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Surface modified polymeric nanoparticles for immunisation against equine strangles

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ABSTRACT

The successful development of particulate vaccines depends on the understanding of their physicochemical and biological characteristics. Therefore, the main purpose of this study was to develop and characterise stable surface modified poly(lactic acid) (PLA) nanoparticles, using polyvinyl alcohol (PVA), alginate (ALG) and glycolchitosan (GCS) containing a *Streptococcus equi* enzymatic extract adsorbed onto the surface. The characterisation of the preparations and a physicochemical study of the adsorption process were performed. The adsorption of *S. equi* proteins is a rapid process reaching, within 1 h, maximum adsorption efficiency values of 75.2 ± 1.9% (w/w) for PLA–PVA, 84.9 ± 0.2% (w/w) for PLA–GCS and 78.1 ± 0.4% (w/w) for PLA–ALG nanoparticles. No protein degradation was detected throughout the formulation procedures. As expected from a complex mixture of proteins, adsorption data suggest a Freundlich-type of equilibrium with regression coefficients (r^2) of 0.9958, 0.9839 and 0.9940 for PLA–PVA, PLA–GCS and PLA–ALG, respectively. Desorption studies revealed a burst release within the first 6 h, for all formulations, followed by a sustained release profile. Nanoparticle surface modification with GCS improved the sustained release profile, as 20% of protein remained attached to the particle surface after 30 days. The results show that adsorption is an alternative method for the production of *S. equi* antigen carriers for vaccination purposes.

1. Introduction

Strangles is an infectious disease that affects the upper respiratory tract, mainly the nasopharynx and lymphatic nodes of the *Equidae* (Waller and Jolley, 2007). Despite extensive efforts regarding prevention of large outbreaks of this endemic disease, *Streptococcus equi* infection remains spread worldwide, causing important economic loss to horse industry, not only due to the cost of treatment and occasional the death of affected animals, but also to the quarantine measures needed to prevent the contamination of other animals (Sweeney et al., 2005). In addition, the recent development of new and sensitive diagnostic tests, which are currently available on the market, strangles long convalescent period, and asymptomatic *S. equi*-infected animals are limiting the effective contribution of these tests for the effective control of the disease (Sweeney et al., 2005; Waller and Jolley, 2007). Due to its complex pathogenesis, it has been strongly recommended that improved strategies to control strangles should include the development of efficient vaccines, as the commercially available ones induce poor and short immunity and frequently produce strong adverse effects (Waller and Jolley, 2007).

S. equi antigens are often weak immunogens, requiring multiple administrations and association with suitable adjuvants (Sweeney, 1996; Flock et al., 2004; Aguilar and Rodríguez, 2007). The use of adjuvants in veterinary vaccines is less restricted than that for humans, and a large number of different types and formulations of adjuvants are currently used in licensed veterinary vaccines, compared to only three adjuvants licensed for human vaccine use. Nevertheless, the emphasis on subunit or inactivated vaccines that meet the desired criteria of a perfect vaccine has resulted in a critical need for better adjuvants and delivery systems (Bowersock and Martin, 1999; Meeusen et al., 2007).

It is well known that some antigens in their soluble form are not recognised by APCs and therefore do not induce a protective immune response (Bramwell and Perrie, 2006). Consequently, antigens responsible for the stimulation of these responses against certain pathogens can be immobilized or associated to controlled release particulate systems. These are taken up by APCs, processed and transported to the lymph nodes and other immuno-competent organs, based on a combination of mechanisms (Ludewig et al., 2000; Eyles et al., 2001; Shen et al., 2006; Aguilar and Rodríguez, 2007).

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The therapeutic use of particulate carriers for the development of protective immune responses is currently one of the most promising strategies to fight infectious diseases (Bramwell and Perrie, 2006). Their particulate nature along with the depot effect will determine the induction of the immune response (Aguilar and Rodríguez, 2007; Eyles et al., 2003). Many publications have justified their application in the induction of local and systemic immune responses, capable of protecting animals against numerous pathogens (Eyles et al., 2001; Bramwell and Perrie, 2006; Shen et al., 2006). However, biodegradable and polymeric particles may present some problems, such as low loadings, loss of antigen activity during the encapsulation process and difficulty in controlling the release of the active protein, especially when encapsulating larger and fragile molecules (Benoit et al., 1999; Zhu et al., 2000). In fact, some limitations have risen as it is necessary for the encapsulation of high amounts of macromolecules, without change of their structural integrity or antigenic activity. The development of new vaccine carriers is therefore 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. 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 encapsulation, since it has been able to induce strong immune responses to several antigens, including those of *S. equi* (Almeida et al., 1993; Bramwell and Perrie, 2006; Aguilar and Rodríguez, 2007; Florindo et al., 2008). When compared to encapsulation, loading by adsorption procedures avoids harsh formulation conditions, such as the contact of protein antigens with organic solvents, high shear agitation and the low pH environment resulting from the degradation of poly(lactic acid) (PLA) and poly(lactide-co-glycolide) (PLGA) (Bramwell and Perrie, 2006; Jiang et al., 2005).

Therefore, the main purpose of this study was to develop and characterise stable polymeric nanoparticles able to be used as *S. equi* antigen carriers and adjuvants. The adsorption of a *S. equi* enzymatic extract onto surface modified PLA nanoparticles was carried out and analysed in order to understand and control the complex interactions involved in the absorption phenomenon.

2. Materials and methods

2.1. Materials

Poly(lactic acid) (PLA, average molecular weight (MW) 2 kDa), polyvinyl alcohol (PVA, MW 13–23 kDa, 87–89% hydrolyzed), alginate low viscosity (ALG), glycolchitosan (GCS) and sucrose were supplied by Sigma–Aldrich Co., UK. Dichloromethane (DCM) was purchased from BDH Laboratory Supplies, UK. Bicinchoninic acid (BCA) kit was provided by Sigma–Aldrich Co. UK. *S. equi* subsp. *equi* (strain LEX) ATCC 53186 was a kind gift from Prof. J.F. Timoney (University of Kentucky, USA).

Nuclear magnetic resonance (NMR) tubes were obtained from Wilmad/Lab Glass and acetic acid-d_4 from Cambridge Isotope Laboratories, Inc., UK.

All other chemicals were of reagent grade and used as purchased.

2.2. Nanoparticle preparation

PLA (2 kDa) nanoparticles were prepared by the double emulsion (w/o/w) solvent evaporation method, as described elsewhere (Conway and Alpar, 1996). Polymer was dissolved in dichloromethane and emulsified with a 10% (w/v) polyvinyl alcohol (PVA) solution by homogenization at 24,000 rpm for 2 min. The w/o emulsion was emulsified into PVA (PLA–PVA) or 0.75% (w/v) low viscosity ALG (PLA–ALG) solutions to prepare the negatively charged nanoparticles, while 10% (w/w) GCS solution was used for the formulation of positively charged nanoparticles (PLA–GCS). The solvent was evaporated for 4 h and particles were harvested by centrifugation (20,000 rpm/20 min, 10–15 °C; Beckman J2-21 Hi speed centrifuge) and freeze dried.

2.3. Physicochemical characterisation

The volume mean diameters (VMD) of PLA nanoparticles were determined by photon correlation spectroscopy (PCS; Malvern ZetaSizer, Malvern Instruments, UK provided wit Dispersion technology software). PLA nanospheres were dispersed in double-distilled and filtered water (0.2 μm Whatman filters; refraction index 1.330; viscosity 0.888 cP, 25 °C) were placed in a cuvette for size measurements. The determination of the zeta potential was performed by anemometry, using the Malvern ZetaSizer (Malvern Instruments, UK) following the dispersion of particles 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%. Each measurement was performed in triplicate.

2.4. Residual solvent quantification

A nuclear magnetic resonance (NMR) spectroscopy technique was used to assess the dichloromethane (DCM) residual solvent present in particles after freeze-drying. Briefly, 1 ml of d_4-acetic acid was added to accurately weight plain nanoparticles (30 mg) and sodium formate (20 mg, Sigma–Aldrich Co., UK). Samples (PLA–PVA, PLA–GCS and PLA–ALG) and standards (5 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 a high field proton NMR (≥400 MHz) was recorded 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 for the current standard and residual DCM were obtained from the spectra.

2.5. S. equi enzymatic extract adsorption

Antigens were prepared as previously described (Florindo et al., 2008). Briefly, an *S. equi* CF 32 (ATCC53185, virulent strain) culture was harvested and treated with mutanolysin and lysozyme (Sigma–Aldrich Co., UK) to extract out the cell wall proteins. Adsorption studies of *S. equi* extract proteins onto plain PLA–PVA, PLA–GCS and PLA–ALG nanoparticles were undertaken in order to evaluate the influence of time and media in the adsorption process. To assess the influence of time, 10 mg of PLA nanoparticles were incubated up to 48 h with 2000 μg/ml of an aqueous *S. equi* extract solution (1 ml). At different periods of time (30 min, 1 h, 2 h, 4 h, 6 h and 9 h), particle suspensions were centrifuged and the amount of proteins adsorbed onto the particles was indirectly determined by the quantification of total protein concentration in the supernatants, using the BCA protein assay (Pierce). Plain nanoparticles (without antigen) were used as a control and a calibration curve was performed using a series of protein standards.

The extension of the *S. equi* protein adsorption was evaluated 1 h after PLA nanoparticle incubation in several media containing a similar amount of *S. equi* extract proteins: double-distilled water, NaCl 0.9% (w/v) pH 7.4, phosphate buffer saline pH 7.4 (PBS) or 20 mM Tris–HCl/NaCl pH 8.0 buffer.

To perform the physicochemical study of the adsorption process, nanoparticles (10 mg) were incubated for 1 h with a *S. equi* extract

solution (50–5000 μg/ml) at 37 °C and the total amount of protein associated to particles was quantified as mentioned above.

2.6. Analysis of S. equi protein extract

The freeze-dried nanoparticles were assessed by SDS-PAGE, in order to evaluate the effects of processing parameters on the structural integrity of the adsorbed proteins. In brief, antigens were 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 h of incubation at 37 °C in an orbital oven shaker. Samples were then vortexed for 30 s in order to get a homogeneous dispersion and then heated for 95 °C/3 min, with the Laemmlie Sample Buffer (Bio-Rad Laboratories, UK). Formulations (20 μl), enzymatic extract proteins prior to adsorption (10 μl of a 2.5 mg/ml solution) and broad range pre-stained 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 min 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.

2.7. Antigen desorption studies

S. equi antigens adsorbed onto PLA–PVA, PLA–GCS and PLA–ALG nanoparticles (10 mg) were dispersed in PBS (pH 7.4) buffer and incubated in an orbital shaker at 37 °C. At each time point, one tube was collected, centrifuged and the total protein concentration was determined in the supernatants by the BCA protein assay.

2.8. Statistical analysis

ANOVA general linear model and multiple comparisons following a LSD post hoc test were performed using SPSS software (Version 13, Microsoft), with values of $P \leq 0.05$ considered as significant.

3. Results and discussion

3.1. Nanoparticle characteristics

Particle size measurements show that all PLA particles produced were within the nanosize range and the differences obtained in size and surface charge before and after adsorption demonstrate the good reproducibility of the formulation process. After adsorption nanoparticles prepared with PVA in the external phase (255.8 ± 10.0 nm) were significantly smaller ($P < 0.02$) than those obtained with GCS and ALG (407.1 ± 57.0 nm and 346.7 ± 18.5 nm, respectively) (Fig. 1A). Although an increase in particle size may be detected after adsorption, the change is not significant (254.7 ± 8.9 nm to 255.8 ± 10.0 nm for PLA–PVA; 384.4 ± 29.9 nm to 407.1 ± 57.0 nm for PLA–GCS; 343.5 ± 21.4 nm to 346.7 ± 18.5 nm for PLA–ALG). As expected, PLA–ALG and PLA–PVA nanoparticles presented a negative surface charge before and after protein adsorption (−41.3 ± 1.6 mV to −39.6 ± 1.1 mV and −30.4 ± 7.2 mV to − 21.0 ± 1.7 mV for PLA–PVA). In contrast, GCS-containing particles kept a positive surface charge throughout the process (Fig. 1B). However, as it was previously observed for PLA and poly-ε-caprolactone nanoparticles, the adsorption of *S. equi* proteins decreases significantly (from +36.4 ± 5.0 mV to +5.2 ± 2.1 mV) the positive zeta potential values conferred by GCS (Florindo et al., 2009a,b). Although the data herein obtained do not allow establishing a clear correlation between the amount of adsorbed protein and the VMD of PLA nanoparticles (Fig. 1A), apparently *S. equi* proteins

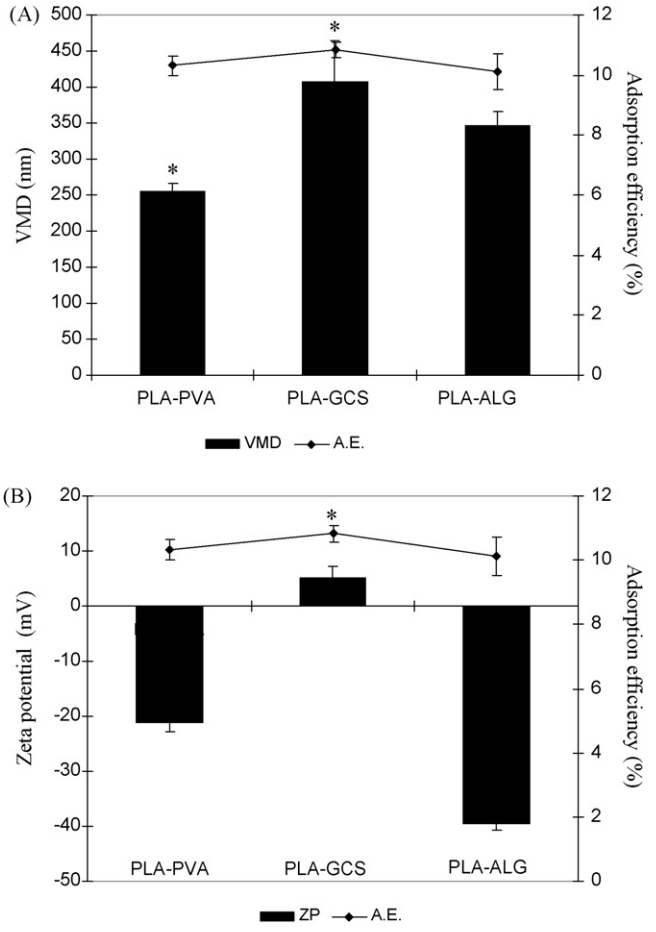

Fig. 1. Adsorption efficiency variation with VMD (A) and zeta potential (B) of PLA nanoparticles (mean ± SD; $n = 3$; $^*P < 0.05$).

adsorb preferentially to the positively charged nanoparticles prepared with GCS. This can be explained by the net electrical charge of this mixture of proteins, which seems to be negative, having in mind the decrease of PLA–GCS particle surface previously mentioned.

3.2. Residual solvent quantification

The use of organic solvents is one of the main disadvantages of the emulsion solvent evaporation methods because only small amounts of these organics solvents are allowed in the final medicinal product. In this particular study, PLA was dissolved in DCM, a Class 2 solvent for which the maximum residual concentration allowed is 600 ppm (i.e. 0.06% w/w) (EMEA, 2006). The DCM residues (% w/w) present in the freeze-dried nanoparticles were assessed by ^{1}H NMR. It is accepted that when proton NMR spectra are run under quantitative conditions, protons give rise to responses which are directly related to their concentrations (Saito et al., 2004). Therefore, precise integrals for the sodium formate reference standard singlet absorption at 8.2 ppm and residual DCM at 5.4 ppm were recorded.

The ^{1}H NMR spectra of the purified DCM were compared with those obtained with the nanoparticles. As it can be seen from Fig. 2A–C, the spectra obtained with the samples are properly phased and completely resolved. The spectra of the three samples are similar and a small and single peak was observed between 5.4 ppm and 5.5 ppm, which can be due to a shift of the pure DCM peak. Nevertheless, the DCM strength should be below 600 ppm, which means that in 30 mg of nanoparticles the maximum amount of this organic solvent that could remain was 18 μl. In the case

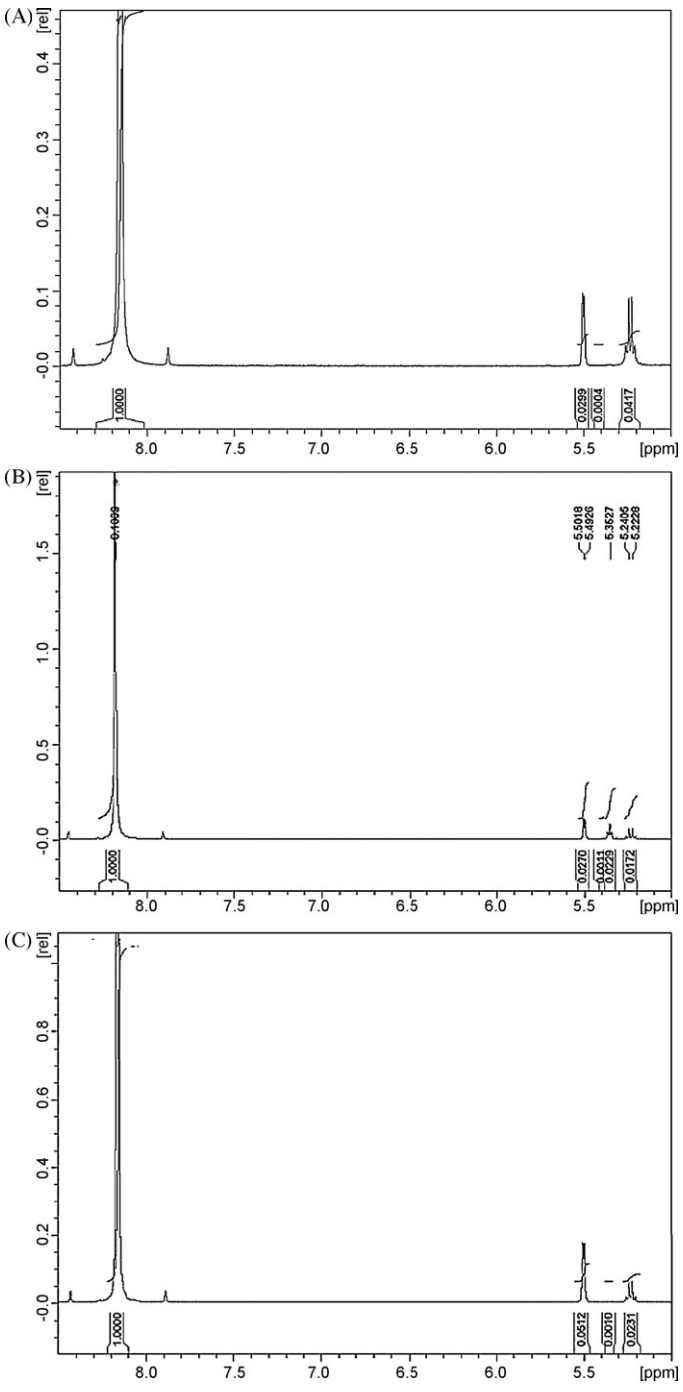

Fig. 2. ^{1}H NMR spectra of nanoparticle formulations: (A) PLA–PVA, (B) PLA–GCS and (C) PLA–ALG.

of identifying the single peak observed between 5.4 ppm and 5.5 ppm as a shift of the pure DCM absorption singlet, the organic solvent strengths found for PLA–PVA, PLA–GCS and PLA–ALG nanoparticles were 0.016% (w/w), 0.017% (w/w) and 0.020% (w/w), respectively, which are well below the accepted limit for DCM residues.

3.3. Analysis of S. equi protein extract

Antigens responsible for the induction of a protective immunity against various pathogens may be suitably attached to particulate systems, which have the capacity to be modified and associated

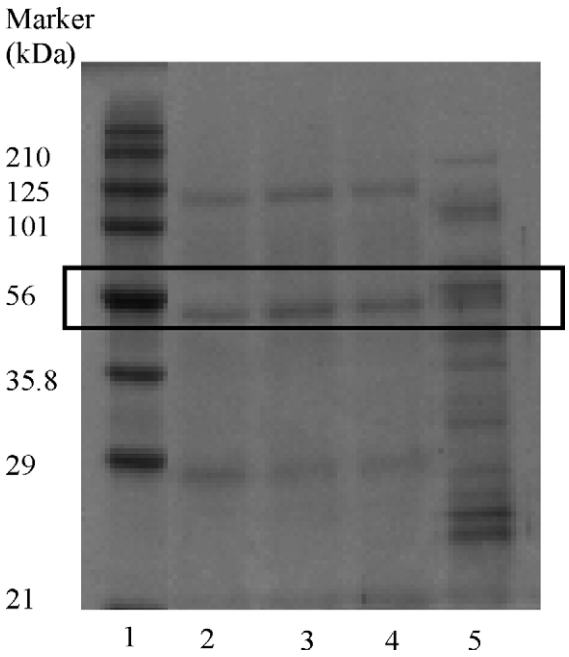

Fig. 3. SDS-PAGE (10% gel) of *S. equi* enzymatic extract solutions before and after adsorption onto PLA nanoparticles. Lanes: (1) Standard MW markers; (2) *S. equi* proteins extracted from PLA–PVA nanoparticles; (3) *S. equi* proteins extracted from PLA–GCS nanoparticles; (4) *S. equi* proteins extracted from PLA–ALG nanoparticles; and (5) *S. equi* enzymatic extract standard solution (2000 μg/ml).

with other adjuvants in order to achieve a prolonged and/or pulsed release of the antigen (Bramwell and Perrie, 2006). However, the prolonged and controlled release of antigens encapsulated and/or adsorbed onto particles surface is crucial to prevent a considerable loss of antigen associated with particles before they are taken up by the APCs (Katare et al., 2005). The double emulsion solvent evaporation is one of the most commonly used techniques to formulate polymeric particles, but it requires the use of mechanical agitation and organic solvents, as DCM, which can affect the antigen structure and its immunogenic properties (Benoit et al., 1999).

In the present work, adsorption was used to associate proteins to PLA carriers in order to avoid protein exposition to those harsh chemical and physical conditions. Nevertheless, structural changes and loss of activity of protein molecules during adsorption and desorption may also occur. Therefore, the adsorption behaviour of proteins is related to their structure stability. Adsorption from aqueous solutions usually involves dehydration of hydrophobic areas of the adsorbate at the solid–liquid interface, which can modify the interfacial properties and the structure of the peptides. The stability of the enzymatic extract was investigated by SDS-PAGE before and after adsorption onto nanoparticles (Fig. 3). No changes in the migration of the proteins could be detected In addition, it shows that the majority of proteins in the extract are adsorbed but the different intensities observed in the bands also suggest different adsorption for those proteins. The selective adsorption does not seem to be dependent on particle surface charge or composition. Interestingly, protective immunity against *S. equi* is believed to be mediated by antibodies directed against the M-like protein (SeM), which is known as its major virulent factor. This is a 54–58 kDa protein, and a thin band can be seen in this area in lanes 2–4, similarly to the standard extract solution (Timoney et al., 1998). Therefore, within the scope of this analytical method, the adsorption procedure appears not to affect the *S. equi* protein antigens. However, such observation does not necessarily indicate that the antigens retain their immunogenicity intact. The ultimate feasibility of the

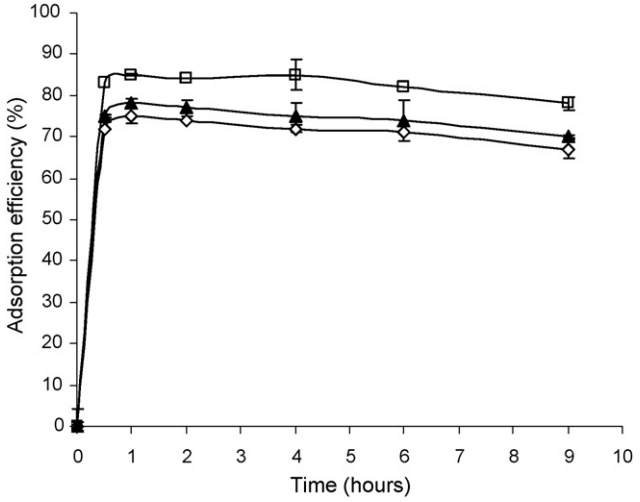

Fig. 4. Effect of incubation time on adsorption of *S. equi* extract proteins onto PLA nanoparticles, in double-distilled water (mean ± SD; $n = 3$): (◊) PLA–PVA, (□) PLA–GCS and (▲) PLA–ALG.

formulation approach herein described is confirmed by recently published *in vivo* studies where *S. equi* antigens adsorbed onto PLA nanosphere surface were able to stimulate humoral, cellular and mucosal immune responses, without requiring the administration of other co-adjuvants in order to achieve balanced Th1/Th2 immune responses (Florindo et al., 2008, 2009a).

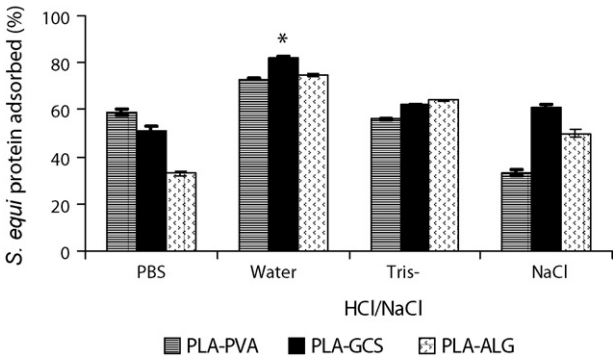

Fig. 5. Effect of different media (NaCl 0.9% pH 7.4; double-distilled water; phosphate buffer saline pH 7.4 (PBS); 20 mM Tris–HCl/NaCl pH 8.0 buffer) on adsorption of *S. equi* proteins onto PLA nanoparticles (mean ± SD; $n = 3$).

3.4. S. equi enzymatic extract adsorption

S. equi protein adsorption is a time dependent phenomenon reaching, within 1 h in double-distilled water, maximum adsorption efficiency values of 75.2 ± 1.9% (w/w) for PLA–PVA, 84.9 ± 0.2% (w/w) for PLA–GCS and 78.1 ± 0.4% (w/w) for PLA–ALG nanoparticles (Fig. 4). As expected, adsorption of *S. equi* proteins was greatly influenced ($P < 0.05$) by the type of media used in the experiment, and the highest amount of adsorbed protein was obtained when incubation was carried out in double-distilled water, confirming the contribution of electrostatic interactions (Fig. 5). As a con-

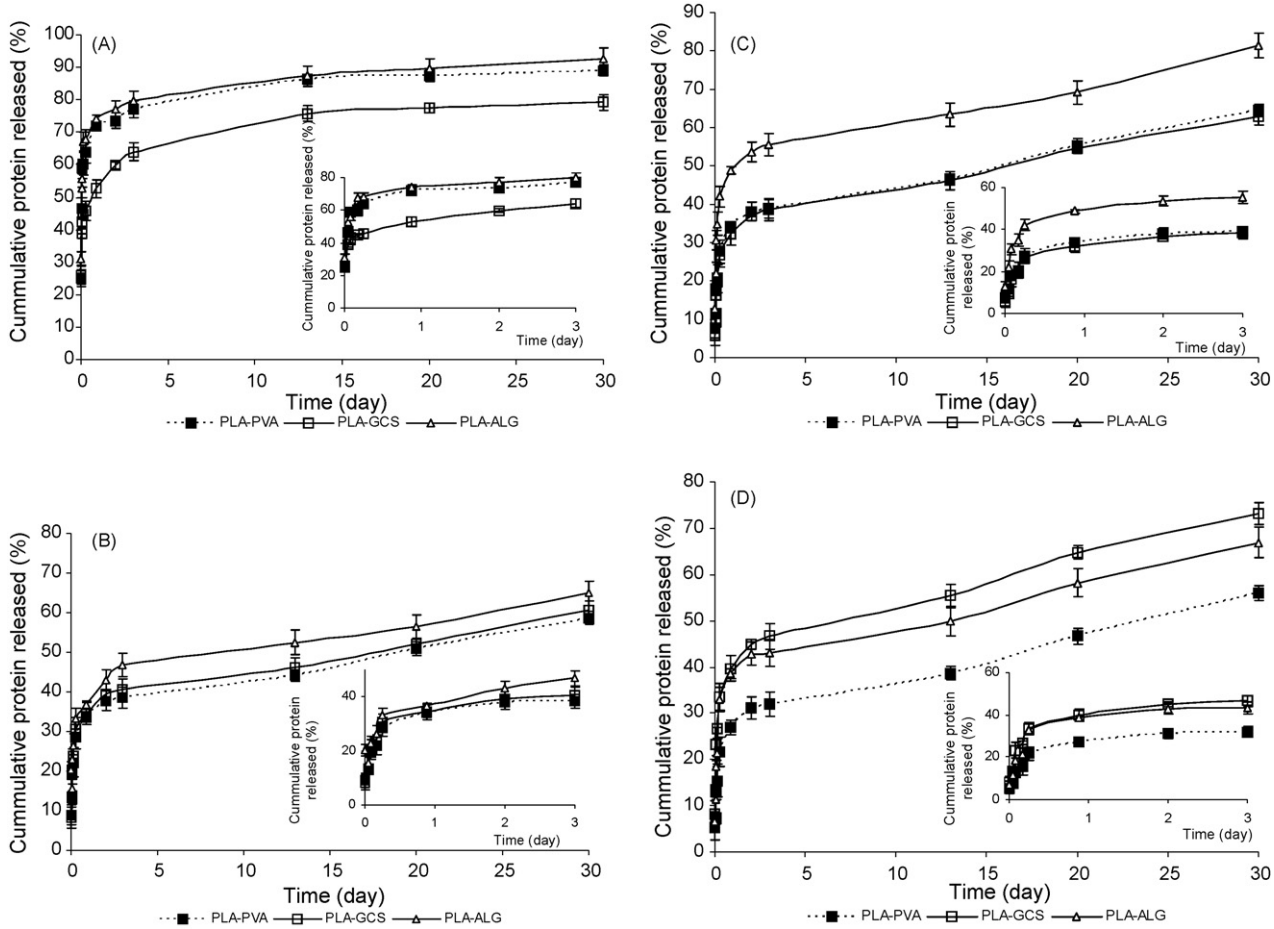

Fig. 6. *In vitro* desorption profile of *S. equi* extract proteins from PLA–PVA (■), PLA–GCS (□) and PLA–ALG (△) nanoparticles, previously adsorbed in (A) water, (B) Tris–HCl/NaCl, (C) PBS and (D) NaCl 0.9% pH 7.4 (mean ± SD; $n = 3$).

Table 1
Parameters of Freundlich and Langmuir isotherms for adsorption of *S. equi* proteins onto PLA nanoparticles.

PLA nanoparticles	Freundlich constants			Langmuir constants		
	K_F	n	r^2	Q_m	K_L	r^2
PLA–PVA	0.0732	0.7551	0.9958	769.2308	0.0629×10^{-2}	0.8440
PLA–GCS	0.4480	0.9984	0.9940	769.2308	0.0543×10^{-2}	0.5868
PLA–ALG	0.1940	0.7505	0.9900	1428.571	0.0372×10^{-2}	0.6196

PLA–PVA: poly(lactic acid)–polyvinyl alcohol nanoparticles; PLA–ALG: poly(lactic acid)–alginate nanoparticles; PLA–GCS: poly(lactic acid)–glycolchitosan nanoparticles.

sequence, the adsorption of *S. equi* proteins in the different PLA nanoparticles, performed simply in double-distilled water, at 25 °C, was selected for the subsequent characterisation studies of the adsorption process.

Regardless of the adsorption medium, desorption studies demonstrated a burst protein release within the first 6 h, for all particulate systems (Fig. 6A–D). However, after 3 days into the experiment, only the formulations prepared with double-distilled water showed a controlled and more sustained desorption over a prolonged period of time (Fig. 6A). Nanoparticle surface modification by the use of the hydrophilic polymer GCS improved not only the adsorption efficiency but also the desorption profile, as 20% (w/w) of protein remained attached to the particle surface over 30 days after the beginning of the study (Fig. 6A).

3.4.1. Adsorption isotherms

The prediction of a protein adsorption from a mixture requires a complete understanding of the competition between charges, depending upon the electrostatic, Van der Waals, and steric interactions of the different types of proteins in the mixture and of these proteins with the surface. The adsorption isotherm shows the relation between the amount of protein effectively adsorbed onto particle surface and the protein concentration that is in solution at equilibrium, and can be described by several models, such as the most commonly used equations of Langmuir and Freundlich (reviewed by Florence and Attwood, 2006). Similarly to other authors, these models were used to characterise the adsorption of the *S. equi* protein antigens onto the nanoparticles' surface (Alpar and Almeida, 1994; Yoon et al., 1998). The Langmuir model can be expressed by (Florence and Attwood, 2006): $\frac{C_e}{Q_e} = \frac{C_e}{Q_m} + \frac{1}{Q_m K_L}$ where C_e is the concentration in equilibrium (μg/ml), Q_e is the amount of protein adsorbed (μg/mg) and Q_m is the maximum adsorption capacity (μg/mg). K_L is a Langmuir constant related to the adsorption capacity.

Usually the deviations from this theory are attributed to the formation of multilayers and are treated by the equation of Freundlich which does not predict a limiting value for adsorption and can be expressed by (Florence and Attwood, 2006; Nadavala et al., 2009):

$$\text{Log}\, Q_e = \log K_F + \frac{1}{n} \log C_e$$

where K_F is the Freundlich constant for the system, whose index n is usually <1, so that the amount adsorbed increases less rapidly than the concentration.

In this study, the experimental data of the adsorption process seems to fit the empirical Freundlich model, as acceptable regression coefficients ($r^2 \geq 0.990$) were obtained from the plot of log Q_e versus log C_e (Table 1; Fig. 7). The constants K_F and n are also presented in Table 1 and again were inferred from the regression equations obtained in the previously mentioned graphs. As expected from a complex mixture of proteins, data suggest a multilayer type of protein deposition onto PLA nanoparticles surface, resulting from the competition of many different protein molecules to the particle surface. The Langmuir monolayer theory does not take into consideration the multiple sites available for adsorption in a surface, also ignoring the interactions protein–protein, therefore this mixture of proteins behaves differently from pure proteins that have been described to adsorb according to Langmuir-type isotherms (Alpar and Almeida, 1994; Yoon et al., 1998). The adsorption capacity of PLA–GCS ($K_F = 0.4480$) is higher than that of the negatively charged PLA–PVA or PLA–ALG nanoparticles, at the same temperature, suggesting a higher affinity of the *S. equi* proteins for the positively charged GCS surface modified nanoparticles (Table 1).

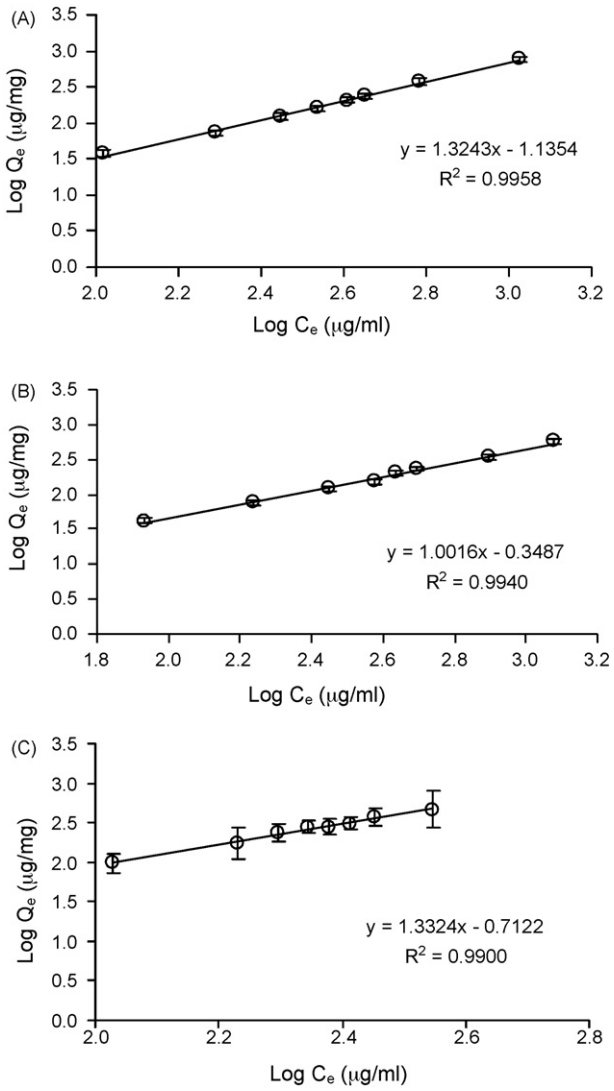

Fig. 7. Freundlich adsorption isotherms of *S. equi* proteins onto (A) PLA–PVA, (B) PLA–GCS and (C) PLA–ALG nanoparticles (mean ± SD; $n = 3$).

4. Conclusions

The successful development of particulate vaccines depends on an intimate understanding of their physicochemical and biological characteristics. To find strategies to reduce or prevent chemical degradation, denaturation and aggregation is a priority in the development of a new drug formulation. The results have shown that PLA particle surface properties, mainly the electrical charge, greatly affect the amount of proteins immobilized onto their surface. In fact, PLA–GCS nanoparticles presented the highest adsorption capacity when compared to the negatively charged formulations. For this *S. equi* extract proteins, the adsorption efficacy was higher when particles were incubated in an aqueous solution. The formulation by adsorption did not untowardly affect the molecular stability of the proteins present in the *S. equi* extract solution. As a result, adsorption remains as a promising alternative method for the production of particulate vaccine carriers for bacterial extracts, as it can avoid the possible degradation of proteins caused by organic solvents commonly used in the solvent evaporation technique and at the same time achieve relative high loadings. The feasibility of the formulation approach herein described is confirmed by the *in vivo* studies that are currently in publication.

Acknowledgments

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