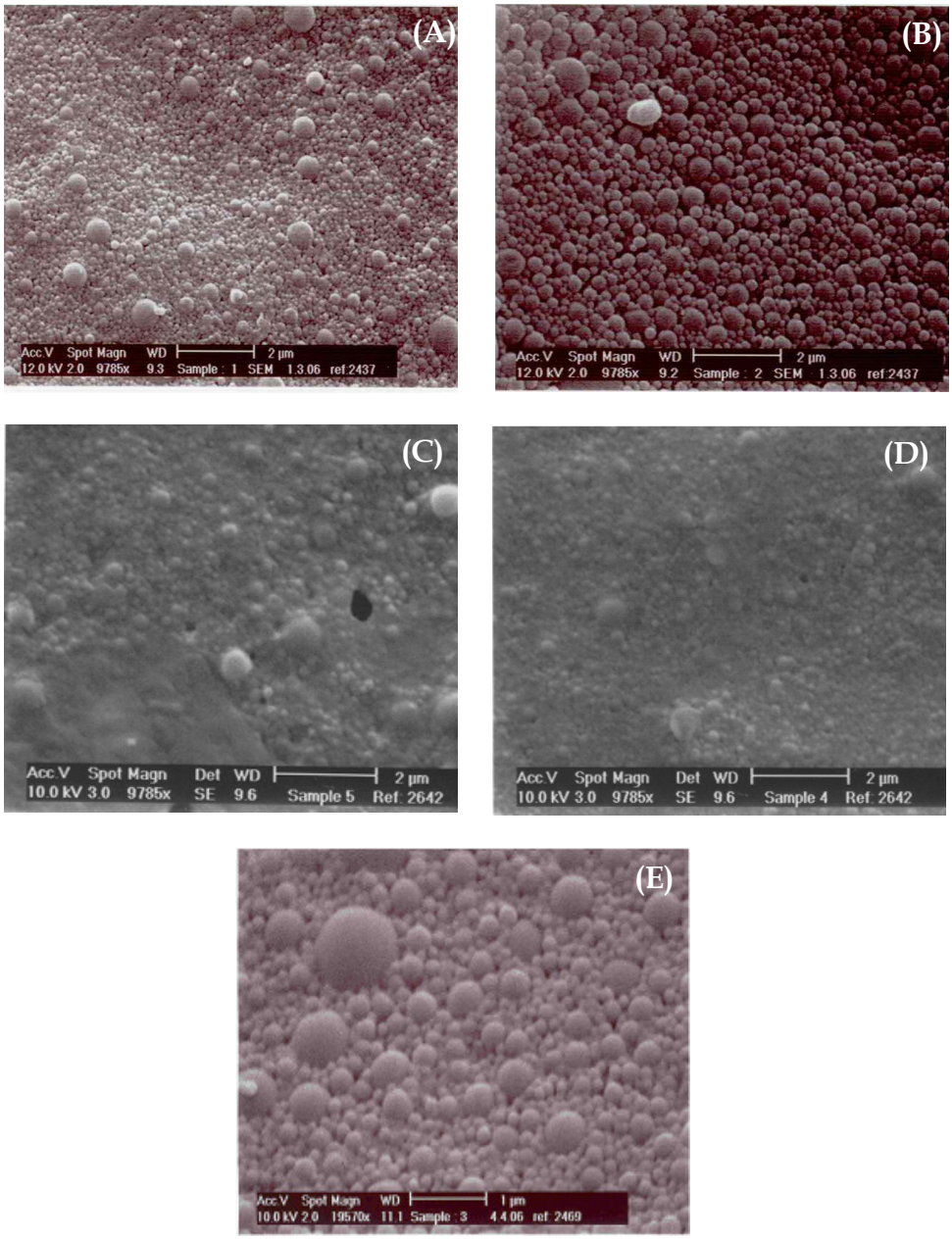


Figure 5.1 – SEM photographs of (A) PLA-PVA, (B) PLA-GCS, (C) PLA-SP, (D) PLA-OA and (E) PLA-ALG nanospheres.

No differences were observed between the surface morphologies of particulate formulations prepared in the present study. PLA low molecular weight solubility in organic solvents, as DCM, and water is higher when compared to higher molecular weight polymers, which reduces particle precipitation after organic solvent evaporation. SEM confirms PLA

nanospheres formation and that their shape was maintained with no detectable aggregation.

The small differences obtained in size and surface charge between the different batches of nanospheres demonstrates the good reproducibility of the production method used (Table 5.3). The size of *S. equi*-adsorbed PLA nanospheres was found to increase from 255.8 ± 10.0 nm for PLA-PVA, to 407.1 ± 56.98, for those formulated with GCS. *S. equi* entrapped-PLA particle size ranges from 205.47 ± 35.99 to 396.0 ± 36.11, being the smallest and highest obtained for PLA-OA and PLA-GCS formulations, respectively. When GCS was added to the inner phase, it was observed an increase in solution viscosity which led to larger emulsion droplets formed during homogenisation, and therefore may justify the difference obtained in particle size. As a result, despite differences in particles diameters, all formulations are suitable for APCs uptake after i.n. administration [24].

All particles present a negative surface charge before and after whichever protein association method used, with an exception for that containing the positively charge polysaccharide (GCS). Even so, PLA-GCS nanospheres zeta potential decreased after *S. equi* protein adsorption, which allows predicting that adsorption actually occurred and consequently the mixture of proteins used seems to have a net negative surface charge.

Chitosan (CS) is one of the few polycations naturally available, which may be advantageous relatively to others vectors, as liposomes, due to its minimal toxicity. In the present study, a CS derivate was chosen for the formulation of positively surface charged PLA nanospheres, as lysozyme used for extract production has the ability to degrade this natural polymer. A previous study made by our group had shown that the dissolution of GCS and *S. equi* enzymatic extract protein did not result in any destabilisation of the inner phase. In addition, GCS kept the biodegradability and biocompatibility attributed to CS, but its solubility in a wide range of pH aqueous solutions overcomes CS low solubility at basic pH values [25].

Table 5.3
In vitro characteristics of *S. equi*-adsorbed and -entrapped PLA nanospheres prepared by double emulsion (w/o/w) solvent evaporation technique.

FORMULATION		VMD[a] (nm)	ZETA POTENTIAL [b] (mV) BP	AP	L.C. [c] (%W/W)
Ads.[d]	PLA-PVA	255.8 ± 10.00	-30.4 ± 7.19	-29.6 ± 8.95	10.32±0.32
	PLA-GCS	407.1 ± 56.98	+36.4 ± 5.03	+5.17 ± 2.05	10.83±0.25
	PLA-ALG	346.7 ± 18.50	-41.3 ± 1.55	-39.63 ± 1.07	10.11±0.60
Entrapped	PLA-PVA	281.1 ± 17.30	-33.7 ± 6.82	-31.63 ± 8.95	6.04±0.24
	PLA-GCS	396.0 ± 36.11	+ 37.1 ± 3.43	+31.83 ± 3.10	7.16±0.56
	PLA-ALG	327.0 ± 57.78	-40.7 ± 2. 23	-50.10 ± 4.49	5.56±0.10
	PLA-SP	267.9 ± 16.41	-22.9 ± 4.45	-24.50 ± 2.95	5.37±0.49
	PLA-OA	205.47 ± 45.99	-28.33 ± 8.09	- 29.30 ± 7.82	7.84±0.27

[a]Particle size as volume mean diameter (VMD) expressed as mean ± S.D. (n=6);
[b]PLA nanospheres surface charge, before (BP) and after (AP) protein association (mean ± S.D. (n=6)).
[c]Loading capacity (L.C.): represents the mean percentage of actual amount *S. equi* extract proteins content ± S.D. (n=6).
[d]Ads.-adsorbed.

As shown in Table 5.3, particles produced by adsorption procedures showed higher antigen loadings than *S. equi*-entrapped PLA nanospheres, with no significant difference in the mean particle diameter. Among adsorbed formulations, PLA-GCS nanospheres presented a similar L.C. when compared to those formulated with PVA (PLA-PVA) or alginate (PLA-ALG), supporting the idea that charge and size do not have an important influence in the adsorption of the *S. equi* protein extract.

L.C. varied from 5.37 % (w/w) on PLA-SP nanospheres, to 7.84 % (w/w) for PLA-OA nanoparticles. Interestingly, OA may have increased the stability of the primary emulsion, resulting in the highest L.C. of all *S. equi*-entrapped polymeric nanospheres studied, besides its lower mean diameter (Table 5.3). Higher antigen loading may be a result of organic phase viscosity increase, which prevents protein migration from the aqueous to the external phase.

S. equi-entrapped PLA nanospheres were easily dispersed in PBS (pH 7.4), especially PLA-SP and PLA-ALG, and therefore it was possible to efficiently administer higher amounts of these particles during immunisation studies. Nevertheless, Johansen *et al*. (2000) demonstrated that the number of particles

administrated is not a limiting factor for the stimulation of the immunological response [26].

For the antigen-entrapped nanospheres to function as controlled release delivery systems for the generation of long lasting immune responses, they should not only contain suitably high antigen levels, but also maintain their antigenicity [27]. Accordingly, the molecular weight and structural integrity of entrapped and adsorbed *S. equi* antigens were studied by SDS-PAGE and their antigenicity was confirmed by western-blot analysis. Despite the relative chemical and physical harsh conditions imposed to antigens during the entrapment method, the structural integrity, antigenicity and immunogenicity of the proteins were conserved, including SeM (58kDa), (Figures 5.2 and 5.3), confirming the potential of these particles for immunisation studies. The adsorption process not only avoids the harsh entrapment process, but also extensively increases the amount of protein associated to the PLA nanospheres. This is even more important for *S. equi* enzymatic extract, as it contains a mixture of several proteins wherein only 2.70 % (w/w) is attributed to SeM [21, 28].

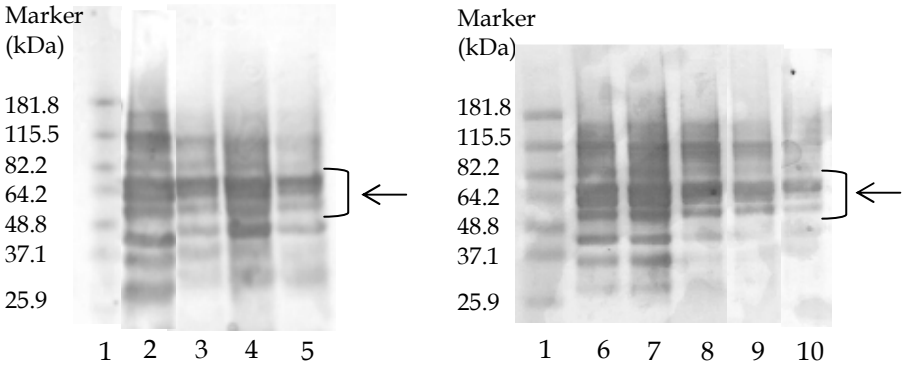


Figure 5.2 - SDS-PAGE (12% gel) analysis of *S. equi* enzymatic extract protein-entrapped or -adsorbed PLA (MW 2 kDa) nanospheres. Lanes: 1) Standard markers; 2) Plain *S. equi* antigens at 1250 µg/ml (control); *S. equi* antigens adsorbed onto 3) PLA-PVA, 4) PLA-GCS and 5) PLA-ALG nanospheres; and encapsulated in 6) PCL-PVA, 7) PCL-GCS, 8) PCL-OA, 9) PCL-SP and 10) PCL-ALG nanospheres.

Figures 5.2 and 5.3 suggest that *S. equi* extract proteins were successfully adsorbed onto PLA nanospheres without changes in molecular stability and antigenic components of the extract, which can be seen by the absence of alterations in the pattern of migration and proteins reaction with specific anti-SeM antibody, being a potential vaccine adjuvant for these antigens.

Our research group has showed that the double emulsion solvent evaporation technique has not affected the activity and immunogenicity of *S. equi* antigens incorporated in the internal phase [20, 21, 29].

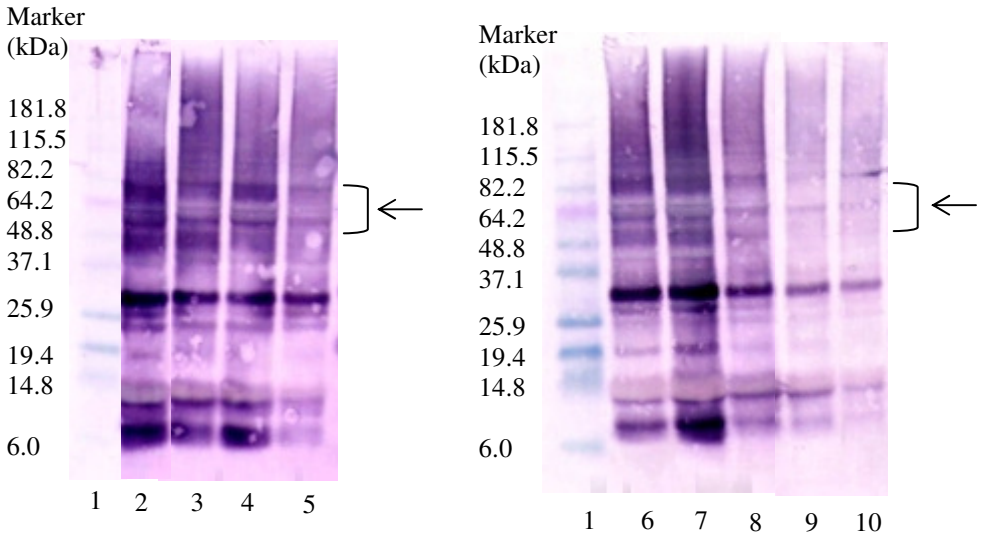


Figure 5.3 - Western blot analysis showing immunoreactivity of *S.equi* antigens adsorbed and entrapped in PLA (MW 2.0 kDa) nanospheres with anti-SeM antibody as described in Materials and Methods section. Lanes: 1) Standard markers; 2) Plain *S. equi* antigens at 1250 µg/ml (control); *S. equi* antigens adsorbed onto 3) PLA-PVA, 4) PLA-GCS and 5) PLA-ALG nanospheres; and entrapped in 6) PLA-PVA, 7) PLA-GCS, 8) PLA-OA, 9) PLA-SP and 10) PLA-ALG nanospheres.

Polymeric composition, particle size, antigen loading, route of administration and their association with other adjuvants, are the main characteristics that influence the interaction with mucosal tissues, particles uptake and their subsequent transport to the lymphatic nodes [26, 30]. Among all of these physicochemical characteristics, size seems to be the main parameter, although it is possible to find contradictory results in the literature. Particle size affects deposition in the nasal cavity and uptake by the mucosa. It is general agreed that particles with mean diameters less than 10 µm are readily

taken up by cells, whereas those in the nanometric range are more efficiently absorbed by lymphoid tissue associated to the nasal mucosa (NALT) [31]. Particles with mean size < 500 nm were found to indeed induce higher immune responses than larger carriers (> 2 μm) [32]. As a result, particulate form, size and successful association to *S. equi* antigens predict that these PLA nanospheres may be extensively taken up by nasal mucosa M cells, as well as by dendritic cells or macrophages. In addition, its antigen depot effect will enhance the processing and presentation of associated *S. equi* antigens, thereby inducing higher immunological responses [31].

In order to increase particles stability and prevent their rapid clearance from the organism, recent approaches involve particle surface modification with hydrophilic polymers, such as CS and ALG. For example, it has been observed an increase in the immune response when the surface of PLGA nanospheres was modified by CS, consequence of its mucoadhesivity. This positively charged particles surface seem to favour interaction between particles and cell membranes [15].

Figures 5.4 and 5.5 show *in vitro* release profiles of *S. equi* antigens adsorbed and entrapped in PLA nanospheres, respectively. It was found 49.25±1.37%, 45.00±1.31 %, 53.83±1.07 %, 41.76±0.368 %, and 50.63±2.60 % burst release (1 day) of *S. equi* extract proteins entrapped in PLA-PVA, PLA-SP, PLA-ALG, PLA-GCS and PLA-OA nanospheres, respectively and tends to a plateau after 10 days, occurring a prolonged antigen release. This burst release has been mentioned in the literature for proteins as BSA and OVA entrapped in PLA low molecular weight nanospheres, which increases with the decreasing of polymer molecular weight [33].

Despite this rapid degradation, PLA-GCS nanospheres presented the slowest release (P<0.04) of the entrapped *S. equi* antigens, which may be due to its higher mean diameter or, most probably, to the stronger interaction established between the positive charge of the surface and proteins. It was interesting to verify that PLA-SP nanospheres, regardless of presenting one of

the lowest particle diameters and having a high hydrophilicity, slowly released *S. equi* antigens.

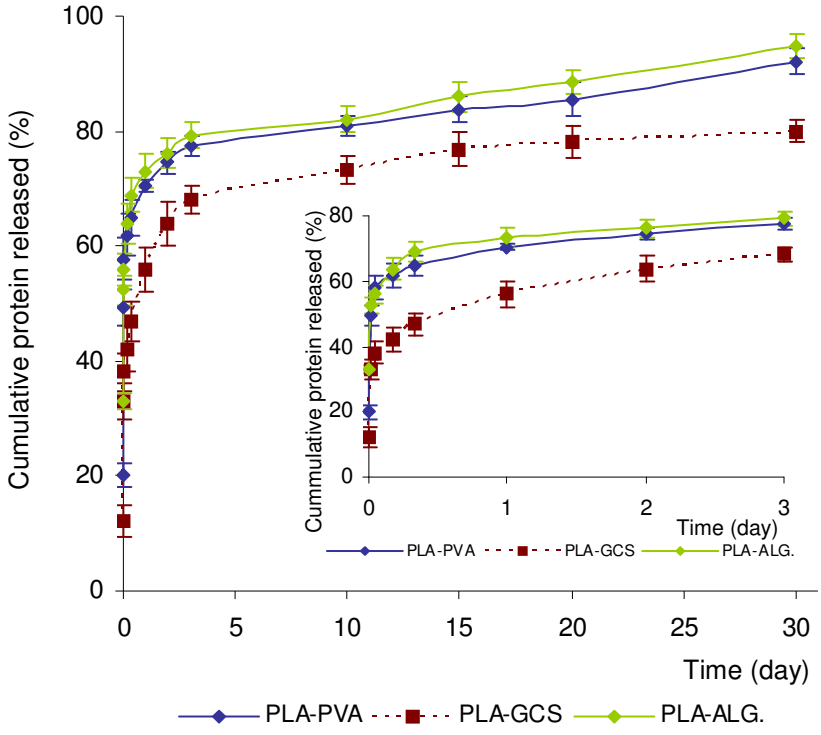


Figure 5.4 - *In vitro* cumulative release of *S. equi* antigens adsorbed onto different PLA-based nanospheres, for a period of 30 days (mean ± S.D.; n=3).

Even 30 days after PLA particles incubation in the release media, 20-34% of the entrapped *S. equi* extract antigens were still associated to these carriers (Figure 5.5).

The prolonged release of the entrapped or adsorbed antigens is vital in order to preserve a considerable amount of antigen in the particles when achieving the APCs, as it is well accepted that the higher amount of antigen in particles elicits longer immune responses and lymphocytes T activation [34].

As it was expected, *S. equi*-adsorbed PLA nanospheres quickly released antigens, but remain potential vectors for nasal immunisation as after 4 hours there was still 36-58% of the protein adsorbed onto particles surface (Figure 5.4). Even so, this study confirmed the stronger interactions established

between PLA-GCS surface and the *S. equi* protein extract, as this system achieved a more prolonged release profile.

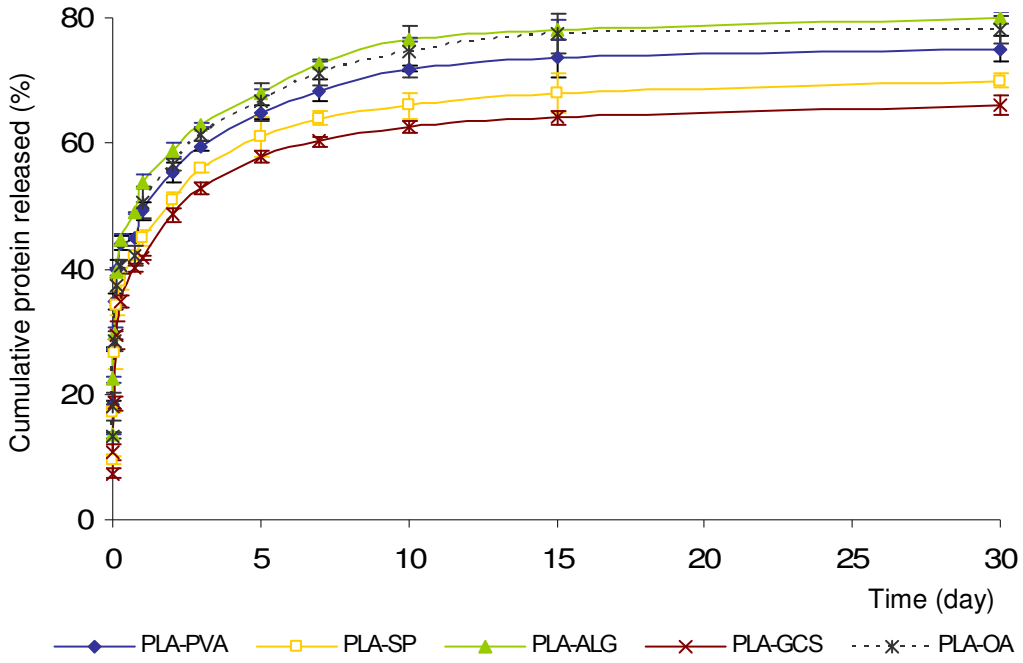


Figure 5.5 – *In vitro* cumulative release of *S .equi* antigens entrapped in different PLA-based nanospheres, for a period of 30 days (mean ± S.D.; n=3).

An interesting approach would be the adsorption and entrapment of these *S. equi* antigens in the same polymeric system, in order to have a higher amount of protein released just after their contact with APCs and simultaneously maintain a prolonged release, contributing for the renovation of major histocompatibility complex (MHC) molecules at APCs surface and therefore guarantee a continuous activation of cells of the immune system.

PLA NANOSPHERES *IN VITRO* CELL VIABILITY ASSAY

Figure 5.6 shows that all PLA formulations did not have cytotoxic effects to BALB/c cells (J774A.1 cell line), which remained more than 90.8 % viable when compared to control, at concentrations as high as 2.50 mg/ml. Furthermore, these cells viability was significantly higher than that presented

by cells treated with PEI (P<0.01). Among PLA formulations, PLA-ALG led to the highest cell viability.

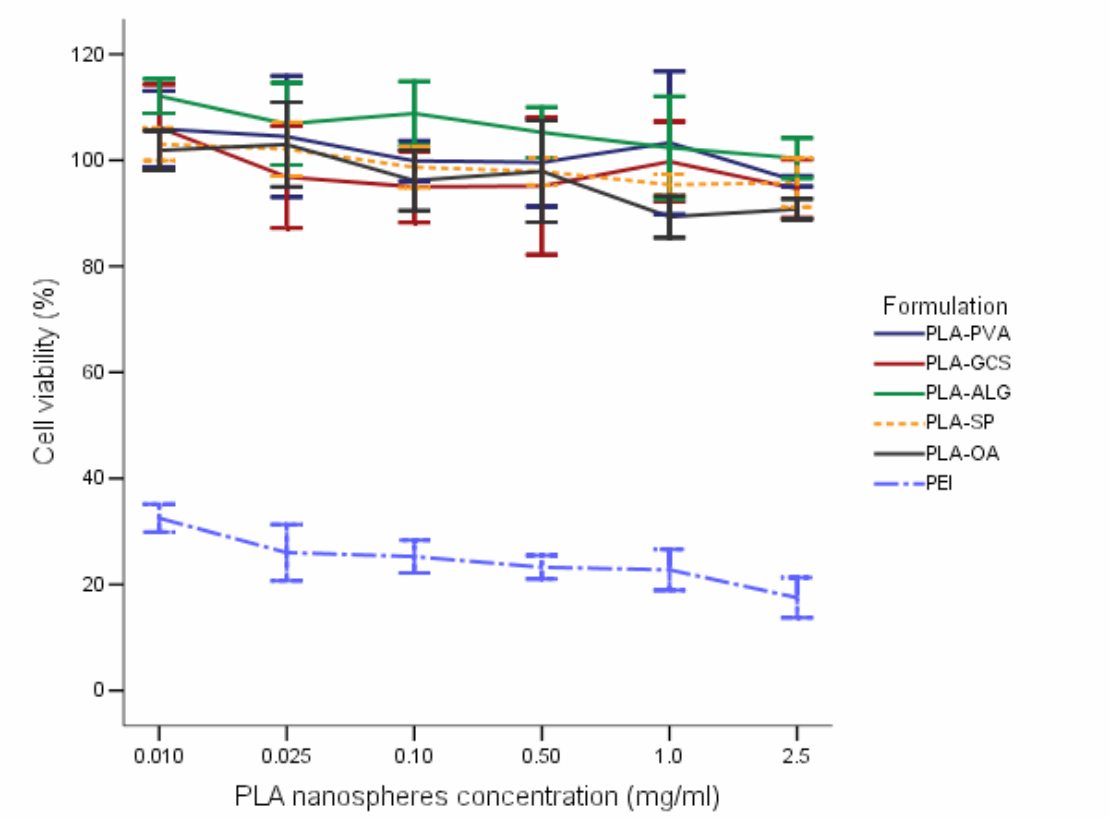


Figure 5.6 - Viability of mouse BALB/c monocyte macrophage cells (J774A.1 cell line) after incubation with increasing concentration of PLA nanospheres (mean ± S.D.; n=3).

SYSTEMIC IgG ANTIBODY IMMUNE RESPONSE

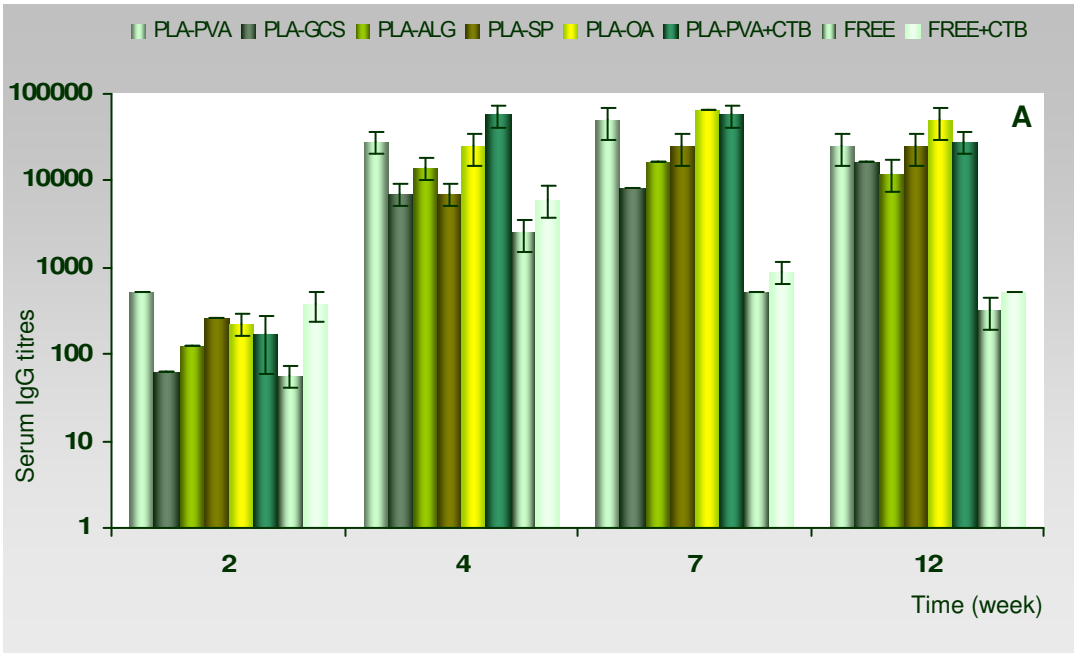
Controlled release of *S. equi* antigens adsorbed and entrapped in PLA carriers resulted in high, sustained levels of *S. equi*-specific IgG antibodies at all time points after boosting (Figures 5.7A and 5.8A). Empty nanospheres induced a baseline humoral immune response, not statistically different from antibody levels observed for the negative control (results not shown). Free *S. equi* antigens delivered in PBS (pH 7.4) resulted in lower levels of anti-*S. equi* IgG antibodies when compared with other groups, being this difference observed 7 weeks after dosing statistically significant when compared with IgG titres induced by all adsorbed particulate systems (P<0.041) and *S. equi* antigens-entrapped PLA nanospheres studied (P<0.036).

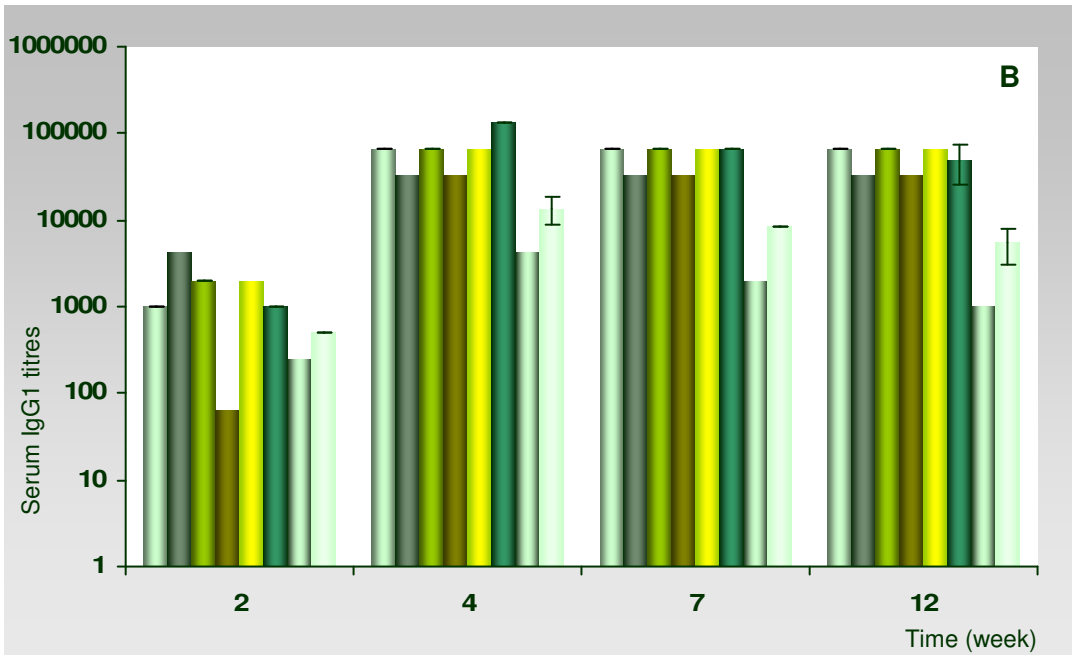
The co-administration of polymeric particles and other adjuvants has been proposed and studied by some investigators in order to increase particles adjuvant activity [35, 36]. Several studies have showed that CTB by itself strongly induces mucosal immune response, but its adjuvant activity was not observed when associated to total antigen extracts from several microorganisms [37, 38]. When *S. equi* was associated to CTB, it was observed an increase in the humoral response, however not as high as IgG antibodies elicited by PLA carriers 7 weeks after animal vaccination (P<0.039).

Anti-*S. equi* IgG levels induced by PLA-PVA+CTB were statistically different from those assessed in serum of mice vaccinated with either antigen-entrapped PLA-ALG (P=0.025) and PVA-OA (P=0.01) nanospheres, or any of the adsorbed particulate systems (P<0.048). On the other hand, *S. equi* antigens adsorbed onto PLA-PVA and co-administrated with CTB induced *S. equi* specific IgG antibodies markedly higher than any of the formulations tested (P<0.025), excepting IgG serum titres elicited by PLA-PVAads and PLA-GCSads systems. In fact, these latter particulate systems, together with *S. equi*-entrapped PLA-OA nanospheres, induced the highest IgG antibodies 12 weeks after i.n. administration, which was statistically different from all other antigen-associated nanospheres (P<0.01).

It is important to point out that boosters elicited a significant increase in antibodies levels, which was maintained at the end of the study by all formulations, with an exception for PLA-GCS, PLA-SP and PLA-OA systems, as it induced a gradual increase during this trial period. These results are in agreement with the *in vitro* release profiles, especially for the GCS adjuvanted nanospheres as this formulation has showed the slowest release rate during that study. Likewise, over the end of the trial period, positively charged nanospheres adsorbed with *S. equi* antigens induced IgG titres higher than those elicited by PLA-ALGads (P=0.008), while not being statistically different from the levels observed for both PLA-PVAads, alone or associated to CTB adjuvant. These observations are similarly related with the *S. equi* proteins

release profile from the surface of these positively charged nanospheres. Even so, comparing *S. equi*-entrapped and -adsorbed PLA carriers, it seems that the latter elicited a higher *S. equi* specific humoral immune response, which can be due to the higher amount of *S. equi* antigens that has reached the APCs. As a result, besides PLA-GCS higher surface positive charge, when compared to the moderate PLA-GCSads charge obtained after proteins adsorption, latter formulation induced statistically higher IgG antibody levels even 9 weeks after animals boosting.





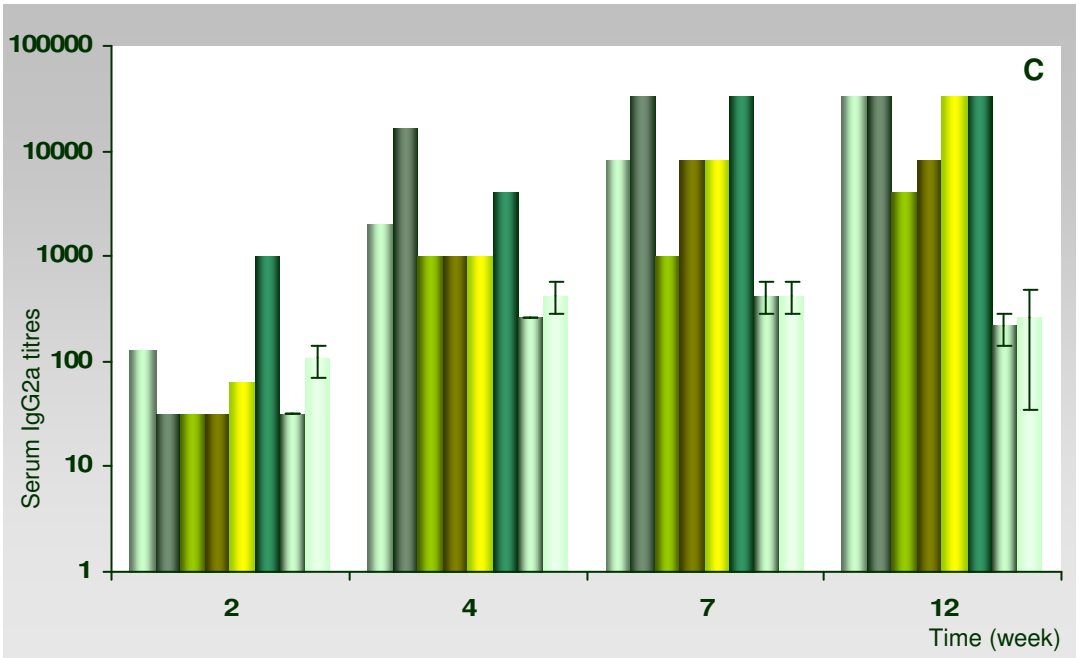


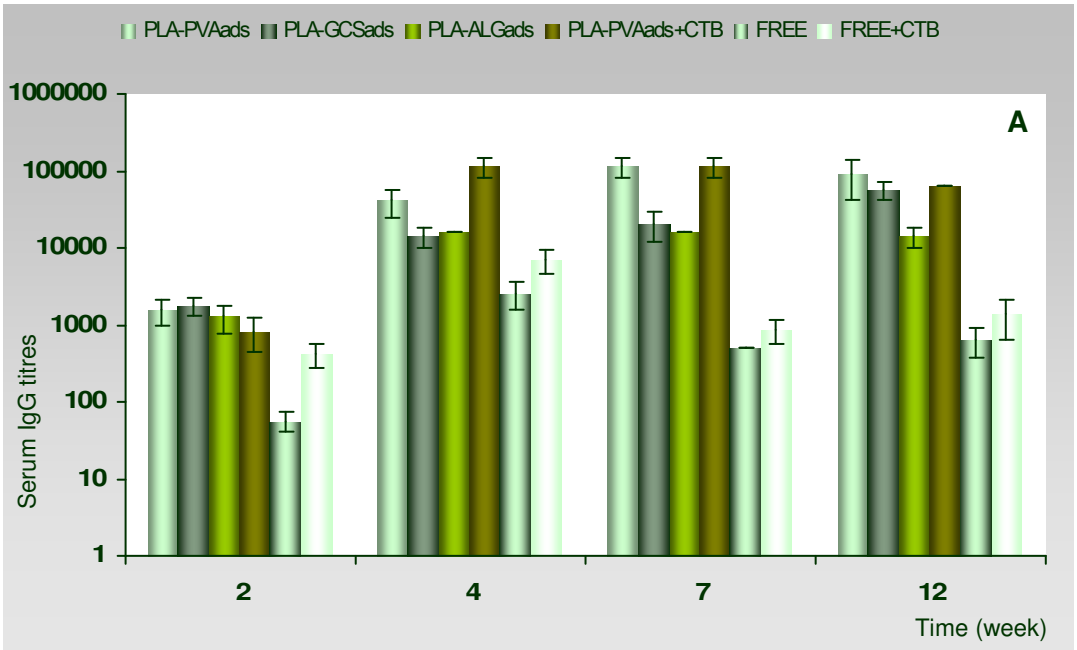
Figure 5.7 - Serum anti-*S. equi* specific IgG (A), IgG1 (B) and IgG2a (C) profiles of mice immunised by i.n. route with different formulations of *S. equi* antigens encapsulated in PLA nanospheres, compared to the free antigen (FREE) and co-administered with CTB (FREE+CTB) immunisations (mean ± S.D.; n=4).

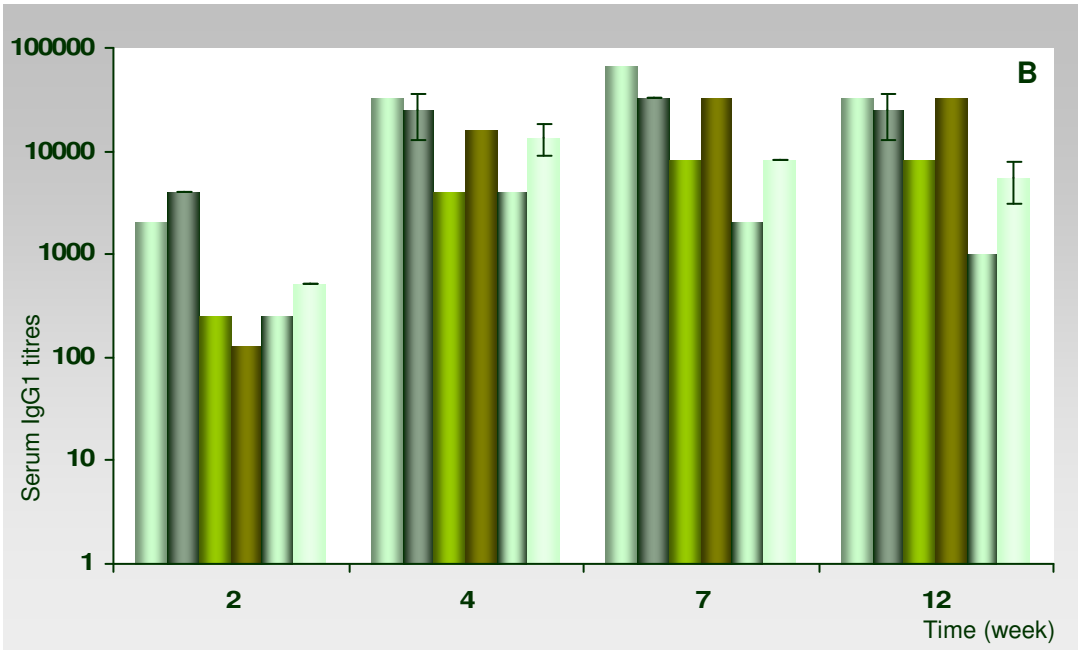
CS has the ability to increase antigen bioavailability due to its mucoadhesion and capacity to open tight junctions between cells, promoting the paracellular transport [39, 40]. In fact, at neutral pH the mucus layer is negatively charged and therefore positively charged particles can interact with mucus, decreasing particle clearance from nasal mucosa [15].

Several studies have confirmed that PLA and PLGA particles are potential carriers for adsorbed or entrapped proteins [27], being able to preserve stability and immunogenicity, in order to stimulate a higher immune response able to be maintained for prolonged periods of time, and therefore avoiding administration of several vaccine doses [34, 41].

By IgG titres assessed in the sera of mice treated with PLA nanospheres associated to *S. equi* enzymatic extract antigens, it seems that the nasal administration of these vehicles, mainly those with adsorbed antigens, contributes to improve uptake and processing of antigens by APCs, as well as more efficiently deliver to peripheral lymph nodes. Comparing these humoral

responses with those reported in a previous study using PCL nanospheres, it is possible to see that the IgG levels at the end of the study were higher for PLA nanospheres, although statistically different only when PCL-PVAads and PCL-GCSads titres are compared with those elicited by PLA-PVAads (P=0.033) and PLA-GCSads (P=0.008), respectively [20].





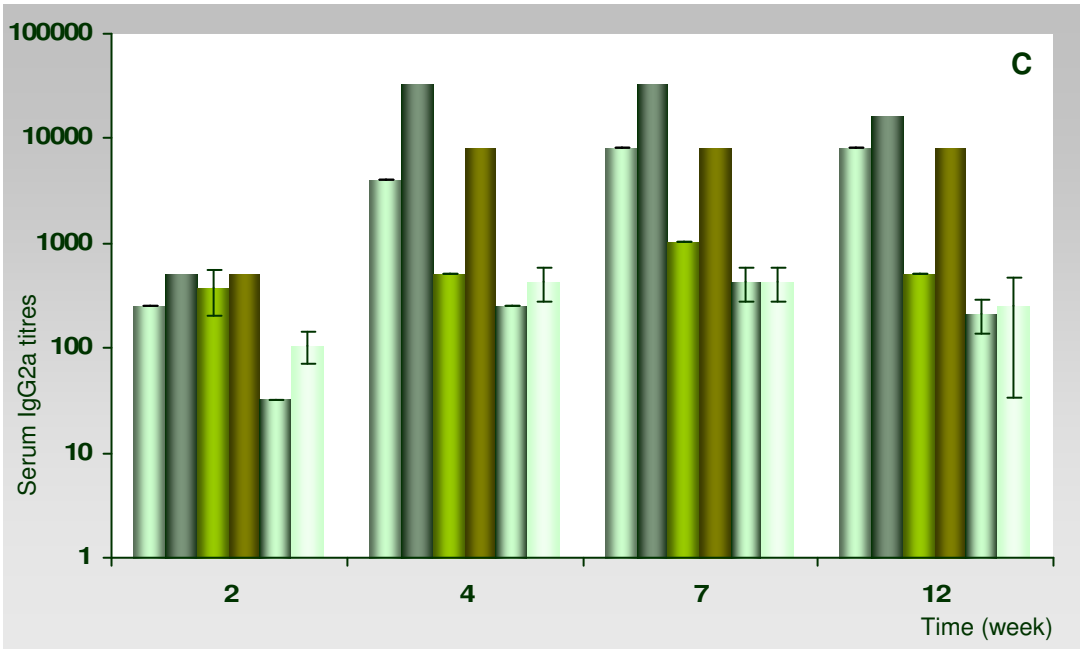


Figure 5.8 - Serum anti-*S. equi* specific IgG (A), IgG1 (B) and IgG2a (C) profiles of mice immunised by i.n. route with different formulations of *S. equi* antigens adsorbed onto PLA nanospheres, compared to the free antigen (FREE) and free co-administered with CTB (FREE+CTB) immunisations (mean ± S.D.; n=4).

Since particles were formulated with the same adjuvants, using the same method of production and presented a similar diameter, it seems that the difference observed might be due to their main polymeric composition and its surface interaction with the *S. equi* extract. *In vitro* release studies have shown a faster release of *S. equi* proteins from PLA nanospheres when compared with the release profile observed for *S. equi* proteins desorption from PCL nanospheres, resulting in a difference of approximately 10% of protein released 30 days after particles incubation in the media. This higher amount of antigen released during this period may be responsible for the stronger immune response obtained in animals treated with these nanospheres. On the other hand, even if *S. equi*-entrapped PCL nanospheres induced lower IgG titres during the first weeks of the study, the total systemic antibody levels elicited by *S. equi*-entrapped PLA nanospheres started to decrease after 12 weeks, in contrast with the humoral immune response obtained with the PCL nanospheres. In fact, at the end of the study, there was a significant difference between the IgG titres induced by PLA-OA *versus* PCL-OA (P=0.008) and also

PLA-ALG *versus* PCL-ALG (P=0.033) nanospheres. These results are in agreement with the predicted slower and more prolonged antigens release from PCL particles due to its higher molecular weight (42.5 kDa vs. 2 kDa), which is reinforced by the *in vitro* release profile obtained in both situations [20]. A detailed difference in the immune response induced by both type of polymeric particles can be more specified by the analysis of IgG subtype antibodies.

IgG SUBTYPE PROFILING

To assess the predominance of Th1 or Th2 T cell immune response to *S. equi* delivered in the two forms of polymeric carriers, the levels of both IgG isotypes were determined, being the IgG2a isotype more related to a Th1 response, whereas IgG1 isotype antibodies are predominant in a Th2 pathway.

Intranasal vaccination with antigen-loaded PLA nanospheres resulted in strong IgG1 and IgG2a immune responses, in contrast to antigen isolated in its soluble form or co-administered with CTB, being statistically different from 4 weeks of immunisation onwards, with the exception of *S. equi* antigens-adsorbed and -entrapped PLA nanospheres formulated with alginate, (Figures 5.7B, 5.7C, 5.8B and 5.8C). In fact, IgG2a titres induced by antigens-adsorbed and -entrapped PLA-ALG nanospheres, dosed in sera collected 7 weeks after immunisation, were not markedly different from those obtained with *S. equi* proteins in solution co-administered with adjuvant CTB.

The administration of PLA-PVA nanospheres co-admixed to CTB induced IgG1 levels, after 12 weeks of animals vaccination, that were not different from those detected in animals treated with antigen loaded PLA-PVA, PLA-ALG and PLA-OA nanospheres.

PLA-PVAads+CTB particles induced high IgG1 subtype levels, although these were not statistically significant when compared with those resulted from i.n. administration of *S. equi* proteins-entrapped PLA-GCS and PLA-SP

nanospheres. On the other hand, Th1 immune response elicited by antigens adsorbed onto PCL-PVA nanospheres and admixed with CTB was not different from that elicited by these carriers isolated and PLA-SP, being inclusively markedly lower than that induced by the positively charged PLA-GCSads nanospheres (P=0.024), PLA-PVA (P=0.014), PLA-GCS (P=0.014) and PLA-OA (P=0.014) nanospheres. *S. equi* antigens released from PLA-PVA, PLA-GCS and PLA-OA nanoparticulate systems induced a Th1 immune response as high as that obtained in animals dosed with PLA-PVA nanoparticles co-administered with CTB.

It is important to mention that while all nanoparticulate formulations studied induced IgG1 levels essentially similar after boosting, high titres of IgG2a were markedly induced after the second dose of rather antigen-adsorbed and -entrapped positively charged PLA nanospheres, and those were further sustained during all the trial period.

Despite *S. equi* PLA adsorbed nanospheres initially result in elevated Th1 and Th2 responses during the first weeks of this *in vivo* study, it was observed an increase of IgG2a subtype levels determined in mice vaccinated with *S. equi* antigen-entrapped PLA nanospheres later from week 7 to 12, probably due to a lower release of the entrapped antigens. In general, it seems that these particulate systems allowed the development of a more continuous and sustained immune response than the correspondent adsorbed ones.

Overall, all particulate systems with an exception for PLA-ALGads nanospheres were able to induce IgG1 and IgG2a titres statistically higher than the isolated form of the antigen (P<0.002) or associated to CTB adjuvant (P<0.004), which strengths the idea that an i.n. administration of *S. equi* antigens polymer formulated are able to induce both Th1 and Th2 levels, being the prevalence of one dependent on particles composition [42]. Interestingly, the ratio of different anti-*S. equi* IgG subclasses (IgG2a/IgG1), by the end of the trial period, indicated that PLA nanospheres formulated with GCS elicited a more balanced immune response (IgG2a/IgG1= 0.83 for

adsorbed and IgG2a/IgG1=1.0 for entrapped antigen), which was more equilibrated than those induced in a previous work with the correspondent nanospheres composed by PCL polymer (IgG2a/IgG1=0.49). Therefore, these positively charged PLA nanospheres can act as a strong immunostimulator for locally administered vaccines. Moreover, CTB adjuvanted formulation induced an immunity as balanced as that obtained after PLA-OA administration (IgG2a/IgG1=0.5), which highlights the role of OA as a potential mucosal adjuvant.

To put it briefly, the i.n. administration of *S. equi* extract associated to PLA nanospheres resulted in a better immune response than the free extract proteins or plain nanospheres, supporting the role of these particles as an adjuvant for these *S. equi* antigens.

LOCAL IgA IMMUNE RESPONSES

An animal recovering from strangles present specific anti-SeM IgG and IgA antibodies in the serum and nasal secretions, which seems to be vital for its protection against future infections, as they will recognise, attach and inactivate the infectious *S. equi* [3-5].

Although the mechanism responsible for horse protection is not completed understood, the disappointing results obtained with the commercially available vaccines seem to result from their failure in stimulate a local immune response and therefore, to the low levels of IgA and IgG sub-classes founded in the nasopharynx, which are believed to be the protective antibodies similar to those found in animals recovering from a natural infection [3]. In fact, the mucosal immune response is believed to have an important role in the protection of the upper respiratory tract from bacterial infections. In addition, even if the immune response to *S. equi* infection is normally characterised by high levels of specific anti-SeM systemic antibodies, as those obtained by *S. equi*-loaded PLA nanospheres in this experiment, they seem not to be the main responsible for animals protection,

being predominantly the mucosal immune response developed. Therefore, IgA locally produced in the respiratory tract are important for prevention and control of this endemic disease, mainly in their port of entry, being apparently complemented by systemic immune response [3, 4].

In order to further characterise the type of immune response in study, it is then essential to assess the mucosal immunity induced by these *S. equi* particulate loaded systems.

Polymeric particles composition modification is one of the most used alternatives, in order to achieve a specific phagocytosis [43, 44]. After phagocytosis, the intracellular phenomena related to antigen processing and presentation will depend on particle polymeric composition [45]. As a result, biodegradable and polymeric nanoparticles associated to mucoadhesive polymers, as CS [14, 15, 39] or ALG [17], to increase time of residence of particles near the cells, and absorption enhancers, like SP [18, 20, 21] and OA [19, 46], are important strategies to overcome nasal barrier [19]. Indeed, as mucus layer is anionic at a neutral pH value, positively charged groups on particle surface can promote interaction between the mucus and particles, and therefore increase the contact time of these surface modified delivery systems with the nasal mucosa [23, 47].

The SIgA assessed in nasal lavages indicate that *S. equi*-loaded particulate systems, either by adsorption or entrapment, stimulated IgA-secreting cells of the NALT and/or draining lymph nodes, as it was obtained levels of SIgA statistically higher than those secreted in consequence of unentrapped antigen ($P<0.003$), or even of its co-administration with the mucosal adjuvant CTB ($P<0.008$) (Figure 5.9). These observations suggest that a mucosal response in several places distant from the site of immunisation was elicited by i.n. antigen exposure, which is characteristic of i.n. immunisation and actually constitutes its main advantage. It has been shown that SIgA can function not only as first defence line at portal of entry of the microorganisms and viruses, but also is more effective in terms of protection than systemic antibodies [48].

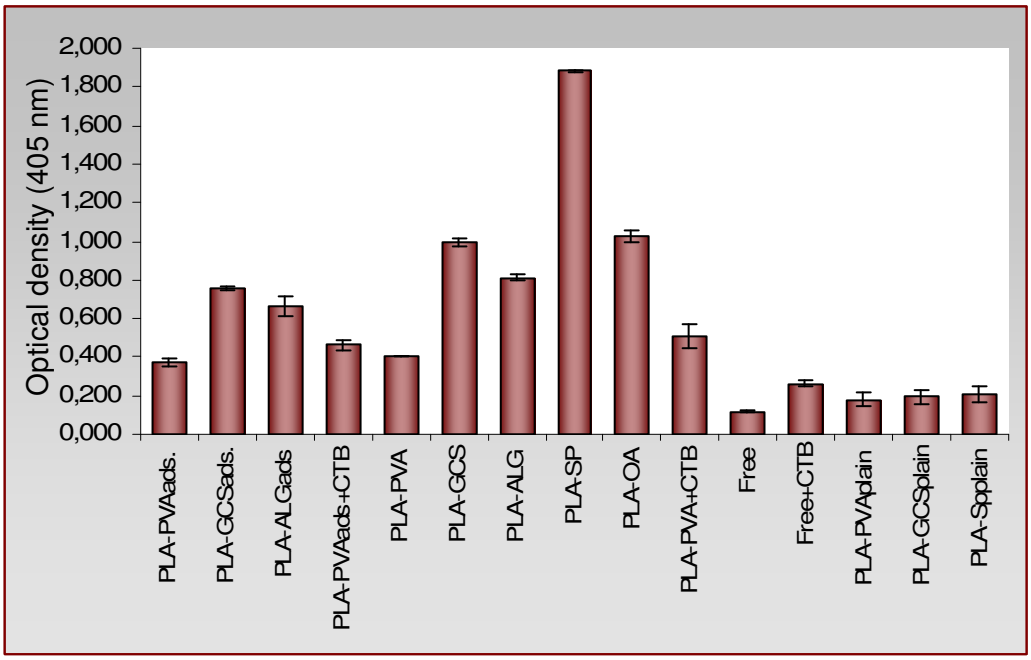


Figure 5.9 – Secretory IgA (SIgA) level in lung washes of mice immunised intranasally with *S. equi* antigens-entrapped or -adsorbed PLA nanospheres. Plain particles were used as control (mean ± S.D.; n=4).

Analysing the systemic and mucosal immune responses obtained after administration of *S. equi* antigens-loaded PLA nanospheres, it is possible to predict that the formulation method used to entrap these antigens preserves the epitopes necessary for the immune response (Figures 5.7 and 5.9).

Among *S. equi*-loaded nanospheres, SIgA titres observed in Figure 5.9 are not markedly different only when those induced by PLA-PVAads and PLA-GCS nanospheres are compared with the ones assessed in lung washes of animals exposed to PLA-PVA and PLA-OA, respectively. It is important to mention that *S. equi*-entrapped PLA-SP nanospheres induced the highest SIgA antibody titres, which was also obtained in the previous study performed by our group with PCL nanospheres formulated with SP, which means that this polyamine, as an absorption enhancer, might have significantly increased particles uptake by M cells in the NALT and subsequently delivered the antigen to the peripheral lymphoid tissues.

Nevertheless, besides not as high as those induced by these particles, SIgA levels identified in nasal lavages of animals exposed to PLA-GCS and PLA-OA were extremely higher than those obtained when PLA-PVA nanospheres were admixed with the mucosal adjuvant CTB ($P<0.01$). These results point out a strong mucosal adjuvant effect of those formulations, mainly those containing GCS and absorbance enhancers, as their i.n. administration effectively induced not only a systemic but also a considerable mucosal immune response, which justifies their potential use as alternatives to CTB adjuvanted systems.

S. equi-loaded PLA carriers did not induce detectable SIgA levels in intestinal contents of animals, which suggests that, though the large volume administered in both nostril, it seems that most of the dose was inhaled and not swallowed (data not shown), as previously demonstrated by Eyles *et al*. (2000) [13].

SPLENOCYTE CULTURE STUDIES

Several studies have shown that antigens immobilised in polymeric particles are absorbed with greater efficiency and have a greater ability to be presented to APCs than soluble molecules, providing a greater concentration of antigen at the inductive site [49]. These phagocytic cells will take up and process the antigens, and their maturation will lead to its migration to secondary lymphatic tissues, where those will be presented to T and B cells by molecules of the MHC system [5, 35, 44]. The transport of antigen to APCs and the way that it is processed by these cells are the main aspects to consider in the definition of the type (Th1/Th2) and duration of the immune response elicited after a vaccine administration. Two subsets of T-CD4$^+$ cells, Th1 and Th2, can be identified on the basis of the cytokines secreted following their stimulation, being the first more involved in the development of a cellular immune response, while the latter influences preferentially the humoral responses. IL2, IFN-γ and tumor necrosis factor β (TNF-β) are mainly secreted

by Th1 cells and induces class-switching in B cells promoting the synthesis of IgG2a, while Th2 cells are essential for antibody-mediated immunity as they produce IL4, IL5, IL6, IL10 and IL13, being the isotype IgG1 the one preferentially secreted after B cells activation wherein this pathway [27].

A previous study performed by our group has demonstrated the immunostimulatory properties of PCL carriers for *S. equi* antigens as these *S. equi* antigen-loaded polymeric particles were able to induce not only high levels of serum antibodies, but also a considerable cellular immune response [20, 21].

The supernatants of spleen cells stimulated with recall *S. equi* antigens were collected to quantify the cytokines produced in response to stimulations, which are shown in Figure 5.10. As it can be seen, i.n. immunisation with polymer formulated *S. equi* antigens was effective at stimulating Th1 and Th2-type cytokines production, being the IFN-γ the one detected in higher amount and the differences observed in these cytokine titres significant when compared with both FREE (P<0.02) and FREE+CTB (P<0.018) vaccinated animals. Both *S. equi* antigens-adsorbed and -entrapped nanospheres elicited cytokines levels significantly higher than those obtained in splenocytes cultures of mice vaccinated with soluble molecules of antigen, isolated or co-administered with CTB, which failed to stimulate elevated cytokine levels, with the exception of IL6 cytokines secreted by spleens of mice pre-immunised with FREE antigen when compared with those determined in PLA-PVAads and PLA-GCSads immunised groups.

The pattern of IL4 production by cultured splenocytes was markedly different from that of the remaining cytokines studied, but still distinct from those secreted by cells following their exposure to the antigen soluble groups (P<0.02).

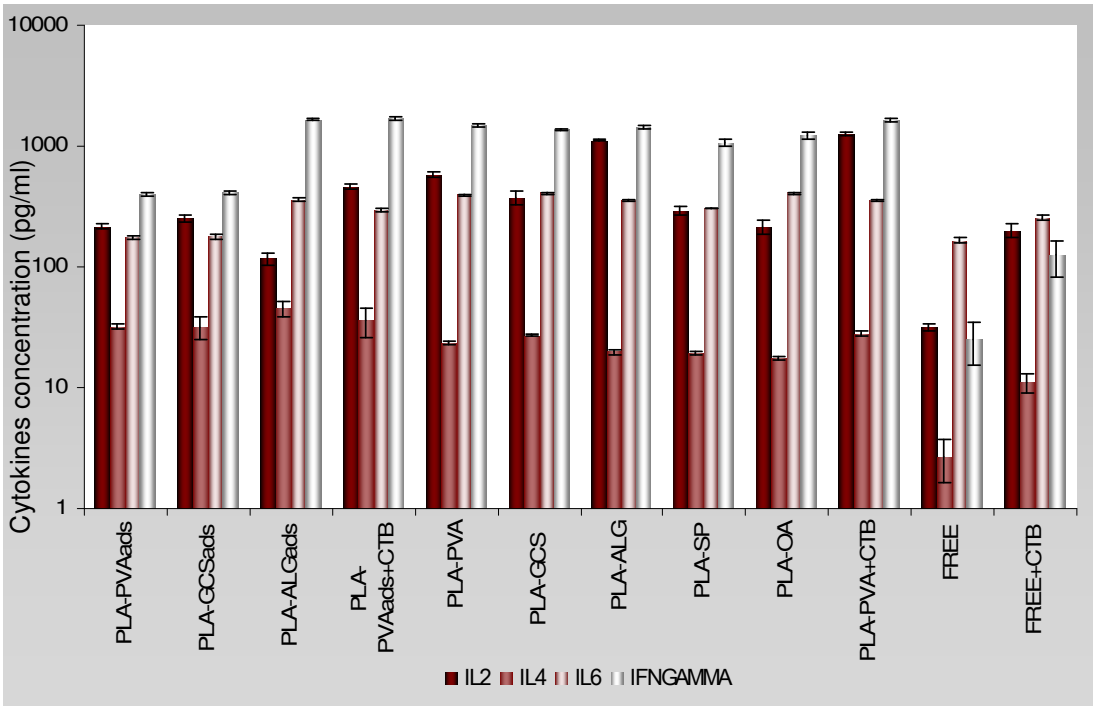


Figure 5.10 – IFN -γ, IL2, IL4, IL6 and IL12 levels produced after stimulation of splenocytes derived from mice immunised with *S. equi* enzymatic extract alone (FREE), co-administered with CTB and associated to different PLA-based nanospheres (mean ± S.D.; n=4).

It is interesting to compare cytokine levels resultant from *S. equi*-loaded PLA nanospheres mice immunisation with the obtained previously with i.n. administration of PCL nanospheres. Therefore, all cytokine titres, mainly IFN-γ and IL2, were statistically higher for antigen-adsorbed PLA nanospheres treated animals, when compared with those induced by splenocytes of PCL immunised groups, obtaining the opposite tendency when *S. equi*-entrapped nanospheres were used. These observations can mean that the hydrophobic character may have distinguished *S.equi*-adsorbed PCL nanospheres by increasing their uptake, which however may not have been enough to overcome the lower amount of antigen released during PCL particles degradation relatively to the correspondent PLA ones, as it was seen by their *in vitro* release profiles (Figures 5.4 and 5.5).

The outcome of adjuvant synergy obtained with a combination of a carrier/depot adjuvant and an immunomodulator adjuvant is a current strategy, as it may result in an enhanced and/or biased/balanced immune response profile (Th1/Th2), depending on the combination investigated, according to the requirements believed to result in a protective immunity [51].

CTB admixed with *S. equi*-loaded PLA-PVA particles elicited Th1 cytokines at a significant level, except when IFN-γ amount obtained in PLA-PVAads+CTB group is compared with the correspondent levels consequence of mice dosing with antigens adsorbed onto PLA-ALG nanospheres. Nevertheless, due to the high levels of cytokines induced by particulate systems, together with humoral and mucosal response, it is possible to conclude that the association of CTB to PLA-PVA nanospheres did not bring a considerable advantage over *S. equi*-loaded PLA nanospheres by themselves.

Altogether, having in mind IgG subtypes and IgA levels, it can be inferred that, for the trial period, nasal immunisation of *S. equi*-entrapped PLA nanospheres induced Th1 and Th2 cytokines profiles, with IgA responses in nasal secretions, more effectively than the adsorbed ones, having the mucoadhesiveness and positive charge of PLA-GCS an important contribution for the immunostimulatory effect observed and may be an useful tool for the induction of systemic and mucosal immune responses, which highlights its role in the development in a strangles vaccine. The immunostimulating effect of PLA nanospheres obtained after their i.n. administration may have resulted from their ability to expose the antigen to nasal mucosa for prolonged periods of time, improving its uptake by M-cells and APCs at this mucosa, to the efficient delivery to mucosal lymph nodes and to the stimulation of APCs after uptake. PLA-GCS especially can prolong the residence of antigen in the nasal cavity by the electrostatic interaction establish between their positive charge and mucus negatively charged, giving more time for antigen to be taken up in nasal tissues.

CONCLUSIONS

The purpose of this study was to study the effect of different mucoadhesive and absorption enhancers on humoral, cellular and mucosal immune responses induced by *S. equi* adsorbed or entrapped into PLA nanospheres. It was shown that both adsorption and entrapment processes can be successfully used for the development of *S. equi* enzymatic extract protein drug carriers with no compromise of its molecular structure or immunogenicity.

The association of antigens to PLA polymeric particles was able to change their processing and/or presentation by the immune system. All taken together, humoral, cellular and mucosal immune responses make it possible to predict that the PLA nanospheres by themselves hold a great promise as a delivery system for i.n. vaccination against strangles and do not require the co-administration of other adjuvants in order to achieve a mucosal and balanced Th1/Th2 immune response, fundamental for animals protection against *S. equi* infection.

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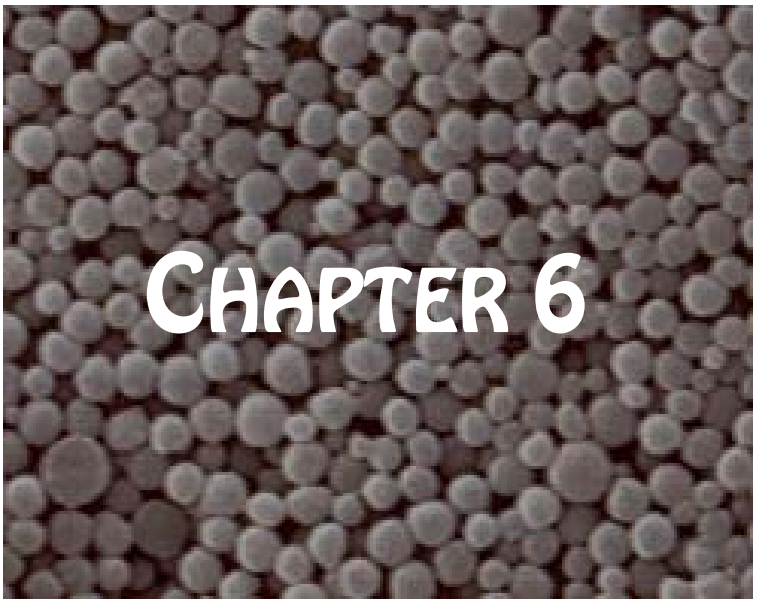

Chapter 6

Enhancement of the immunogenicity and the efficacy of *S. equi* antigens (SeM and enzymatic extract) by poly-L-lactic acid nanospheres

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ABSTRACT

Strangles is an infectious disease caused by *Streptococcus equi* subspecies *equi* that affects the upper respiratory tract of the *Equidae*. The control of this disease seems to be dependent on its earlier detection and prevention, but prolonged animals protection without development of strong and severe side effects was not yet achieved. Convalescent horses present a protective immune response, mainly against SeM (58 kDa), an antiphagocytic and opsonogenic *S. equi* M-like protein, known as the major protective antigen against strangles. SeM and *S. equi* protein extract-entrapped poly(lactic acid) (PLA) nanospheres were developed and their adjuvant potential was studied. The effect of absorption enhancers (spermine, oleic acid) and mucoadhesive polymers (alginate, glycol-chitosan) were also evaluated. Spherical nanometric particles of less than 500 nm were developed, despite particle composition, and protein structure was not affected by the solvent evaporation method. The humoral immune response induced by nanospheres was markedly higher than that elicited by soluble antigens, isolated or co-admixed with CpG. IgG and IgG subtypes, along with cytokines titres, indicated that nanospheres composed by glycolchitosan developed a more balanced Th1/Th2 response for both SeM and *S. equi* enzymatic extract proteins, although those induced by the pure antigen-entrapped particles were higher than the *S. equi* tested vaccines composed by total antigens entrapped in polymeric nanospheres.

Keywords: Poly(lactic acid), nanospheres, SeM, vaccines, immune responses.

INTRODUCTION

Strangles, from Latin *strangulina*, is an infectious disease that affects the upper respiratory tract, more specifically, the nasopharynx and lymphatic nodes drainage of horses, donkeys and mules [1-3]. Nevertheless, it is not exclusive of the *Equidae* family, as it was as well identified in two humans and one dog [4-6]. It is a highly infectious and worldwide endemic infection, representing 30% of all horse diseases and is caused by *Streptococcus equi* subspecies *equi* (*S. equi*) [2, 7]. These bacteria cross the oral and nasal mucosa, lodge in the head and neck lymphatic nodes, leading to the formation of abscesses that, with progression of the disease, will become painful and consequently prevent horses from eating, resulting in a general weak condition [2, 7]. *S. equi* infected horses present high fever, acute rhinitis and pharyngitis, and swallowed lymph nodes, which after being mature, will disrupt and release a mucopurulent discharge. This liquid contains a high level of infectious bacteria that can be inhaled or ingested by healthy horses [2]. The convalescence period is usually long, but in most of the cases, 4 to 6 weeks after abscess rupture and complete drainage, animals start to recover and will completely eliminate *S. equi* from their organism. However, in some circumstances, pharyngeal abscesses content can lodge in horse guttural pouch for months, without causing the development of any clinical sign of the disease [2, 7]. These apparent healthy horses constitute *S. equi* carriers, able to release the infectious bacteria for their throat and consequently to the environment, representing a high risk of contamination for other non-infected horses during that period, as they are not easily detected by the diagnostic methods commonly used [2, 7, 8]. Therefore, these infectious *S. equi* carriers are the most important cause for the long lasting maintenance of the disease, as the causal agent only survives in the environment for some weeks. Moreover, strangles is still a worldwide endemic disease with an important economical drawback as, after being identified, infected animals require not only the conventional treatment (pain killer, analgesics and, in some

situations, antibiotics), but also their maintenance in quarantine or with a controlled mobility until the complete elimination of the microorganism is confirmed by diagnostic methods. Even so, it is important to follow animals for prolonged periods of time as it was already detected the presence of *S. equi* in horses that did not show any clinical sign of the disease for the last 12 months [2].

S. equi infected animals treatment seems not to be effective when animals already present signs of the disease. In addition, some clinicians believe that antibiotics will delay the abscesses maturation, contributing for the systemic dissemination of the microorganism to other regions of the body, leading to the most dangerous situation of this disease called "bastard strangles", resulting in most of the cases in the death of the animal [7].

Besides being an endemic disease, few is known about *S. equi* virulence, but most of the work developed in this field has been directed to the study of secreted substances or bacterial cell wall proteins [9, 10]. On the other hand, *S. equi* resistance to phagocytosis seems to be the main factor responsible for its infectious character. Hyaluronic acid capsule, cell wall SeM (58 kDa) and secreted IdeE and Se18.9 proteins are the main factors that contribute for *S. equi* phagocytosis resistance [10]. SeM and IdeE limit C3b complement factor deposition on bacterial surface, while Se18.9 blocks the bind complement regulatory factor H [11, 12]. In addition to their antiphagocytic properties, SeM proteins are the most protective antigens as they can also induce the production of specific opsonogenic antibodies [11]. In fact, convalescent animals present a protective immune response mainly directed to SeM, presenting IgG and IgA anti-SeM immunocomplexes, both in serum and nasal secretions, that have the ability to recognise, bind and inactivate *S. equi* in further infections. Nevertheless, these opsonogenic antigens are not fully protective by themselves in horses and, therefore, anti-SeM immunity may be complemented by neutralising antibodies against IdeE and Se18.9. On the other hand, mucosal antibodies may be more effective than systemic ones,

and therefore also this possibility must be taken into consideration [10]. Even if the complete protective mechanisms are not yet completely clarified and the immunogenic *S. equi* proteins are not fully identified, it seems that to combat and prevent strangles it is mandatory the development of an effective vaccine [1, 2, 7, 13].

In recent studies performed by our group, it was demonstrated that polymeric particles were able to change *S. equi* protein extract processing and/or presentation by the immune system, inducing a mucosal and balanced Th1/Th2 immune response fundamental for animal protection against *S. equi* infection [14-16]. Therefore, the aim of the present work was to evaluate and compare the humoral and cellular immune responses induced by SeM and a mixture of *S. equi* cell wall extracted proteins, entrapped in PLA nanospheres, after mice intramuscular (i.m.) vaccination. In order to assess the effect of mucoadhesive polymers and absorption enhancers in the resultant immunity, glycolchitosan, alginate, spermine and oleic acid were included in particle formulations. It is important to emphasise that this is the first study that evaluates the adjuvant potential of polymeric particles associated to recombinant *S. equi* proteins.

Materials and Methods

PLA nanospheres preparation

Poly(L-lactic acid) (PLA, 2.0 kDa) with an average of 2000 Da molecular weight (MW) was obtained from PolySciences, Inc., UK. Polyvinyl alcohol (PVA, MW 13 23 kDa, 87-89% hydrolysed), alginate low viscosity (ALG), spermine (SP), oleic acid (OA), glycolchitosan (GCS), sucrose were purchased to Sigma Aldrich Co., UK. Dichloromethane (DCM) was obtained from BDH Laboratory Supplies, UK. SeM (58 kDa) was obtained from Prof. J.F. Timoney (University of Kentucky, USA), being produced in *E. coli* following a previously published protocol [11]. To prepare *S. equi* enzymatic extract, N-acetyl muramidase, lysozyme and mutanolysin (Sigma Aldrich Co., UK), were used to extract proteins from *S. equi* cell wall as previously described [14].

S. equi enzymatic extract proteins (10 mg) and recombinant SeM protein (0.5% theoretical loading) were entrapped in different types of PLA nanospheres by double emulsion (w/o/w) evaporation method, as described elsewhere [14]. Briefly, antigens were dissolved in 10 % (w/v) PVA solution, and then emulsified with a solution of PLA (2 kDa) in DCM (100 mg in 6 ml), by homogenisation using an ultra turrax (T25 Janke & Kunkel, IKA Labortechnik). This primary w/o emulsion was further added to 30 ml 1.25 % (w/v) PVA or 0.75% (w/v) ALG solution and homogenised for 7 min at 10,000 rpm with the Silverson homogeniser (Silverson model L4RT, UK) to form a secondary emulsion (w/o/w). The emulsion was then left under magnetic agitation at room temperature to allow DCM evaporation. The particles were afterwards washed, frozen and freeze-dried in the presence of the lyoprotectant sucrose (10%, w/v), under vacuum, for two days. To study the effect of OA, SP and GCS inclusion in the adjuvant properties of PLA nanospheres, an OA solution (10 mg/ml in 1 ml of ethanol) was added to the

organic phase, while SP and GCS (10 mg/ml) were dissolved in the 10 % (w/v) PVA solution, prior to the w/o primary emulsion formation.

PLA nanospheres volume mean diameter (VMD) was determined by photon correlation spectroscopy (PCS; Zetasizer, Malvern Instruments, UK). PLA nanospheres dispersed in double distilled water, containing 0.02% Tween 20 were placed in a cuvette for size measurements. The zeta potential for *S. equi* antigen-entrapped nanospheres was measured using a Malvern zeta analyser (Zetasizer, Malvern Instruments, UK).

To prepare nanospheres for *in vivo* studies, particles containing required doses of SeM (10 μg) were weighed and dispersed in 100 μl PBS (pH 7.4) buffer, just before mice immunisation.

IMMUNISATIONS

Animals were allowed to acclimate to the new environment for 1 week prior being vaccinated and their care and treatment carried out during these *in vivo* studies were performed in strict accordance with the UK 1986 Animals (Scientific Procedures) Act. Female 6-8 weeks old BALB/c mice (n=4/group; The London School of Pharmacy, UK) were intramuscularly immunised on day 1 and boosted on day 21, with 100 μl of either 10 μg of SeM or the equivalent amount of *S. equi* enzymatic extract proteins dissolved in saline, entrapped in PLA nanospheres, or admixed with CpG adjuvant (10 μg; ODN1826 22.86 mg/ml, Coley Pharmaceutical group, UK). The immunogenicity of the *S. equi* antigen-entrapped PLA nanospheres and CpG was also assessed. Tail vein blood samples were collected 2, 4, 7 and 12 weeks after immunisation, and spleens were isolated at the end of the experiment for immunological assays. Serum was prepared from clotted blood and stored at -20^{o}C until analysis.

QUANTIFICATION OF ANTIGEN SPECIFIC IgG AND SUBTYPES BY ELISA

The antibody responses (IgG, IgG1 and IgG2a) to SeM or *S. equi* cell wall extracted proteins were determined based on a method previously reported [14]. Plates (Immulon 2, flat bottom plates, Dynatech, UK) were coated overnight with 1.0 μg/ml SeM protein (or equivalent *S. equi* extract proteins) in 0.1M sodium carbonate buffer (pH 9.6), washed and afterwards blocked with a 2% (w/v) bovine serum albumin solution (Sigma Aldrich Co., UK). Plates were again washed and sera were tested at 1:32 and 1:64 followed by serial two-fold dilutions. Sera obtained from naive mice were used as a control. Horseradish peroxide conjugate goat anti-mouse IgG, IgG1 and IgG2 (diluted 1:1000) were applied as secondary antibody (Sigma, Pool Dorset, UK). Finally, dissolved 2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) [ABTS] (Sigma Aldrich Co., UK) in citrate buffer, along with H_2O_2 was used to develop the plates, the colour reaction was stopped after 15 minutes, by adding 5N H_2SO_4 to the wells, and absorbance was read at 405 nm. The titres reported are the reciprocal of serum dilutions that gave an optical density 5% higher than the strongest negative control reading.

CYTOKINES ASSAYS

Spleens were aseptically removed 12 weeks after mice immunisation and cell homogenisates were prepared as previously described by our group [14]. Supernatants obtained 48-h after splenocytes stimulation with 2.0 μg/ml of soluble SeM, or equivalent *S. equi* extract proteins, were used for cytokines (IFN-γ, IL2, IL4 and IL6) concentration measurement, by a enzyme-linked immunosorbent assay (ELISA; DuoSet® ELISA Development kit, R&D Systems Europe, UK). The concentrations, expressed as pg/ml, were determined by reference to different cytokines standard curves.

STATISTICAL ANALYSIS

SPSS software (Version 13, Microsoft) was used to analyse the collected data. One way analysis of variance (ANOVA) was applied to study the difference in antibody response and cytokine profiles between SeM and *S. equi* enzymatic extract proteins particulate formulations. The IgG and IgG subtypes immune responses, as well as the cytokine levels secreted by splenocytes induced by soluble antigen were compared with those induced by their polymer formulated form. If ANOVA results were found significant ($P<0.05$), a LSD multicomparison post hoc test was performed in order to specify the difference between the several groups.

RESULTS

PLA NANOSPHERES CHARACTERISATION

In a recent work developed by our group, it was reported the formulation and characterisation of *S. equi* cell wall extracted proteins successfully entrapped in PLA nanospheres containing PVA, GCS, OA, SP or ALG, and no evidence of cytotoxicity was obtained for particles suspension, as macrophages viability of approximately 100% was observed in all tested sets. Particles appeared to be spherical, smooth and fairly homogeneously distributed, without the presence of collapsed particles [15]. Table 6.1 summarises the main physicochemical characteristics of SeM-loaded PLA nanospheres with (PLA_{SeM}).

Table 6.1
In vitro characteristics of SeM protein-loaded PLA nanospheres

FORMULATIONS		VMD[a] (nm)	ZETA POTENTIAL[b] (mV)	L.C.[c] (%)
SeM	PLA-PVA_{SeM}	264.1 ± 7.85	-31.8 ± 1.05	0.247 ± 0.004
	PLA-GCS_{SeM}	403.5 ± 4.96	+45.9 ± 2.32	0.384 ± 0.007
	PLA-ALG_{SeM}	360.6 ± 5.55	-50.8 ± 2.39	0.380 ± 0.017
	PLA-SP_{SeM}	282.7 ± 5.98	-22.4 ± 0.56	0.326 ± 0.029
	PLA-OA_{SeM}	252.6 ± 4.84	-29.0 ± 1.27	0.456 ± 0.010
S.equi protein extract[d]	PLA-PVA_{Ext}	281.1 ± 17.3	-31.6 ± 8.95	6.04 ± 0.242
	PLA-GCS_{Ext}	396.0 ± 36.1	+31.8 ± 3.10	7.16 ± 0.561
	PLA-ALG_{Ext}	327.0 ± 57.8	-50.1 ± 4.49	5.56 ± 0.104
	PLA-SP_{Ext}	267.9 ± 16.4	-24.5 ± 2.95	5.37 ± 0.494
	PLA-OA_{Ext}	205.47 ± 45.9	- 29.3 ± 7.82	7.84 ± 0.268

[a]Particle size measured by laser diffraction expressed as volume mean (mean ± S.D.; n=6) of 50% particles in that range (VMD); [b]Zeta potential as a measure of PLA nanospheres surface charge (mean ± S.D.; n=6); [c]Loading capacity (L.C.) representing the amount (mean ± S.D.;n=6) of *S. equi* antigens recovered after hydrolysis of PLA nanospheres; [d]data discussed in Chapter 5.

Similarly to *S. equi* enzymatic extract-entrapped PLA particles (PLA_{Ext}, Table 6.1), PCS showed that the VMD of SeM-loaded PLA nanospheres was lower than 500 nm, although larger particles were obtained when GCS was incorporated in the internal phase. It is important to mention that the formulation of SeM-entrapped PLA nanospheres showed a higher reproducibility between batches, as it can be observed by lower standard

deviation (S.D.) obtained with multiple size measurements, compared with those resultant from the analysis of PLA_{Ext} [15]. Significant differences were obtained for PLA nanospheres containing GCS (PLA GCS_{SeM}) and ALG (PLA-ALG_{SeM}) when compared with the size presented by the remaining sets of particles (P<0.005). When PLA_{Ext} and PLA_{SeM} nanospheres are compared, no differences in their size are observed concerning the type of antigen entrapped in these carriers. The surface charge of PLA nanospheres containing SeM protein re-constituted in 10 mM potassium chloride (KCl) solution was found to be markedly different between particles with different composition, except for the zeta potential obtained for OA formulated PLA nanospheres (PLA-OA_{SeM}) compared to -22.4±0.56 mV and -31.8±1.05 mV, respectively for PLA containing SP (PLA-SP_{SeM}) and PVA (PLA-PVA_{SeM}) vehicles. Opposite to the VMD, *S. equi* type of antigen clearly influenced the charge of PLA-GCS nanospheres, as the PLA-GCS_{SeM} presented higher positive charge when compared with the correspondent *S. equi* enzymatic extract-entrapped PLA nanospheres (PLA-GCS_{Ext}; P<0.001). The entrapment efficiency (E.E.) of SeM varied from 91.2 % (w/w) to 49.4 (w/w), respectively for PLA-OA_{SeM} and PLA-PVA_{SeM} nanospheres, which corresponded to a SeM loading of 4.56±0.167 μg/mg and 2.47±0.004 μg/mg (Table 6.1).

Table 6.2
Vaccination strategies in BALB/c mice

ANTIGEN	CARRIERS	ANTIGEN	CARRIERS
S. equi enzymatic extract	PLA-PVA_{Ext}	SeM protein (58 kDa)	PLA-PVA_{SeM}
	PLA-GCS_{Ext}		PLA-GCS_{SeM}
	PLA-ALG_{Ext}		PLA-ALG_{SeM}
	PLA-SP_{Ext}		PLA-SP_{SeM}
	PLA-OA_{Ext}		PLA-OA_{SeM}
	PLA-PVA_{Ext}+CpG		PLA-PVA_{SeM}+CpG
	Ext		SeM
	Ext + CpG		SeM+CpG

BALB/c female mice 6-8 weeks old were vaccinated by i.m. route at weeks 0 and 3. Tail veins blood samples were collected at weeks 2, 4, 7 and 12 for antibodies titres analysis by enzyme-linked immunoassay.

Accordingly, as observed for PLA_{Ext} nanospheres, PLA-OA gave the highest entrapment of SeM, among all the PLA nanospheres studied. The evaluation by SDS-PAGE demonstrated that, similarly to *S. equi* cell wall proteins [15], SeM (58 kDa) retained its structural integrity after the entrapment process, which is shown in Figure 6.1, by the maintenance of protein profile before and after being loaded in polymeric nanospheres.

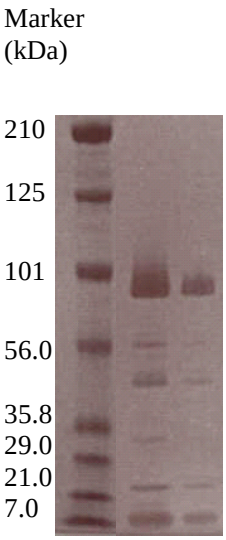
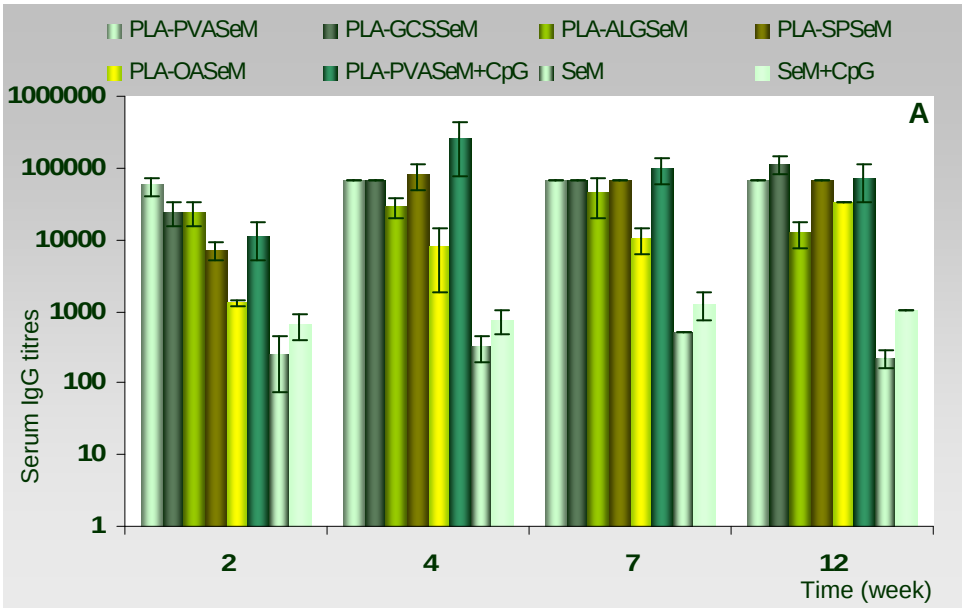


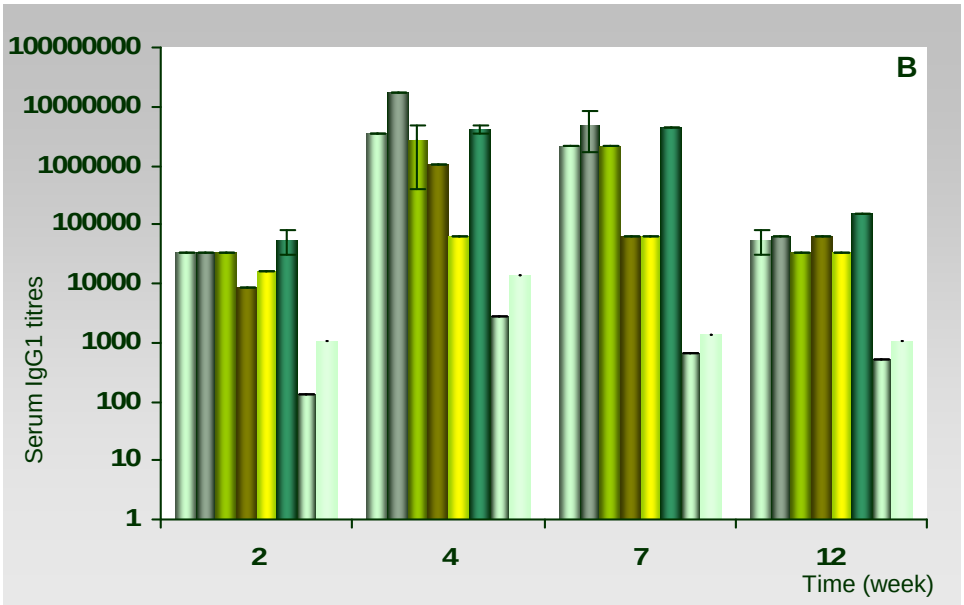
Figure 6.1- Assessment of structural integrity of extracted SeM from SeM-entrapped PLA (MWT 2.0 kDa) nanospheres using SDS-PAGE (12% gel, 100 V, 1h30min). Lanes: 1) Standard markers (broad range Biorad® prestained MW marker: 7.1-209 kDa); 2) Control sample of unentrapped SeM at 950 μg/ml (same as loaded samples); 3) SeM extracted from PLA-PVA nanospheres.

HUMORAL IMMUNE RESPONSES

Mice IgG and IgG subtypes serum antibodies elicited after animals' i.m. vaccination on days 1 and 21, with *S. equi* antigens alone, admixed with the potent adjuvant CpG or entrapped in PLA nanospheres, are summarised in Figures 6.2 and 6.3. At two weeks post-immunisation, anti-SeM IgG antibody levels elicited by $PLA\text{-}PVA_{SeM}$, $PLA\text{-}GCS_{SeM}$ and $PLA\text{-}ALG_{SeM}$ nanospheres were above those resultant from the immune response to SeM alone, or even co-administrated with the potent adjuvant oligodeoxynuleotide with repeating C and G motives (SeM+CpG) (P<0.001) (Figure 6.2A). On the other hand, polymeric formulated *S. equi* extract, along with AO and GCS, induced IgG levels statistically higher than those measured in the sera of mice treated with the soluble form of the antigen and with its admixture with CpG (P<0.026). By this time, $PLA\text{-}PVA_{SeM}$ nanospheres induced the highest

immune response among all SeM sets (Figure 6.2A), while PLA-OA_{Ext} formulation allowed the production of the highest IgG antibodies titre overall *S. equi* enzymatic extract, in solution or in a particulate form (Figure 6.3A). Apparently, the size differences did not noticeably affect antibody production. Levels of antibodies induced by PLA-PVA_{SeM}, PLA-SP_{SeM} and PLA-GCS_{SeM} were maintained statistically different than those observed with the same dose of SeM and SeM+CpG, one week after boosting.





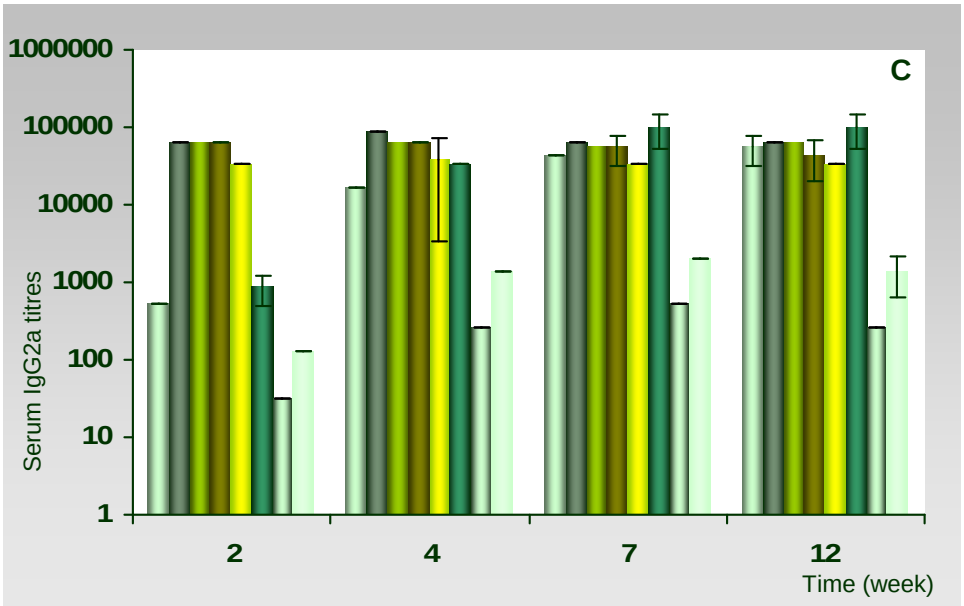
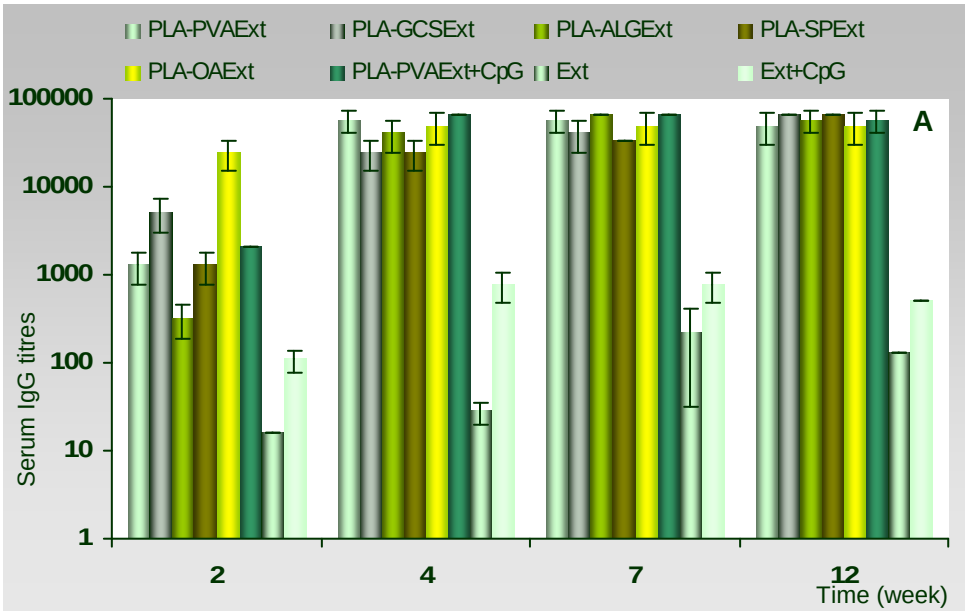
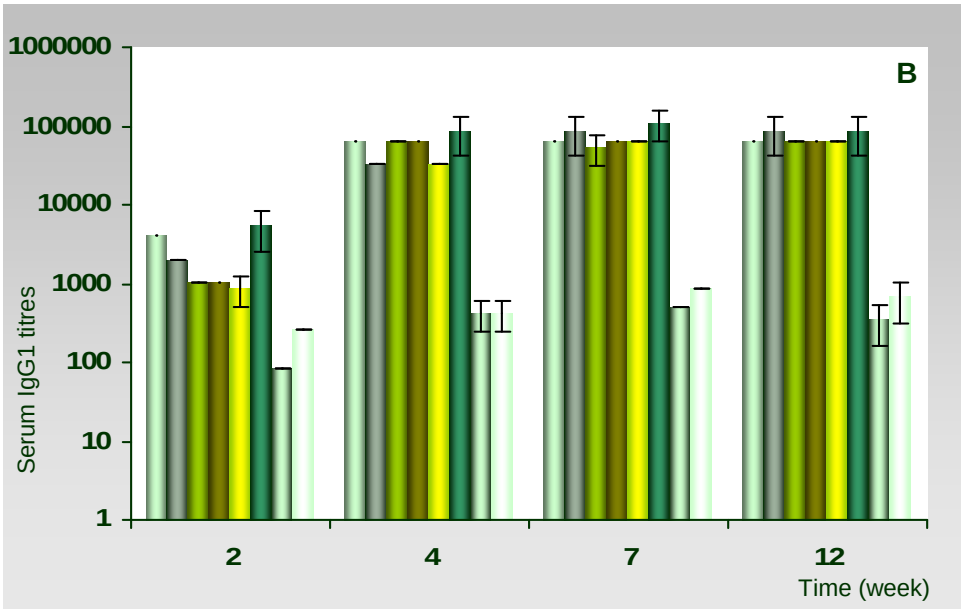


Figure 6.2– Geometric means (mean ± S.D.; n=4) of anti-SeM IgG (A), IgG1 (B) and IgG2a (C) concentrations induced by intramuscular immunisation with different PLA-based nanospheres entrapping SeM (10 μg/dose), compared to the free antigen (SeM) and SeM co-administered with CpG (SeM+CpG) immunisations.

Antibody levels highly increased between weeks 2 and 4 in the group immunised with PLA-PVA$_{SeM}$ co-administered with CpG (PLA-PVA$_{SeM}$+CpG), being at this time point significantly higher than those measured in any other group (P<0.011) (Figure 6.2A). In *S. equi* extract-vaccinated groups it was noticeable a considerable increase in IgG levels at week 4 and PLA-PVA$_{Ext}$, which along with PLA-OA$_{Ext}$ gave higher titres than PLA-GCS$_{Ext}$ and PLA-SP$_{Ext}$ particulate systems (Figure 6.3A). Despite these differences, responses assessed in serum of animals treated with any of the PLA$_{Ext}$ nanospheres were statistically higher than those induced by *S. equi* extract antigens in solution (P<0.006). By the IgG antibody levels induced in mice vaccinated with PLA nanospheres, it can be observed that, at this time point, the type of antigen did not induce any significant difference between particles with similar composition (Figures 6.2A and 6.3A). Nevertheless, PLA-PVA$_{SeM}$ particles suspension co-administered with CpG adjuvant (PLA-PVA$_{SeM}$+CpG) resulted in IgG production higher than any of those obtaining in the remaining immunisation groups (P<0.001).

At week 7, only PLA-OA_{SeM} immunisation group gave IgG titres not different from those obtained with SeM in solution, alone or associated with CpG (Figure 6.2A). By this time, although the response elicited in animals vaccinated with PLA-PVA_{SeM} nanospheres co-admixed with CpG has dropped very noticeably, IgG levels were still the highest among all other SeM related vaccination groups (P<0.008). On the other hand, all *S. equi* extract-entrapped formulations raised the immune response to antibody titres statistically different from those obtained 7 weeks after i.m. administration of *S. equi* cell wall protein in solution, alone or with CpG (P<0.001) (Figure 6.3A).





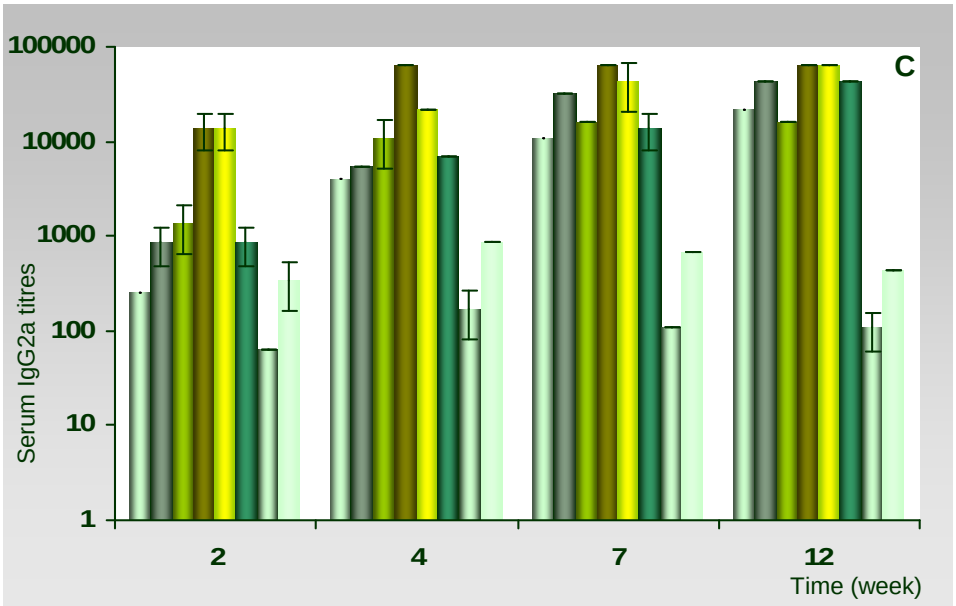


Figure 6.3– Anti-*S. equi* specific IgG (A), IgG1 (B) and IgG2a (C) titres elicited by immunisation groups. Animals were inoculated twice (days 0 and 21) intramuscularly with one of the following formulations: *S. equi* enzymatic extract proteins loaded 1) PLA-PVA$_{Ext}$, 2) PLA-GCS$_{Ext}$, 3) PLA-ALG$_{Ext}$, 4)PLA-SP$_{Ext}$, 5) PLA-OA$_{Ext}$ and 6) (PLA-PVA$_{Ext}$+CpG) nanospheres; 7) *S. equi* extract alone (Ext); 8) (Ext+CpG) mixture (n=4, mean±S.D.).

Among *S. equi* extract-entrapped particulate systems, PLA-PVA$_{Ext}$ gave higher IgG antibody titres than those elicited by PLA-GCS$_{Ext}$ and PLA-SP$_{Ext}$ formulations, although not being statistically different from those obtained with PLA-ALG$_{Ext}$ and PLA-OA$_{Ext}$ adjuvantised samples. IgG antibody levels remained the highest in animals vaccinated with PLA-PVA$_{SeM}$+CpG (P<0.001) (Figure 6.2A). It seems that, at this time point it starts to be evidenced that the immune response is dependent on the type of antigen, for particle similar composition, as responses obtained with PLA-GCS$_{SeM}$ and PLA-SP$_{SeM}$ were statistically higher than the correspondent particles entrapping *S. equi* extract (P<0.014 for PLA-GCS$_{SeM}$ vs PLA-GCS$_{Ext}$, P<0.001 for PLA-SP$_{SeM}$ compared with PLA-SP$_{Ext}$) (Figures 6.2A and 6.3A). An opposite tendency was obtained for *S. equi* extract-entrapped PLA-ALG and PLA-OA particles, which induced higher IgG levels than SeM-loaded PLA nanospheres with similar composition (P<0.038 for PLA-ALG$_{Ext}$ vs PLA-ALG$_{SeM}$, P<0.001 for PLA-OA$_{Ext}$ compared with PLA-OA$_{SeM}$).

At the end point of the study, PLA-GCS$_{SeM}$ formulation gave IgG levels higher than all formulations tested, inclusive PLA-PVA$_{SeM}$+CpG group (P<0.005), while PLA-ALG$_{SeM}$ formulation did not elicited IgG levels different from those obtained with the i.m. administration of the recombinant protein (Figure 6.2A). On the other hand, PLA$_{Ext}$ nanospheres immunisation groups, besides inducing IgG response higher than those raised by groups vaccinated with antigen in soluble form (P<0.001), gave levels of comparable magnitude among particulate vaccinated groups. Besides these differences observed between SeM and *S. equi* extract groups during the *in vivo* study, at the end of the experiment significant differences were observed just when those antigens were entrapped in PLA-GCS and PLA-ALG. More specifically, levels of IgG antibodies elicited by PLA-ALG$_{Ext}$ were markedly higher than those resultant from PLA-ALG$_{SeM}$ suspension administration (P<0.001). On the contrary, PLA-GCS$_{SeM}$ nanospheres, did not only rise IgG titres noticeably higher than those assessed in the serum of mice vaccinated with PLA-GCS$_{Ext}$ (P=0.001), but also elicited the highest response among all immunisation groups (P<0.001) (Figures 6.2A and 6.3A).

IgG SUBTYPE PROFILING

Figures 6.2B, 6.2C, 6.3B and 6.3C show the evolution of IgG subtypes specific responses for 12 weeks against *S. equi* antigens, elicited after mice i.m. vaccination. At two weeks post-immunisation, anti-SeM IgG subtypes antibody levels elicited by SeM-entrapped PLA nanospheres were higher than those assessed when antigen in soluble form was administered to mice, with an exception of IgG1 titres induced by PLA-SP$_{SeM}$ nanospheres (Figures 6.2B and 6.2C). The enhancement of IgG1 antibodies using PLA-PVA$_{SeM}$, PLA-GCS$_{SeM}$ and PLA-ALG$_{SeM}$ nanospheres suspension was similar, besides lower than that observed for PLA-PVA$_{SeM}$+CpG group (P<0.01) (Figure 6.2B). At this time point, PLA-GCS$_{SeM}$, PLA-ALG$_{SeM}$ and PLA-SP$_{SeM}$ nanospheres improved IgG2a antibody titres, and these were statistically different (P<0.001) from that measured in the remaining immunisation groups (Figure

6.2C). After boosting, all SeM-entrapped formulations highly enhanced the IgG1 response, exception for PLA-OA_{SeM} formulation that slightly increase the immune response between week 2 and 4 (Figure 6.2B). Nanospheres containing SP, PLA-PVA_{SeM} and PLA-ALG_{SeM} induced similar IgG1 antibodies levels. In the case of PLA-GCS_{SeM}, the improvement in IgG1 antibody response was the highest, being statistically different from all other immunisation groups (P<0.001). This formulation also induced the strongest IgG2a response, although not different from PLA-ALG_{SeM} and PLA-SP_{SeM} (Figure 6.2C). At week 7, IgG1 antibody response profile obtained for different PLA nanospheres was maintained, except for PLA-SP_{SeM}, which antibody titres drastically decreased (Figure 6.2A). Furthermore, the IgG1 responses obtained with PLA-ALG_{SeM}, PLA-GCS_{SeM}, PLA-PVA_{SeM} and PLA-PVA_{SeM}+CpG were similar 7 weeks after animals' vaccination. On the other hand, at weeks 7 and 12 the enhancement of IgG2a antibody levels using SeM-entrapped nanospheres was higher than that observed for the soluble form of the antigen (P<0.04), despite its admixture with CpG (P<0.049) (Figure 6.2B). CpG co-administration with PLA-PVA_{SeM} nanospheres induced the highest IgG2a titres from week 7 until the end of the experiment, but those were not different from those elicited by PLA-GCS_{SeM} formulation. At the end of the experiment, PLA-PVA_{SeM}, PLA-GCS_{SeM} and PLA-SP_{SeM} improved IgG1 antibodies titres at the same level, but statistically higher than that obtained for groups vaccinated with SeM antigen, alone or admixed with CpG (P<0.04) (Figure 6.2A). The inclusion of the additional adjuvant CpG in PLA-PVA_{SeM} nanospheres had a beneficial impact, as this formulation was the one that elicited the highest IgG1 antibody levels for prolonged periods of time. Even though, the improvement of IgG2a levels induced by this formulation was not different from that observed for PLA-GCS_{SeM} and PLA-ALG_{SeM} nanospheres (Figure 6.2B).

The IgG subtype titres demonstrated that *S. equi* enzymatic extract-entrapped nanospheres led to differential degree of enhancement in antibody titres, when compared with SeM-entrapped carriers. At two weeks

post-immunisation, IgG1 antibodies elicited by PLA-PVA_{Ext} formulation were statistical different from those assessed in serum collected from mice immunised either with soluble antigen (P<0.001), or *S. equi* extract polymeric formulation (*P*<0.009) (Figure 6.3B). Nevertheless, the IgG1 response obtained with PLA-PVA_{Ext} nanospheres were not different from those observed when admixture of those particles with CpG was used for vaccination. The enhancement of IgG2a antibodies using PLA-SP_{Ext} and PLA-OA_{Ext} was higher than that observed for the remaining immunisation groups (*P*<0.001) (Figure 6.3C). A marked increase was obtained in IgG1 and IgG2a levels induced after boosting, and antibody titres were sustained at high levels throughout the post-immunisation period (Figures 6.3B and 6.3C). IgG1 responses comparable in magnitude were elicited by PLA_{Ext} nanospheres from week 7 until the end of the experiment (Figure 6.3B). IgG2a titres enhancement induced by PLA-SP_{Ext} nanospheres was statistically significant among all other groups (*P*<0.001), and these differences persisted until week 12, being only similar to antibody levels obtained at the end of the study for PLA-OA_{Ext} particles suspension (Figure 6.3C). Besides these differences, PLA-GCS_{Ext} elicited IgG2a response that was markedly higher than those determined in PLA-PVA_{Ext} (*P*<0.017) and PLA-ALG_{Ext} (*P*<0.004) groups, and not different from that resultant from PLA-PVA_{Ext} co-administration with CpG. It is important to state that at the end of this study, all particulate formulations induced IgG2a levels higher than those induced by the soluble antigen, even when associated to CpG adjuvant (*P*<0.001).

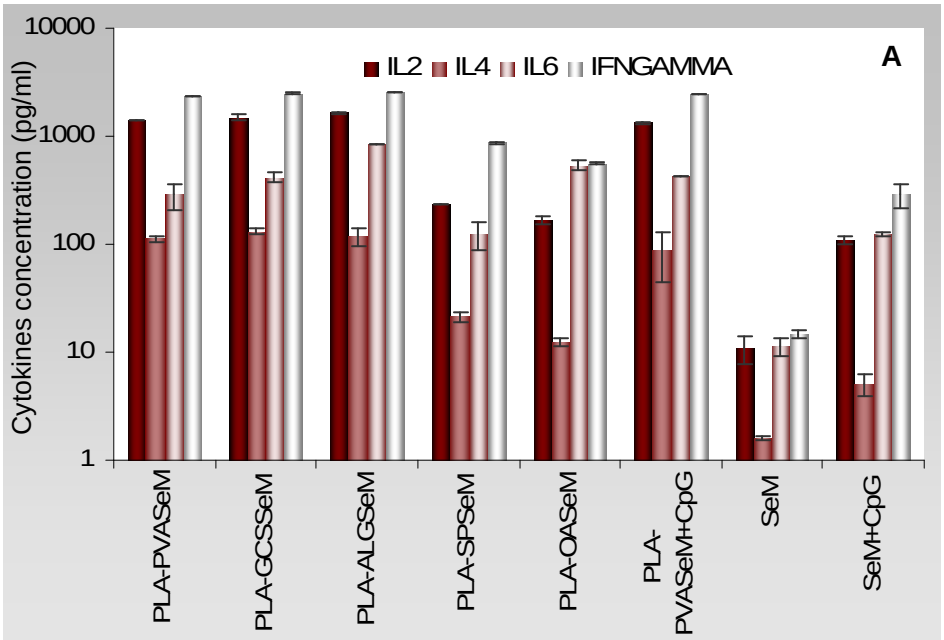
Over the entire period, no improvement was obtained in antibody titres when immunising admixture of particles with CpG, as this formulation induced IgG1 levels comparable to those assessed in serum of animals vaccinated with whichever particulate formulation. On the other hand, PLA-SP_{Ext} and PLA-OA_{Ext} nanospheres gave in fact higher IgG2a titres than those obtained by the addition of CpG to PLA-PVA_{Ext} nanospheres (*P*<0.017), which were not

different from those elicited by the cationic preparation (Figures 6.3B and 6.3C).

CYTOKINES PROFILES

Cytokine levels can be used to characterise the type of Th2/Th1 immune response [17]. From Figure 6.4 it can be seen that formulations successfully induced the production of cytokines Th1 and Th2-related.

Similarly to the IgG2a/IgG1 relation, IL2, IL6, IFN-γ cytokines secreted by splenocytes of mice pre-immunised with PLA-ALG$_{SeM}$ formulation were markedly different from those obtained in splenocyte cultures of mice vaccinated with any other formulation (P<0.003). It is possible to distinguish a preferential secretion of Th1 pathway-related cytokines by cells pre-stimulated with whichever SeM-entrapped PLA nanospheres. Even so, lower difference between the Th1/Th2 type of immune response was observed in cytokines quantified in the culture of splenocytes of PLA-OA$_{SeM}$ nanospheres-immunised group, as the lowest IL2 and IFN-γ titres were detected when mice were treated with these formulation (P<0.031).



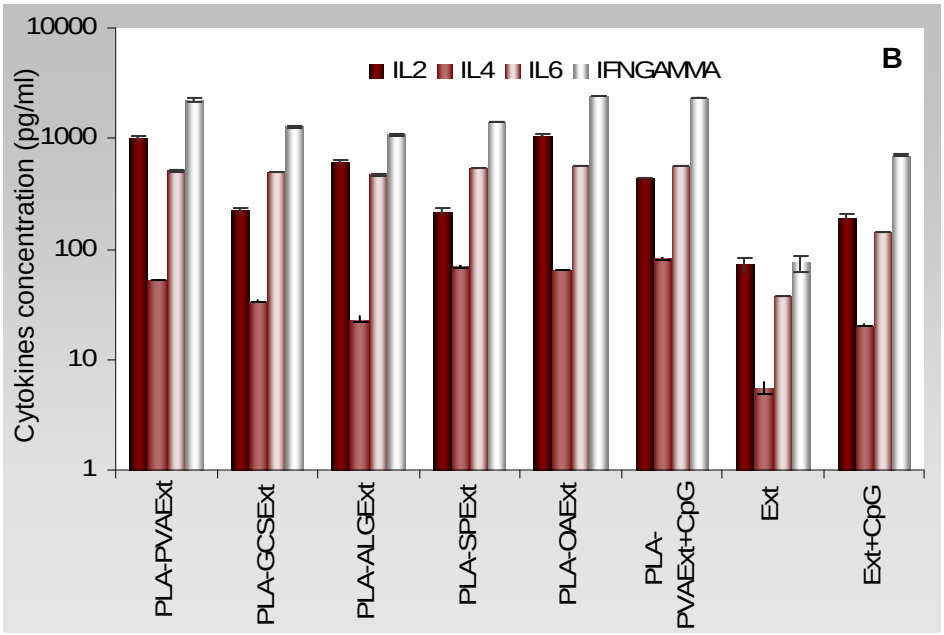


Figure 6.4– Cytokines (IFN -γ, IL2, IL4, IL6 and IL12) titres in supernatant of cultured splenocytes following their stimulation for 48 hours with 2 μg/ml of recombinant SeM (A) or *S. equi* extract (Ext, B), accordingly to antigen-loaded PLA nanospheres composition (mean ± S.D.; n=4).

Similarly to SeM-entrapped nanospheres, a mixed Th1 and Th2 immune responses were obtained in animals vaccinated with particles containing *S. equi* enzymatic extract, as all of these vaccine formulations induced detectable IL2, IL4, IL6 and IFN-γ levels. PLA-OA_{Ext} immunised groups produced the highest amount of the Th1 cell cytokines IL2 and IFN-γ quantified in all *S. equi* extract groups tested (P<0.007), which is in agreement with IgG2a titres (Figures 6.3C and 6.4). The improvement of IL6 quantified in splenocyte cultures of mice that have been vaccinated with PLA-OA_{Ext} test group was statistically significant among all vaccination groups, except when compared with PLA-PVA_{Ext}+CpG nanospheres immunised mice. IL4 highest concentration was quantified in mice treated with latter formulation (P<0.015) (Figure 6.4).

DISCUSSION

The development of an efficient vaccine is needed to guarantee horse protection against *S. equi* invasion, as 75% of those animals in convalescence present a protective immunity that can persist for 5 years or longer, mainly direct to SeM, although the nature of this defence is not yet fully understood [1, 2, 7, 13]. SeM binds to fibrinogen and prevents C3b factor deposition on bacterial surface, resulting in its antiphagocytic properties [11, 12, 18]. This *S. equi* M-like protein is therefore an antiphagocytic protein known as the most protective *S. equi* antigen, due to the specific anti-SeM opsonogenic antibodies found in convalescent animals. Consequently, an extensive research has been directed to the development of a vaccine based in this protective *S. equi* antigen and it is generally recommend horse vaccination against this disease. Nevertheless, despite the development of a high number of research projects, since the 1980s, directed to the production of an efficient vaccine, the results so far obtained with commercially available and widely used preparations are disappointing [2, 11]. These vaccines, frequently based on inactivated *S. equi* or SeM-rich extracts, alone or co-administered with adjuvants, have not only not contributed for the control of strangles, and consequently for horse protection against *S. equi* infection, but also induced strong adverse reactions [2, 7, 19]. Strepguard® co-administered with Havlogen® (Intervet Ltd), *Equi*vac® S and *Equi*vac 2 in 1 (Pfizer Animal Health Pty Ltd) are vaccines based on *S. equi* enzymatic extract proteins administered to animals by an i.m. injection in their necks. Since 1997, an avirulent *S. equi* genetically modified (Pinnacle®, Fort Dodge) is being commercialised in USA market. This intranasal (i.n.) vaccine has been able to reduce the clinical signs usually developed upon a *S. equi* infection, and was developed in order to stimulate systemic and mucosal immune responses comparable to that observed in convalescent animals. Despite eliciting the production of nasopharyngeal antibodies, Pinnacle® treated animals develop not only a short protective immune response, but also strong adverse reactions, which prevented its acceptance by EMEA [10, 18, 20]. In 2004, a vaccine consisting of an

attenuated strain of *S. equi* (Equilis StrepE®, Intervet Ltd) was approved in the UK and USA. The administration of *Equi*lis StrepE® resulted in a total protection of animals, but important adverse reactions were developed in the local of injection. As a result, this vaccine is now administered by submucosal injection in the upper lip. Besides, a short protection has been achieved in 50% of treated animals, which might limit its usefulness [2, 9, 10]. In fact, all Equilis StrepE® batches have been recently removed from the market, and its utilisation is only authorised in Africa [21]. Therefore, the development of an efficient and safe strangles vaccine is still to be achieved.

Some researchers believe that the effective protection of horses against strangles depends on the stimulation of multiple epitopes to induce high levels of local and systemic antibodies, which can be achieved by the administration of a mixture of *S. equi* cells wall immunogenic proteins [2, 22]. On the other hand, others try to clarify the bacterial infection mechanisms in order to identify further virulence factors as potential vaccine candidates [1, 18, 23].

The extensive research based on the study of mechanisms involved in the recognition of microorganisms and in their interaction with cells of the immune system, such as macrophages and dendritic cells, has contributed for the crescent identification of new adjuvants and consequently for the development of alternative vaccines [24, 25]. More recently, researchers are trying to identify new *S. equi* potent antigens, such as bacterial cell surface proteins, alone or in association, suitable for the induction of high level of antibodies and animals protection [1, 22].

Our study compares the immune responses elicited by an economic alternative *S. equi* extract to the highly purified recombinant SeM, when carried by polymeric particles. Their particulate form, along with their antigen depot effect is the main aspect to consider in the potential adjuvant of polymeric nanospheres, which has been extensively referred in the literature [24, 26]. The stimulation of a prolonged immune response is dependent on the

maintenance of structural integrity of proteins and immunogenicity during formulation, storage and also throughout their release to the biological fluids.

Spherical PLA particles containing PVA, GCS, OA, SP or ALG, presented a mean size diameter lower than 500 nm. Larger particles were obtained when GCS was used in particles formulation, which can be attributed to the higher viscosity of the primary w/o emulsion aqueous inner phase. As it was expected, in contrast with all other PLA nanospheres prepared in the present work, PLA-GCS nanospheres presented a positive charge, but all values were higher which means that the particles did not tend to agglomerate [27]. Surface charge influences particle uptake, being the positively charged those that are quickly phagocytosed, which seems to be a result of the ionic interactions established between positively charged particles and the negative charge of phagosomes. Particles containing OA presented the highest loading capacity, which in accordance with the results previously obtained by our group, when *S. equi* extract was entrapped in PLA nanospheres with similar composition [15]. This absorption enhancer was dissolved in the organic phase of the double emulsion w/o/w, and therefore the higher resultant viscosity might have prevented protein diffusion from internal aqueous phase, contributing for higher entrapment efficiency. PLA nanospheres surface modification by ALG is able to stabilise the secondary emulsion by limiting interchanges between external aqueous and the organic phase of the first emulsion, resulting in an improvement on antigen entrapment as it was obtained in our study, comparative to PVA stabilised carriers, which were the ones with lower amount of protein entrapment [25, 27]. Therefore, the inclusion of additional adjuvants had a beneficial effect in protein entrapment. Electrophoresis demonstrated that the protein integrity was maintained after entrapment (Figure 6.1). Once again it was evidenced that the double emulsion (w/o/w) solvent evaporation technique is safe and adequate to preserve *S. equi* proteins structural integrity and, as shown in previous studies by immunoblotting analysis, their immunogenicity [14-16]. Several groups have already demonstrated that solvent evaporation technique

does not change the activity and immunogenicity of antigens entrapped in the inner phase of the double emulsion. Particle physicochemical characteristics are in agreement with those normally presented by these types of nanospheres [27, 28].

Previous studies developed by our group have demonstrated the adjuvant potential of PLA nanospheres, adsorbed or encapsulated with *S. equi* enzymatic extract proteins, after their i.n. administration, especially when compared with those obtained with the *S. equi* antigens alone [15]. These promising results have encouraged us to investigate the use of this vaccine delivery system as the first polymeric carriers for the recombinant antiphagocytic SeM. Therefore, for the first time, recombinant SeM proteins were entrapped in polymeric particles and their immunogenicity was evaluated and compared to those elicited with *S. equi* cells wall proteins. Polymeric particles are extremely versatile systems and their formulation with polymers with complementary characteristics, their association to adjuvants with distinct mechanisms of action, and the entrapment of multiple antigens are currently some of the commonly used approaches in order to optimise these carriers to obtain the desirable immune responses [24, 29]. As a result, PLA nanospheres containing the mucoadhesive polymers GCS [30] and ALG [27] to improve the time of particles residence nearby cells, and absorption enhancers SP [31] and OA [32, 33] were used in order to evaluate their effect in the cellular and systemic immune responses developed against the *S. equi* antigens studied.

Despite particles composition and the type of antigen, humoral immune responses were generally improved by nanospheres suspension, when compared with free antigens, and it was not observed the development of adverse reactions in any group of test. In order to evaluate the synergic effect of the inclusion of a second adjuvant, CpG was chosen as it is a potent humoral, but also mucosal and cellular immune responses adjuvant by increasing preferentially the activity of Th1 cells. Nevertheless, at the end of

the experiment, IgG levels obtained with PLA-GCS$_{SeM}$ nanospheres were statistically different from those elicited by CpG adjuvantised formulation ($P<0.005$), and similar levels were induced by all *S. equi* extract-entrapped particulate systems. These results also reflect that the adjuvant effect of polymeric carriers tested, more specifically their influence in Th1/Th2 bias in immune response, was dependent in part on the type of antigen that is entrapped in particles with similar composition, which is generally agreed and has been extensively discussed in the literature [34]. Chitosan, besides its positive charge, has the ability to open tight junctions, increasing the paracellular transport and therefore the amount of antigen taken up by cells [35]. Moreover, particles polymeric composition, size, antigen load, route of administration and association to other adjuvants are factors that mainly influence the uptake of these carriers, their transport to lymphatic nodes and interaction with tissues [36]. It is possible to state that, at the end of the study, PLA-GCS$_{Ext}$ nanospheres induced IgG1 titres statistically higher than those elicited by PLA-ALG$_{SeM}$, but no other difference was obtained among all other particulate carriers. PLA-PVA$_{SeM}$, PLA-ALG$_{SeM}$ and PLA-OA$_{SeM}$ formulations elicited higher IgG2a antibodies levels than the correspondent *S. equi* extract-entrapped particles.

A more balanced IgG2a/IgG1 immune response was obtained after i.m. administration of PLA-SP$_{SeM}$, PLA-GCS$_{Ext}$, PLA-OA$_{SeM}$ and PLA-OA$_{Ext}$ particulate systems. The CpG admixture with *S. equi* antigens entrapped in PLA-PVA nanospheres increased mainly the Th1 immune response pathway, contributing for a more equilibrated immunity than of particles alone. Overall antibody response, it seems that the co-administration of CpG along with PLA nanospheres stabilised with PVA did not led to differential degree of enhancement in antibodies titres. Even so, PLA-PVA$_{SeM}$+CpG induced the highest IgG1 titres among all other immunisation groups, despite particles composition or type of antigen. CpG biased the immune response towards a Th1 response relatively to the PLA-PVA nanospheres encapsulated either with SeM or *S. equi* extract proteins, as measured by IgG subclass analysis.

Therefore, the addition of this potent adjuvant to the PLA nanospheres provided an improvement of antibody response elicited by *S. equi* antigens-entrapped PLA-PVA nanospheres alone. Even so, mainly formulation containing the mucopolysaccharides GCS and ALG, in the case of SeM, or GCS and both absorption enhancers SP and OA, for those entrapping *S. equi* extract, were able to improve IgG2a responses when compared with that elicited by PLA-PVA+CpG formulation. From these results it seems that the immune response elicited by nanospheres was not influenced by particle size differences, as PLA-GCS_{SeM} and PLA-ALG_{SeM} were larger than the remaining PLA carriers.

Cytokines secreted by splenocytes of mice pre-immunised with polymeric formulations corroborate the IgG subtypes titres measured in the serum of animals. A comparable pattern was obtained after i.n. immunisation of *S. equi* extract-entrapped PLA nanospheres, in a previous study performed by our group [15]. IL4 cytokine levels were the lowest when compared with other cytokines assessed in splenocytes supernatants, while the concentration of IFN-γ was the highest for all particulate formulations. These results indicate a predominant Th1 induction, which is in accordance with results previously obtained for these *S. equi*-entrapped carriers after their i.n. administration [15]. In fact, other researchers have reported that antigens carried in a particulate form favour the stimulation of Th1 immune response [37, 38]. No significant levels of cytokines was detected in culture of splenocytes collected from mice treated with antigen in the soluble form, which supports that the antigen co-delivery in nanospheres was vital for the stimulation of measurable Th1, but also Th2 response throughout the period of study.

CONCLUSIONS

The present studied has showed that vaccination with SeM and *S. equi* extract antigens-entrapped in PLA nanospheres containing GCS, OA, SP and ALG gave a considerable antibody and cellular immune responses, after i.m. administration in mice. In addition, *S. equi* extract and SeM-loaded PLA-GCS nanospheres induced not only IgG and IgG2a subtypes levels, but also IL2 and IFN-γ cytokines titres at least as high as those elicited by PLA-PVA nanospheres admixed with the potent CpG. Results suggest that these PLA carriers are suitable to target phagocytic cells such as macrophages or dendritic cells, being promising candidates for an effective and safe vaccine against strangles, as it did not develop adverse reactions as a consequence of *S. equi* antigens loaded PLA nanospheres.

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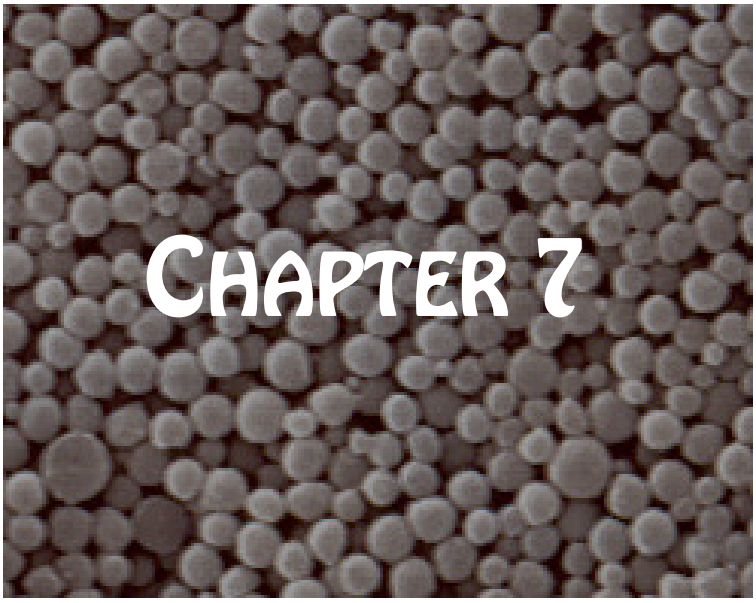


Concluding remarks and perspectives

OVERVIEW

A review of the literature brings together some recent ideas about possible strategies to control strangles (Chapter 1). In particular, it seems that despite extensive efforts regarding prevention of large outbreaks of this endemic disease, *S. equi* infection remains spread worldwide, causing important economic loss to horses industry, important for the financial equilibrium of some countries, not only due to the cost of treatment and occasionally the death of affected animals, but also quarantine measures needed to be adopted in order to prevent the contamination of other animals.

Affected populations present high morbidity levels and infection may cause chronic illness. Despite the recent development of new and sensitive diagnostic tests which are currently available in the market, strangles long convalescent period and *S. equi* infected animals presenting no signs of the disease are limiting the effective contribution of these tests for the control of this endemic infection in horses.

Several research groups in industry and academic institutions all over the world are trying to understand the complex pathogenesis of strangles, which along with *S. equi* and *S. zooepidemicus* genome-sequencing, are contributing for the identification of several virulence factors and better understand their roles at different stages of this infection. It has been strongly recommended that improved strategies to control strangles include the development of efficient vaccines, as the commercially available ones have not made major improvements in addition to frequently produce strong adverse effects and induce poor and short immunity.

Previous studies have suggested that blood circulating antibodies (systemic immune response) are not as important for animal protection as those locally produced in the nose and throat mucosae, suggesting the nasal mucosa as a promising immunisation route, even if the first response complements the latter. As a result, protective immune response may be triggered by using *S. equi* antigens, combined or isolated, associated to a mucosal adjuvant.

The therapeutic use of particulate drug carriers for immunisation purposes is one of the most promising strategies to combat infectious diseases. These vehicles, among other advantages, are able to release antigen in a sustained manner, which allows a depot effect and consequently enhances their immunoadjuvant properties. However, the association of protein antigens to polymeric particulate carriers often presents limitations related to antigen integrity and long-term stability, but previous experiments have shown that adequate modifications of those formulation methods are able to minimise antigen instability and preserve its biological activity. In fact, *S. equi* whole cells-entrapped in PLGA microparticles maintained their antigenicity as they protected mice against an experimental *S. equi* infection. These results obtained in a previous work by our research group have shown that particulate systems may be a potential adjuvant for *S. equi* antigens. Moreover, the delivery of *S. equi* antigens need to receive further attention in order to fully characterise the elicited immune responses, as well as to develop alternative carriers, cheaper but still effective, for new *S. equi* antigens, as PLGA is an expensive polymer that may compromise the future success of this veterinary vaccine.

Therefore, the major goal of the studies herein presented has been to develop alternative polymeric carriers able to be associated to *S. equi* antigens, isolated or in the form of SeM-rich extracts, without compromising their structure integrity and antigenic properties, not only during formulation processes, but also after their administration *in vivo*. After obtaining those polymeric particles with the desired physicochemical characteristics, *in vivo* studies were performed to assess the effect of polymeric composition, antigen type of association, administration route, *S. equi* protein antigen nature and co-administration of different adjuvants in the immune response.

To the best of my knowledge, Chapter 3 describes for the first time the adsorption of *S. equi* protein antigens onto the surface of particulate carriers. Non-aggregate PCL microspheres (< 3 μm) were formulated by (w/o/w)

emulsification solvent evaporation method and *S. equi* enzymatic extract proteins were successfully associated to their surface, with no evidence of any change in protein pattern of migration. Therefore, non-toxic positively and negatively charged particles, stabilised respectively by CS and PVA, were administrated by i.m. route to mice in order to evaluate the adjuvant properties of those adsorbed systems. This was in fact a preliminary study, in order to confirm their immunoadjuvant properties after association to *S. equi* antigens. The systemic (IgG and IgG subtypes) antibody and cytokine levels confirmed that PCL microspheres, despite their surface charge were able to enhance significantly the immune response, when compared to the soluble antigens. Moreover, cytokines attributed to a predominant Th1 immune response were more pronounced when the CS adjuvanted PCL microspheres were used. Therefore, once it was confirmed the potential adjuvant of these polymeric carriers over 300 days and after one single dose, it was important to test those PCL carriers for nasal immunisation in a mice model.

As size matters, it was performed a scale-down in particles size (Chapter 4), and the effect of mucoadhesive polymers (GCS and ALG) and absorption enhancers (SP and OA) in their adjuvant properties was further evaluated. Composition influenced particle size, but all formulations presented VMD lower than 500 nm. In this study, all *S. equi* antigen-adsorbed or -encapsulated PCL nanospheres were able to increase the level of concentration of systemic and mucosal antibodies over 12 weeks. Similarly, cytokines produced by splenocytes obtained from those immunised groups, after being co-stimulated with *S. equi* proteins, indicated a mixed Th1/Th2 response. As it was expected, the antibody profile levels were distinct for adsorbed and entrapped systems, which is in accordance with the *in vitro* release studies performed with those nanospheres.

Besides being extensive and complex the integrations and complete comparison of humoral, local and cellular immune response elicited by *S. equi*-adsorbed and -entrapped PCL nanospheres, it is possible to conclude that

along with particles composition, the type of antigen association determined the nature of the immune responses obtained. In fact, particles presenting exactly the same polymeric composition exhibited a different antigen profile accordingly to the type of antigen association. Therefore, *S. equi* antigen-entrapped PCL-ALG particles seem to be the one that overall induced a more equilibrated immune response, while among the adsorbed systems that role seems to better suit the antibodies and cytokines levels induced by PCL nanospheres formulated with GCS. It is interesting to see that, besides all particulate systems did not fail to induce immune response against *S. equi*, the mucoadhesive polymers presented an additional adjuvant effect over the absorption enhancers tested. Moreover, it seems that the formulated PCL nanospheres are potent adjuvants as it was not identified a synergic adjuvant effect when CTB was co-administered with PCL-PVA carriers.

The i.n. administration route and nanometric PCL particles (Chapter 4) elicited antibodies titres higher than those obtained when PCL microspheres were administered by s.c. route (Chapter 3). Therefore, the next experimental study was performed using PLA nanometric particulate carriers (Chapter 5). The aim was to compare the adjuvant potential of more easily formulated PLA particles for *S. equi* antigens, with that presented in the previous study for the hydrophobic PCL (Chapter 4). Similarly to PCL nanospheres, this study confirmed the potential adjuvant of *S. equi* antigens-loaded PLA nanospheres, and again the mucopolysaccharide GCS induced the most prominent immune response. Locally produced IgA antibodies elicited by PLA nanospheres were higher than those detected in lung washes of mice immunised with loaded PCL nanospheres. Similar tendency was observed for systemic antibodies. Therefore, the positive relation between hydrophobicity and cellular uptake could not be confirmed by our studies, which may be due to a slower release of the antigen from PCL nanospheres, as the immune responses were followed only till 12 weeks after priming. In order to confirm these observations, a long-term study should have been performed, which unfortunately was not possible due to time and study cost limitations. In

addition, a thorough hydrophobicity characterisation could have helped to clarify this point.

New generation antigens are obtained by biotechnological methods and include the production of recombinant proteins previously identified as potential virulence factors of strangles causal agent. SeM is still known as the most protective *S. equi* antigen and apparently a protective immunity requires the production of specific anti-SeM antibodies, although the nature of this defence is not yet fully understood. This protein has been extensively studied and characterised by Prof. Timoney, Kentucky USA. Due to its high cost, a small amount was purchased, just the enough to perform a comparative and preliminary study (Chapter 6). Besides the nasal mucosa being the desired target, the first association of the pure recombinant SeM to polymeric particulate carriers would have to be evaluated by i.m. route. Fortunately, particle production formulation protocol allowed the formulation of all particulate systems used in previous chapters, with a theoretical loading of 1% (w/w). At the end of the experiment, PLA-GCS and PLA-OA nanospheres containing *S. equi* enzymatic extract constituted alternative cost-effective formulation to those loaded with the more expensive antigen product SeM, being inclusive able to induce a balanced IgG2a/IgG1 immune response. Similarly to CTB, CpG co-administered to *S. equi* antigens loaded PLA-PVA nanospheres generally did not led to differential degree of enhancement in antibodies titres when compared with the remaining groups vaccinated with particulate systems, except PLA-PVA ones.

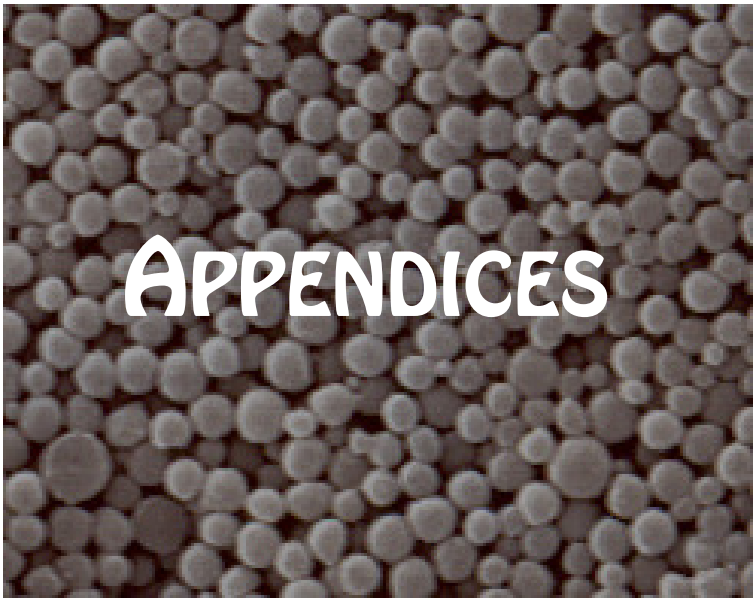
Finally, it is possible to conclude that the main goal of this thesis was achieved as an alternative polymeric carrier for delivery of *S. equi* antigens able to induce both mucosal and systemic immune responses was developed and characterised.

FUTURE DIRECTIONS

Taken together, the results of this thesis confirmed the versatility of polymeric particles and indicated their ability to protect *S. equi* antigens from extracellular degradation, but also to be taken up by APCs. Systemic, mucosal and cellular immune responses assessed 12 weeks after animal vaccination show that polymeric nanoparticles were able to carry and sustain release *S. equi* antigens over an extended period. They also modulate antigens processing and/or presentation by the immune system. Additionally, the delivery of *S. equi* antigens associated to polymeric nanoparticles should receive further attention in order to characterise the mechanisms responsible for their uptake by APCs and strong activity after being loaded in particles. It would be interesting to study the body distribution of those particles after being administered by i.n. route.

The particulate carriers developed during this PhD thesis are promising adjuvants for a safe vaccine against strangles, with no toxicity issues associated to their utilisation, in contrast to other adjuvants that have been associated to *S. equi* antigens. These studies bring new insights into the strangles prevention field, but unfortunately the study was not concluded. In fact, the results could have been improved if a challenge study had been performed. The potent adjuvant properties of polymeric particles formulated with GCS could have been confirmed by the experimental infection of vaccinated animals, in order to assess in fact, the desired conclusion: the formulation is protective! Unfortunately, those infectious studies were not allowed to be conducted in the facilities of London School of Pharmacy, one of this PhD host universities, where the *in vivo* work was developed. Even so, these challenge studies must be done in the future in order to further expand their use in the development of a strangles vaccine. As it is an expensive and time-consuming experiment, GCS adjuvanted polymeric formulation should be selected to confirm if the obtained immunity is protective.

It is as well accepted that the immunological responses induced by mice and horse are different, and therefore the choice should be done in the target animal. Therefore, the next step would be the test of this selected *S. equi* antigen-loaded polymeric formulations in a horse model. In fact, this work has already contributed for the establishment of a interesting collaboration with Prof. Ian-Ingmar Flock, Karolinska Institut, Sweden, whose lab has been extensively involved in the identification of potent *S. equi* protein antigens (SclC, CNE, EAG, FNZ, SFS), which have been tested in mice and horses. Further application of these particles is expected to be the study of their adjuvant properties for those recombinant *S. equi* antigens, as well as the test of the best formulation obtained in this thesis for less expensive *S. equi* enzymatic extract in horses.


APPENDICES

APPENDIX I

RESIDUAL SOLVENT QUANTIFICATION

In order to assess the dichloromethane (DCM) residual solvent present in particles after freeze-drying, a Nuclear Magnetic Resonance (NMR) spectroscopy technique was undertaken. Briefly, 1 ml of acetic acid-d4 was added to plain particles (30 mg) and sodium formate (20 mg; Sigma Aldrich Co., UK) previously accurately weighed into the same vessel. Both samples (PCL-PVA, PCL-CS0.75L, and PCL-ALG) and standards (5 µl, 10 µl, and 15 µl of DCM) were completely dissolved by sonication for 15-30 min, and the solution obtained was transferred into a 5 mm NMR tube. The ^{1}H-NMR spectra were acquired on a Bruker spectrometer in which the temperature was set to 300 K and was recorded a high field proton NMR (≥400 MHz) using a 45 degree pulse and a 60 second pulse repetition time. The spectrum was referenced by setting the chemical shift of the methyl signals due to residual acetic acid to 2.03 ppm. Accurate integrals were obtained for the current standard and residual DCM from the spectra.

STRUCTURAL INTEGRITY OF ADSORBED ANTIGENS

The freeze-dried microspheres were assessed by SDS-PAGE, in order to evaluate the effects of processing parameters on the structural integrity of the adsorbed proteins. In brief, antigen was extracted from the different preparations by dispersing 10 mg of particles in 200 µl of 5% (w/v) PBS-SDS (pH 7.4), which were followed by 2 hours of incubation at 37^{o}C in an orbital oven shaker. Samples were then vortexed for 30 seconds in order to get a homogeneous dispersion and then heated for 95^{o}C/3 min, with the Laemmlie sample buffer (Bio-Rad, UK). Formulations (20 µl), *S. equi* enzymatic extract proteins prior to adsorption (10 µl of a 2.5 mg/ml solution) and broad range of prestained SDS-PAGE standards (10 µl; Bio-Rad, UK) were loaded onto

10% (w/v) polyacrylamide (Bio-Rad, UK) mini-gel and run with a constant voltage of 100 V for 120 minutes using a Bio-Rad 300 power pack (Bio-Rad, Hercules, CA, USA). The extracted proteins are shown on the gel prior to adsorption as a series of broad bands. Gels were visualised using SimplyBlue™ SafeStain solution (Invitrogen, USA), and imaged using a UVP gel scanning camera.

IN VITRO CYTOTOXICITY OF PCL MICROSPHERES

Cytotoxicity of PCL microspheres, specifically PCL-PVA, PCL-ALG, PCL-CS0.75L, PCL-CS0.75M and PCL-CS0.75H, to Mouse BALB/c monocyte macrophage cells (J774A.1 cells line, American Type Culture Collection; ATCC#TIB-67) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, in which the absorbance was measured at 570 nm by Dynex MRX Microplate Reader (Dynex, UK). All the experiment was done in triplicates.

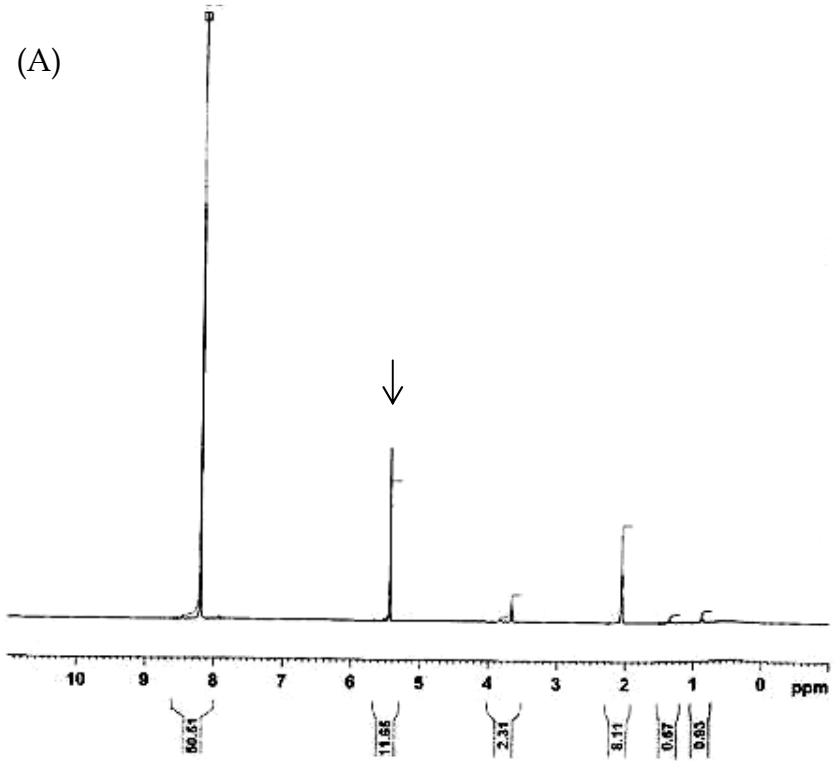
These cells were maintained in 75 cm^2 vented tissue culture flasks, in Dulbecco`s modified Eagle's (DMEM; Sigma Aldrich Co., UK) medium with 4 mM glutamine (Sigma Aldrich Co., UK) plus 10% fetal bovine serum (FBS; Gibco BRL, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, Poole, Dorset, UK). Briefly, cells (100µl) were seeded in 96-well plate (Fisher, UK), at 5000 cells/well in a complete DMEM, and let to attach for 24 hours in a humidified incubator at 5% CO_2. The media was then removed, and 100 µl of diluted antigen, PEI (polyethyleneimine) and plain particles, all previously dissolved or suspended in serum free DMEM at different concentrations (5 mg/ml, 2.5 mg/ml, 1 mg/ml, 0.5 µg/ml, 0.1 µg/ml, 0.025 µg/ml and 0.01 µg/ml), were loaded onto cells, shaken gently and left for 24 hours at 37°C in a humidified incubator at 5% CO_2. A solution (25 µl) of MTT (5 mg/ml; Sigma Aldrich Co., UK) was added to cells and the plate was again left in the CO_2 incubator at 37°C, for 4 hours. The media was then removed with a needle and syringe and the cells were washed with 200 µl of sterile PBS (OXOID,

UK). After that, 200 µl of dimetilsulphoxide (DMSO; Sigma Aldrich Co., UK) were added and pipetted up and down in order to dissolve the eventually formed crystals. The plate was subsequently incubated at 37ºC for 5 minutes to dissolve air bubbles and after that transferred to a Dynex MRX Microplate Reader (Dynex, UK). The optical density of solubilised formazan product was determined using a spectrophotometer (with a 550 nm and 620 nm filter as a reference). Results were expressed as the percent of cell survival (OD of exposed *versus* OD of control non exposed cells).

APPENDIX II

^{1}H-NMR spectra of standards ((A) 5 µl, (B) 10 µl and (C) 15 µl DCM) and samples ((D) PCL-CS0.75L, (E) PCL-ALG, (F) PCL-PVA microspheres) (Chapter 3).

(A)



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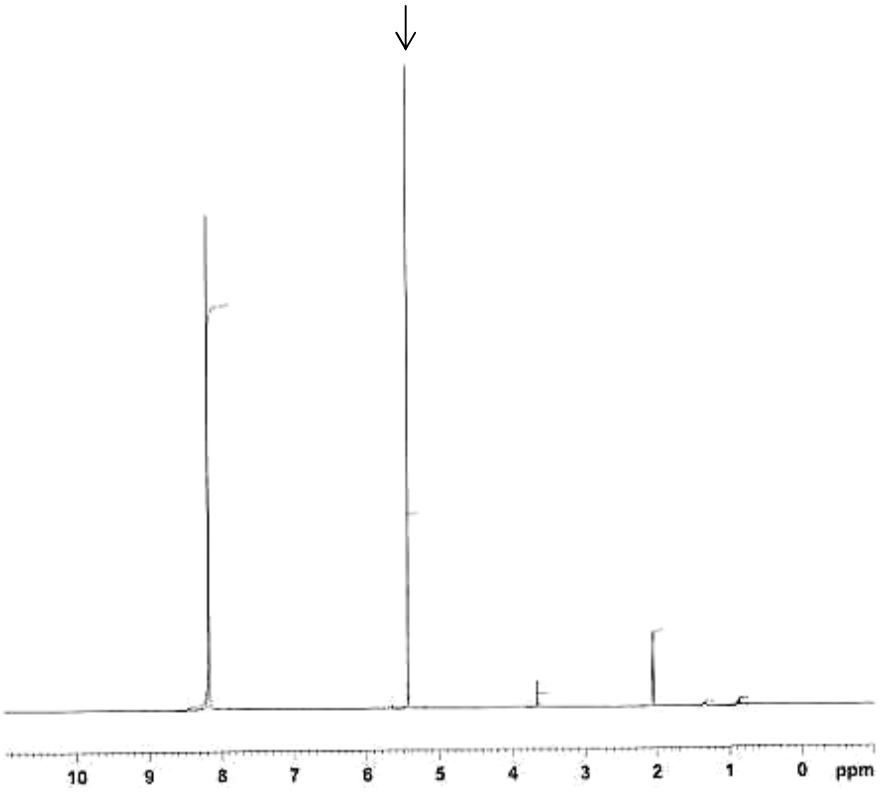
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4
3
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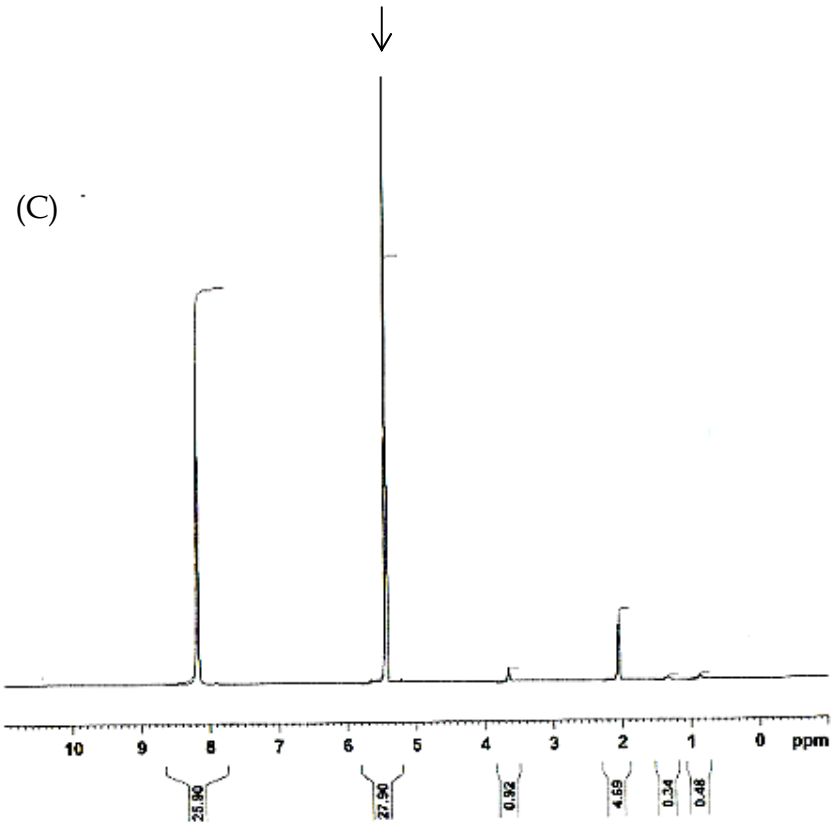
1
0
ppm

(C)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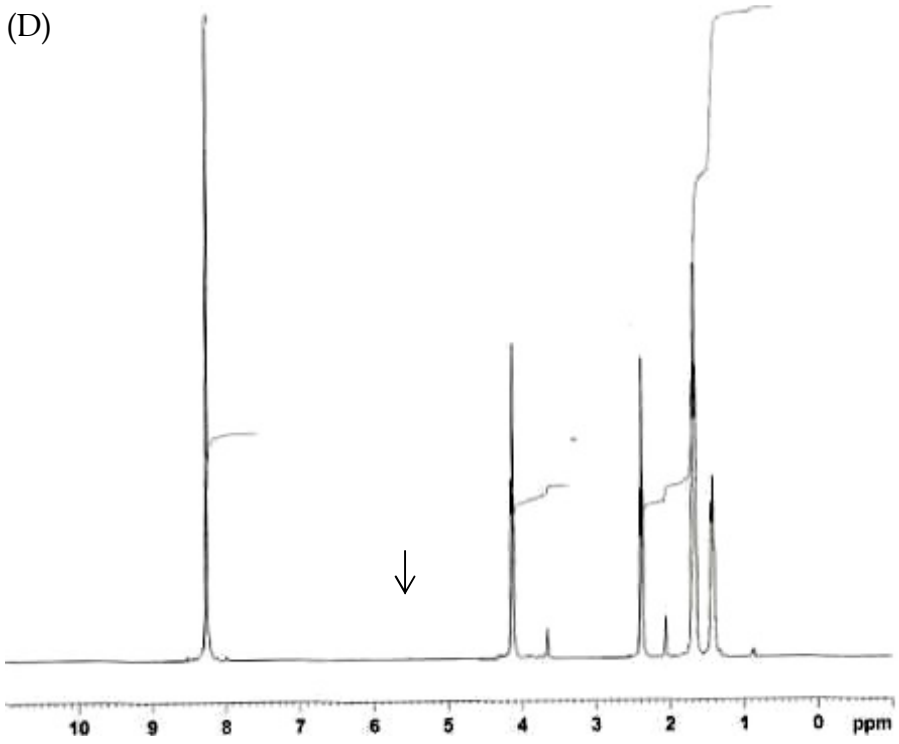
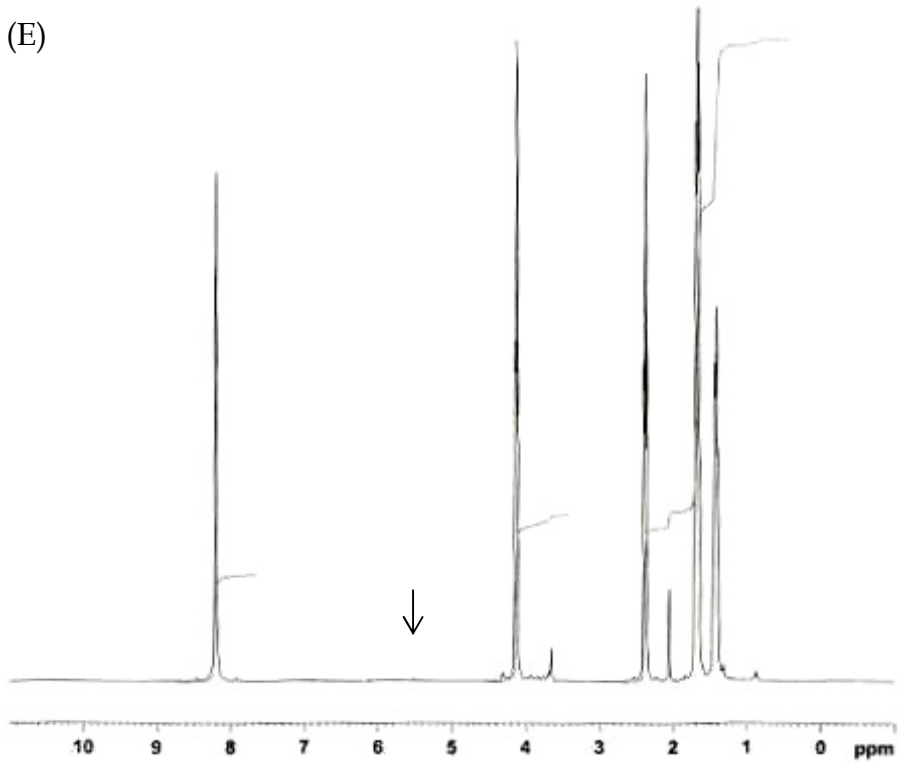
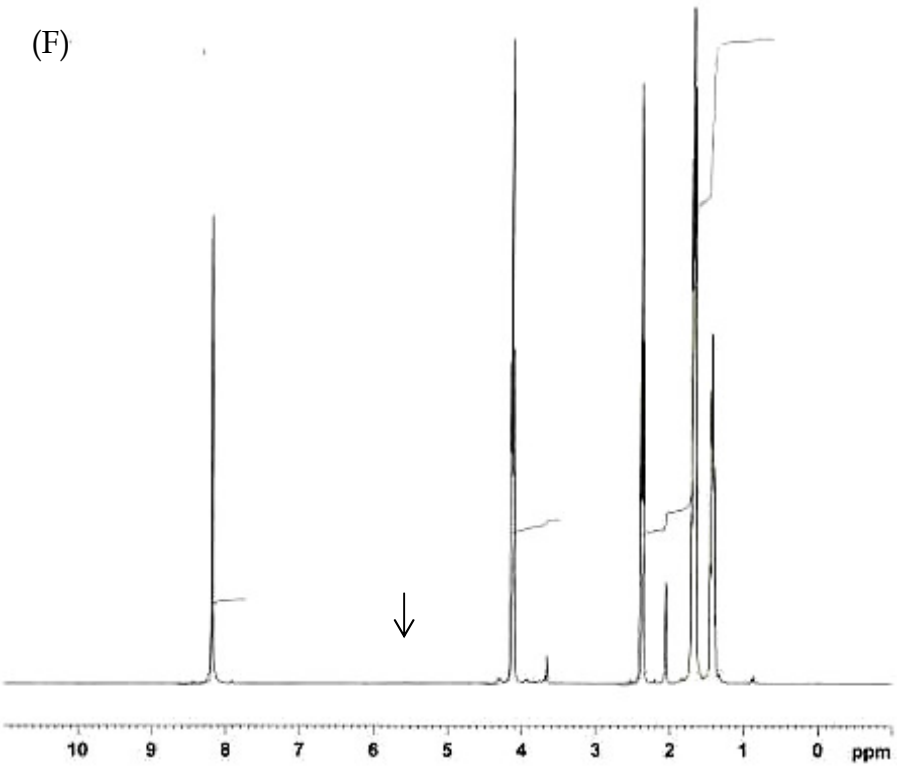
(D)

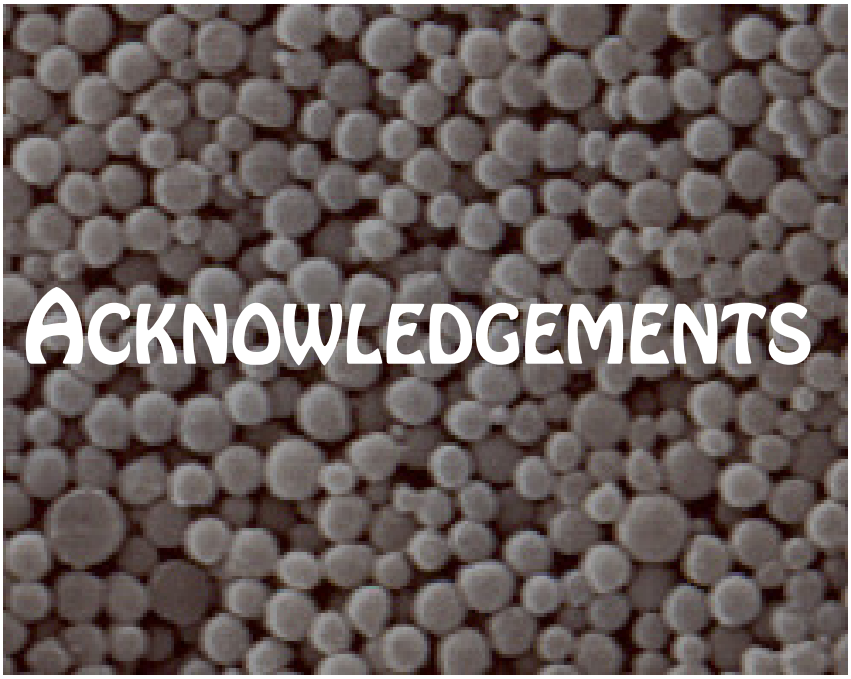


(E)



(F)

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