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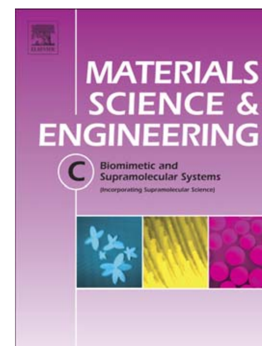

Spray dried microspheres based on chitosan: A promising new carrier for intranasal administration of polymeric antigen BLSOmp31 for prevention of ovine brucellosis

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Spray dried microspheres based on chitosan: a promising new carrier for intranasal administration of polymeric antigen BLSOmp31 for prevention of ovine brucellosis

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1. Introduction

Brucella ovis produces infertility in rams, abortions in ewes and increased perinatal mortality in lambs, causing severe economic loss on sheep husbandry [1, 2]. Ovine brucellosis occurs in most sheep-raising countries and vaccination is recognized as the most suitable tool for *B. ovis* control in endemic situations [2].The smooth attenuated *B. melitensis* Rev 1 strain is the best vaccine available against *B. ovis* [3]. However, Rev1 displays a large number of drawbacks, including residual virulence, pathogenicity for humans and interferences with serodiagnosis [4, 5]. Consequently, there is a need to develop new vaccines against brucellosis [6].

Subcellular vaccines display important advantages to face the classical handicaps of live attenuated vaccines [7]. However, due to its non-replicating nature, immunogens require the use of adequate adjuvants or delivery systems [8, 9].

The bacterial enzyme lumazine synthase identified in *Brucella* spp. (BLS) has been considered as a protein carrier for antigen delivery based on its physicochemical properties [10]. BLS is a remarkably stable dimer of pentamers, with ten N-terminus sites of linkage. Structural analysis of this molecule showed that it was possible to insert peptides and protein domains without disturbing its conformation. The recombinant chimera BLSOmp31 consists of BLS decorated with a protective epitope derived from an Outer Membrane Protein of 31 kDa (Omp31) from *B. melitensis.* This protein has exhibited significant thermodynamic stability including low and high extreme pH conditions with a theoretical isoelectric point of 6.4 [11].

Polymeric antigen BLSOmp31 formulated in oil adjuvant and administered by parenteral route conferred significant protection against *B. ovis* in mice and rams [12, 13, 14]. Moreover, its lack of interference in the diagnostic tests, its intrinsic adjuvanticity properties and its innocuousness have converted BLSOmp31 into an attractive anti-*Brucella* vaccine candidate [13, 15].

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Brucella ovis needs to go through the mucosa during the early steps of the infection. While venereal is the primary route of transmission, the infection can be initiated when *B. ovis* gains entry through mucosal tissues such as the gastrointestinal tract, the respiratory airways and/or the surface of the eye [1, 16]. Therefore it is desirable to obtain a local mucosal immune response as a result of vaccination to block both infection (i.e. colonization) and disease development. Parenterally administered vaccines mainly stimulate systemic responses, whereas the delivery of immunogens through mucosal surfaces is of remarkable interest due to the fact that it both mimics the bacteria behaviour and generates immunity at the major portals of entry for this microorganism [17]. In addition, administration of vaccines by a mucosal route is very practical, non-invasive and is also associated with lower rates of side effects [18].

The mucosal immune system exhibits a high degree of anatomic compartmentalization related to the migratory patterns of lymphocytes activated at different mucosal sites [19]. This compartmentalization imposes constraints on the selection of vaccine administration route against ovine brucellosis. Different studies in mice and human models have demonstrated that intranasal immunization resulted in the generation of immune effectors cells in genital mucosa [20, 21, 22]. Mostly, intranasal route is attractive in ruminants because avoids the rumen and a possible loss of immunogen potency [23].

Intranasal immunisation with toxoplasma tachyzoite antigen encapsulated into a poly (D,L-lactide-co-glycolide) microparticle delivery system [24] or intranasal delivery of ISCOMATRIX® adjuvanted influenza vaccine has been demonstrated to induce both systemic and local immunity in sheep [25] although this strategy has not yet been evaluated in sheep against *Brucella*, the nasal mucosa offers an attractive route for the delivery of recombinant immunogens such as BLSOmp31.

Nasal vaccines have to overcome several limitations, including the inefficient uptake of soluble antigens across the nasal mucosa, which is generally poor due to the mechanical

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protection provided by the tight junctions and mucus layer, plus the mucociliary action of the nasal mucosal epithelium which transfers most delivered particles or substances via mucus streams into the gastrointestinal tract [24, 26, 27]. In addition, development of nasal vaccines based on purified protective antigens requires potent adjuvants and delivery systems to enhance their immunogenicity. Some natural polymers, such as chitosan, have demonstrated that they could enhance the immunogenicity of antigens in solution or in microsphere formulations [28]. Chitosan is the deacetylated form of chitin comprising copolymers of glucosamine and N-acetyl glucosamine linked by β-(1-4) linkages. Chitosan is a cheap, hydrophilic, biocompatible, biodegradable, and nontoxic natural polymer suitable for many biomedical applications [29]. Chitosan microspheres (CMs) are good vehicles for encapsulating proteins, and can achieve high protein loading efficiency and protect them from degradation [30]. Currently, several protein-loaded CMs have been successfully developed [31, 32, 33]. Recent research showed that the bioactivity of nerve growth factor was retained in CMs [34] indicating that CMs are promising candidates for encapsulating growth factors.

In addition, different authors have demonstrated that this cationic polysaccharide behaves as a bioadhesive material with a transient effect on open tight junctions [35]. Consequently, chitosan is capable of slowing down the rate of mucociliary clearance through interactions with the nasal mucosa and thus increase the time available for immunogen absorption [36]. Both properties may aid in the contact and uptake of antigen by immunocompetent cells of Nasal Associated Lymphoid Tissue (NALT) which mediate the induction of mucosal immune responses resulting in strong, long-term, local cellular and humoral immune responses [28, 36, 37].

Different studies have demonstrated that particles with sizes similar to the pathogens are readily taken up and processed by antigen presenting cells (APCs) which lead to enhanced vaccine responses. Besides, CMs were found to be cleared even more slowly

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than chitosan solution from the nasal cavity in humans and sheep [38, 39]. Consequently, the preparation of CMs might be the best alternative for mucosal vaccination.

Chitosan microspheres can easily be prepared by spray-drying. Particles obtained by this method are characterized by high sphericity, positive charge and specific surface area, properties that make chitosan suitable for delivery of immunogens via the nasal route [40,41]. Immunogens can be encapsulated or loaded by passive adsorption onto the CMs avoiding the exposure of the protein antigen to the harsh environment of organic solvents which may cause denaturation as seen in microencapsulation processes [42].

Therefore, the aim of this work was to report preparation, characterization and *in vivo* evaluation of spray-dried CMs as a new carrier for intranasal immunization of chimera BLSOmp31 for prevention of ovine brucellosis.

2. Materials and Methods

2.1 Materials

Chitosan (deacetylation degree 90%, Mv = 1.61 × 10^5 g/mol) was supplied by Parafarm® (Argentina). Glutaraldehyde aqueous solution 25% w/w and Mucin Type III: Partially Purified from porcine stomach were purchased from Sigma® (USA). Bicinchoninic acid protein Assay (BCA) kit was obtained from Thermo Fisher Scientific Inc. (USA). SDS-PAGE Molecular Weight Standards, Broad Range Catalog Number 161-0317 was purchased from Bio-Rad (USA). All other reagents and solvents were of analytical grade and were used as provided.

2.2 Recombinant BLSOmp31 production

Recombinant BLSOmp31 was expressed in *Escherichia coli* BL21 (Stratagene, La Jolla, CA) and was purified by fast- protein liquid chromatography in a Mono-Q and Superdex-

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200 column (GE Healthcare) according to the procedure previously developed by Laplagne et al. (2004) [11]. Purity was assessed by Coomassie Brilliant Blue stain as reported elsewhere. Recombinant protein was adsorbed with Sepharose-Polymyxin B to eliminate lipopolysaccharide (LPS) contamination. Purified protein contained less than 0.05 endotoxin units per 1mg of protein, as assessed by Limulus Amebocyte Lysate Analysis kit (Sigma, St Louis, MO).

2.3 Preparation of CMs

Chitosan microspheres (blank microspheres) were prepared according to the previously studied procedure [39] based on the spray-drying technique. Briefly, chitosan was dissolved in 0.5% aqueous acetic acid to give a polymer concentration of 0.5% w/v. A 4% aqueous solution of glutaraldehyde was added dropwise as crosslinking agent. This mixture was kept at 20ºC under continuous magnetic stirring for 90 minutes and then homogenized by using Ultraturrax T18 (IKA) (6000 rpm). The solutions were then spray-dried using a laboratory-scale Mini Spray Dryer (Büchi B-290, Büchi Labortechnik AG).

A two-fluid nozzle with cap orifice diameter of 0.5 mm was used. The atomizing air flow rate was 600 L/min, and the flow rate was 80 mL/h. The inlet temperature was set at 130ºC and the outlet temperature was 76ºC. The production yield was calculated as the weight percentage of the final product after drying, with respect to the initial total amount of chitosan.

2.4 BLSOmp31 adsorption to CMs

The BLSOmp31 loading of preformed blank CMs was performed by incubating 1% (w/v) chitosan microspheres and 0.5% (w/v) BLSOmp31 (2:1 ratio) in Phosphate-Buffered Saline (PBS; pH 7.2) under shaking conditions (60 rpm) at 37ºC. In order to determine the rate and quantity of BLSOmp31 adsorbed onto CMs at 0, 1, 2 and 4 h, the suspension was

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centrifuged and an aliquot was withdrawn from the supernatant. Finally, after overnight incubation, the suspension was once again centrifuged (3000 rpm for 15 min) to remove the unloaded protein and the pellet was lyophilized (0.1 mbar, 24 h) (Thermovac). The BLSOmp31-adsorption profile and the loading efficiency was calculated by quantifying the non-bound BLSOmp31 in the supernatant with the BCA protein assay method using a microplate reader with a 570 nm filter (Titertek, Multiscan EX, Labsystems).

$$AE (\%) = (m_t - m_s) / m_t x100$$

where AE is the adsorption efficiency, m_t is the total amount of BLSOmp31 added to the system, and m_s is the amount of BLSOmp31 in the supernatant after incubation.

2.5 Characterization of CMs

2.5.1 Size and Zeta potential

Chitosan microspheres (CMs) with and without BLSOmp31 (CMs-BLSOmp31) were dispersed in NaCl solution 10mM (Ph 7.0) and size and surface charge (zeta potential) were analyzed by Delsa™ Nano C Particle Analyzer and Liquid Particle Counting System, HACH Ultra Analytics®, HIAC 9705.

2.5.2 Morphology

CMs and CMs-BLSOmp31 were dispersed in deionized water and dried at 45ºC. Then, the particles were coated with a thin gold-palladium layer using a sputter-coater and the results were examined using FEG-SEM, Sigma, Carl Zeiss.

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2.5.3 Fourier transform infrared spectroscopy (FT-IR) analysis

Chitosan, CMs with and without BLSOmp31 were characterized through FT-IR.The infrared spectra of the samples dispersed at 1% of sample in KBr discs were recorded in a NICOLET FTIR spectrometer (Thermo / Nicolet 360FT-IR E.S.P.Thermo Fisher Scientific, USA). The samples were scanned from 4000 to 400 cm^{-1}. The recording conditions were as follows: resolution of 8.0 and sample scan of 40. Data were analyzed using the OMNIC® software (Thermo Fisher Scientific, (USA).

2.6 In vitro BLSOmp31 release from CMs-BLSOmp31

To determine the rate of release of BLSOmp31 from the CMs, 10 mg of lyophilized CMs-BLSOmp31 were suspended in 1 mL of PBS pH 7.2 at 37ºC and at 60 rpm agitation. Duplicate samples were run. At different time intervals (0, 5, 10, 15, 30, 45, 60, 90, 120 y 180 min) tubes were centrifuged. Samples (0.1 mL) were withdrawn whose volumes were replaced with PBS. The amount of protein in the supernatant was determined by BCA protein assay. The absorbance reading value was corrected subtracting the average absorbance obtained from the supernatant of unloaded CMs prepared under the same conditions.

2.7 SDS-polyacrylamide gel electrophoretic (PAGE) analysis of released BLSOmp31

Released BLSOmp31 from CMs-BLSOmp31 was analyzed by gel electrophoresis to determine possible protein degradation in this process. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out of 12% acrylamide [43] on 1.5 mm thick slabs, cast and run in Tris–Glycine buffer (Bio-Rad, USA). After migration, the gel was stained with 0.1% Coomassie Brilliant Blue in 10% of acetic acid in a solution of methanol:water (1:1) to reveal the antigen.

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2.8 In vitro evaluation of mucoadhesive properties of CMs and CMs-BLSOmp31

Adsorption of mucin on the surface of CMs and CMs-BLSOmp31 was measured in order to evaluate their mucoadhesive properties [44]. Briefly, 1 mL of mucin solution (5 mg/mL) was stirred with 1 mL of CMs (5 mg/mL) and CMs-BLSOmp31 at 5 mg/mL for 1 h at 37ºC. Then, the suspension was centrifuged at 4000 rpm for 10 min at 4ºC. The amount of free mucin was determined from the difference between total and free mucin, using Bradford colorimetric method [45] in order to assess the amount of mucin adsorbed on the microspheres. Mucin standard solutions (0.3125, 0.625, 1.25, 2.5 and 5 mg/mL) were prepared for mucin calibration curve. Bradford reagent was added to the samples and incubated at 37ºC for 10 min. Then, solutions were detected by UV absorption at 595 nm.

The data obtained were interpreted using the following equation:

$$\text{MAE}(\%) = (C0 - Cs) / C0 \times 100\%$$

where MAE is the mucin adsorption efficiency, $C0$ is the initial concentration of mucin and Cs is the concentration of free mucin in supernatant after incubation with the CMs and CMs-BLSOmp31 [44].

2.9 In vivo immunological response

2.9.1 Experimental design

Systemic and mucosal immune responses induced by intranasal immunization with CMs-BLSOmp31 were studied in rams. Corriedale 5-month-old rams (n=8) were used, following the protocols approved by The Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Facultad de Veterinaria, (Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina; http://www.vet.unicen.edu.ar). Rams were immunized intranasally with 1 mL of a suspension of CMs-BLSOmp31 (0.5 mL/nostril). An

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unvaccinated control group was included in the experiment (n=8). Animals were immunized three times at 0, 21 and 42 days.

2.9.2 Samples

Samples of serum and nasal secretions were obtained at 0 and 60 days after the first immunization. Blood samples were collected via jugular venipuncture into tubes without anticoagulant for serum. Nasal secretion samples were obtained from each ram using soft foam sponges trimmed to an appropriate size to fit within a nostril. The sponge was inserted into a nostril and left in place for 1 minute. After the sponge was removed from the nostril, it was placed into a 5-mL syringe from which the plunger had been previously removed. Then, the sponge was embedded in sterile saline by aspiration. The plunger was inserted into the syringe and used to squeeze nasal secretions from the sponge into a polypropylene tube [24].

2.9.3 Systemic and local antibody immune response

Samples were clarified by centrifugation at 6000 rpm at 4ºC for 10 min and supernatants were stored at -20ºC until they were processed. Specific anti-BLSOmp31 IgG and secretory IgA (sIgA) was quantified using indirect ELISA as previously described [13]. Briefly, plates (NUNC Maxisorp, Denmark) were coated with BLSOmp31 and incubated with samples of nasal secretion (1:50) and sera (1:200). Both of them were diluted in blocking solution. Bound antibodies were detected by adding a donkey anti-sheep IgG (whole-molecule) conjugated to horseradish peroxidase (Sigma, St. Louis, MO) and a rabbit anti-sheep IgA conjugated to horseradish peroxidase (AbD Serotec), respectively. The reaction was developed with 2.2'-azino-di (3-ethylbenzothiazolinesulphonic acid) (ABTS)-H_2O_2. Absorbance was measured at 405 nm using a microplate reader. Antibody

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levels (IgG and IgA) were expressed as the arithmetic mean ± SD of the Optical Density (O.D.) obtained for rams from each group as we have done in previous works.

2.10 Statistical analysis

Data from indirect ELISA against BLSOmp31 were analyzed by student's t-test. $P<0.05$ was considered statistically significant. Graphs were performed using Graph Pad software, version 5.0, San Diego, CA.

3. Results and discussion

3.1 Characteristics of CMs and CMs-BLSOmp31

The CMs were obtained with a "suspension crosslinking technique" by using glutaraldehyde as a crosslinker compound. The yield of spray-drying process was approximately 70%. The physicochemical characteristics of the microspheres showed good sphericity and were positively charged with values of +32.18 ± 1.26 mV for CMs and 1.49 ± 0.04 mV for CMs-BLSOmp31 (mean ± SD; n=3). The size of CMs and CMs-BLSOmp31 was found to be 5.28 ± 0.125 μm and 8.66 ± 0.03 μm, respectively (mean ± SD; n=3) (**Figure 1**).

The small differences in size and zeta potential between the different batches of microspheres demonstrated the good reproducibility of the method for microsphere preparation.

Several investigations have demonstrated the importance of the size and zeta potential of microparticles to generate both systemic and local effective immune responses when these are administered by mucosal routes [24,46]. One of the most important properties of chitosan microspheres as a vaccine delivery system is to offer an optimum particle size to ensure the uptake of the immunogen by M cells from the NALT or by the sub-epithelial

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dendritic cells. Small particles (<10 μm) appeared be more immunogenic than larger ones (>10 μm) [27, 47]. Thus, the optimal size of BLSOmp31-loaded chitosan microspheres would be effective to deliver this immunogen to the immune competent cells of nasal mucosa. Another important point to take into account in the design of a nasal vaccine is the zeta potential of microparticles [27]. It is known that positively charged particles are easily associated to the mucin of the mucus layer overlying the nasal epithelium. Thus, CMs with positive charge could increase the residence time and its bioadhesivity; and may facilitate the appropriate uptake of antigen loaded onto the microspheres by longer contact time between the vaccine and the mucosal surface.

3.1.2 Morphology of CMs and CMs-BLSOmp31

The morphology of CMs prepared by the spray-drying method was examined by SEM. **Figure 2a** shows SEM images for chitosan microspheres of about 1‐10 μm average size and spherical and smooth surface without aggregation and uniform porosity. The volume of glutaraldehyde and crosslinking time did not alter the surface morphology of the microspheres (data not shown). The SEM image of BLSOmp31 immobilized on chitosan microspheres (**Figure 2b**) illustrates the physical characterization for BLSOmp31, and the image clearly shows the adherence of individual BLSOmp31 (non-aggregated) to the surface and within the pores of the support microspheres (**Figure 2b**).

3.2 BLSOMP31 loading of CMs

Chitosan microspheres were loaded with BLSOmp31 by incubation of a 1% (w/v) chitosan with 0.5% (w/v) of recombinant protein in PBS. A period not exceeding 18 h loading efficiency of BLSOmp31 onto CMs was 45.19% ± 4.2 (n=2) **(Figure 3)**. The adsorption was of a chemical nature, ie it was because there was an ionic interaction between

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BLSOmp31 (pI 6.4) having a net negative charge at pH 7.2, and the positive charge of microsphere surface. FT-IR results show the presence of an amide group is assigned to the vibrational band at 1665 cm^{-1} [ν (C=O)]. The IR band at 1596cm-^{1} corresponds to combined deformations of the primary amine groups[-(NH_2)], deformation of the primary ammonium cation, and an asymmetric stretching band for C-N in the amide [ν (C¬-N)], and the amide II **(Figure 4a)** [48,49]. The attenuation of the (-NH_2) band at 1596 cm^{-1} is attributed to deprotonation of the ammonium cation and cross-linking with glutaraldehyde. Two new signatures appear at 1560 cm^{-1} (C=C) and 1655 cm^{-1} (C=N); providing support that cross-linking between the amine groups of CH and the aldehyde groups of glutaraldehyde has occurred. Additional evidence of a Schiff base adduct is supported by the new band at 1400 cm^{-1}. This vibrational band is analogous to the deformation of the C-H band observed for aldehydes; however, the signature is for a Schiff base adduct (52Pratt) **(Figure 4b)**. The significant decreasing in transmittance at 1610-1550 and 1400 cm-1, assigned to the vibrations of asymetric streching of CO_2^- and symetric streching CO_2^- group, respectively [50] as shown in **(Figure 4c)**, confirmed the formation of new chemical bonds between the residue amino groups of chitosan microspheres and the carbonyl groups of BLSOmp31 molecules [51].

3.3 BLSOmp31 release from CMs

The *in vitro* release profile of CMs-BLSOmp31 is shown in **Figure 5**. The initial fast release of BLSOmp31 from CMs was 60%, which occurred in the first hour and was followed by a sustained release of this protein.

To investigate if the BLSOmp31 could be degraded during manufacturing processes and/or release experiments, SDS-PAGE was performed. As depicted in **Figure 6**, gel electrophoresis of released BLSOmp31 from loaded CMs-BLSOmp31, blank CMs and native BLSOmp31 in PBS were included in the assay. No difference was observed

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between the released BLSOmp31 and native BLSOmp31 and no protein staining was detected in the lane with empty CMs. Since these results did not show any differences in molecular weight it is assumed that no substantial degradation of BLSOmp31 occurred during the loading and release processes.

3.4 Mucoadhesion of CMs and CMs-BLSOmp31 in vitro

Mucin is the major component of mucus, which is primarily responsible for the viscoelastic properties of the mucus [52]. Positively charged chitosan has electrostatic interaction with negatively charged mucin secreted from epithelial cells [53]. Therefore, the mucoadhesive property of CMs and CMs-BLSOmp31 was determined by the extent of mucin adsorption on the surface of microspheres. As illustrated in **Figure 7**, the amount of mucin adsorbed on the surface of CMs-BLSOmp31 was lower than on the surface of blank CMs at neutral pH mimicking natural features of nasal cavity [54]. These results suggest that blank CMs would interact better with mucus glycoprotein because the presence of BLSOmp31 around CMs could decrease the electropositivity of microspheres and reduce interaction with mucin. Consequently, the mucoadhesiveness of CMs-BLSOmp31 was significantly lower at neutral pH of nasal mucus ($p<0.003$). However, according to the *in vivo* results CMs-BLSOmp31 were retained enough time to induce a specific immune response when administered by intranasal route. This mucoadhesiveness could substantially reduce the rate of clearance of CMs-BLSOmp31 from the nasal cavity and increase their residence time, enhancing uptake by M cells, allowing BLSOmp31 to be taken up specifically by antigen-presenting cells.

3.5 Specific antibodies in serum and nasal secretion of immunized rams

Levels of IgG and sIgA anti-BLSOmp31 were determined by indirect ELISA in serum and nasal secretions of rams before and after intranasal immunization with CMs-BLSOmp31.

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As shown in **Figure 8**, immunization elicited significant higher IgG antibody levels (*p<0.0001*) against BLSOmp31 in serum at 60 days after the first vaccination. Therefore, anti-BLSOmp31 sIgA (**Figure 9a**) as well as IgG antibodies were substantially higher *(p<0.0001)* in samples of nasal secretion (**Figure 9b**). The unvaccinated control group did not show humoral immune response in any samples.

Whereas parenterally administered immunogens mainly stimulate systemic responses, vaccines administered by mucosal route can lead to both efficient mucosal and systemic immune responses [55]. With regard to mucosal administration of vaccines, microspheres are well known for their controlled delivery formulation, which would significantly enhance the effectiveness of the immune response against infectious diseases such as atrophic rhinitis in pigs [41]. Thus, in our study, intranasal administration of CMs-BLSOmp31 in sheep was able to induce mucosal and systemic immune responses which could prevent colonization and infection by *B. ovis*. In contrast, in previous studies, we have demonstrated that BLSOmp31 in PBS or blank CMs administered by different routes of immunization did not induce immune response when each of them was administered individually in mice or rams (data not shown).

4. Conclusion

The major challenge to effective vaccination lies in the formulation and delivery of the antigens and the vaccines will not reach their full potential unless they are formulated and delivered properly. Our results suggest that the spray-drying method may be a promising way to produce good spherical microspheres with a narrow range of particle size, good bioadhesivity and positive charge. In addition, the induction of systemic and local immune responses clearly indicates a promising potential of CMs for the delivery of polymeric antigen BLSOmp31 intranasally. It is well known that the nasal mucosa is rich in lymphoid

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tissue (NALT) and it provides a practical and safe way for immunization in domestic animals. In future studies, we will evaluate the immune response induced in other mucosal secretions (i.e. preputial secretion) and protection conferred against *B. ovis* in rams.

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Appendix A: Figure Captions

Figure A.1. Frequency distribution of particle size values of chitosan microspheres prepared by spray-drying before (■) and after BLSOmp31 adsorption (■).

Figure A.2. Scanning electron micrographs of chitosan microspheres prepared by spray drying after dispersed in deionized water and dried at 45ºC: (a) blank chitosan microspheres and (73450X) (b) BLSOmp31-loaded chitosan microspheres (120990X). Black arrow indicates BLSOmp31 on surface of a microsphere.

Figure A.3. BLSOmp31 loading onto preformed spray-dried chitosan microspheres at different times. Passive adsorption of BLSOmp31 was performed by incubating 1% (w/v) chitosan microspheres and 0.5% (w/v) BLSOmp31 (2:1 ratio) in PBS (pH 7.2) at 37ºC. Data are expressed as mean±SD of 3 experiments.

Figure A.4. FT-IR spectra of (a) Chitosan, (b) Chitosan microspheres and (c) Chitosan microspheres with BLSOmp31.

Figure A.5. *In vitro* release of BLSOmp31 from spray-dried chitosan microspheres was determined in PBS (pH 7.2) at 37ºC at different times by BCA protein assay. Data are expressed as mean±SD of 3 experiments.

Figure A.6. Electrophoretic analysis on SDS- PAGE with Coomassie Brilliant Blue staining of the BLSOmp31 released from chitosan microspheres. Lane 1: Standard Molecular Weight (kDa); Lane 2: blank chitosan microspheres; Lane 3: native BLSOmp31 solution (control); Lane 4 to 10: BLSOmp31 released from spray-dried chitosan microspheres at 10, 30, 45, 60, 90, 120 and 180 min, respectively.

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Figure A.7. Adsorption of mucin onto blank chitosan microspheres and BLSOmp31-loaded chitosan microspheres with respect to the amount of mucin added. The amount of free mucin was determined using the Bradford assay method. Values significantly different from blank chitosan microspheres are indicated by ***$p<0.003$.

Figure A.8. Anti- BLSOmp31 IgG induced in serum by intranasal administration of loaded-BLSOmp31 chitosan microspheres. Data are expressed as arithmetic mean ± SD absorbance units of animals per group (n=8). Values significantly different from unvaccinated group (n=8) are indicated by ***$p<0.0001$.

Figure A.9. 9a) Anti-BLSOmp31 secretory IgA and **9b**) IgG induced in nasal secretion by intranasal administration of BLSOmp31-loaded chitosan microspheres. Data are expressed as arithmetic mean ± SD absorbance units of animals per group (n=8). Values significantly different from unvaccinated group (n=8) are indicated by ***$p<0.0001$.

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Appendix B: Math formulae

Eq. B1: AE (%) = $(m_t - m_s) / m_t \times 100$

Eq. B2: MAE (%) = $(C0 - Cs) / C0 \times 100\%$

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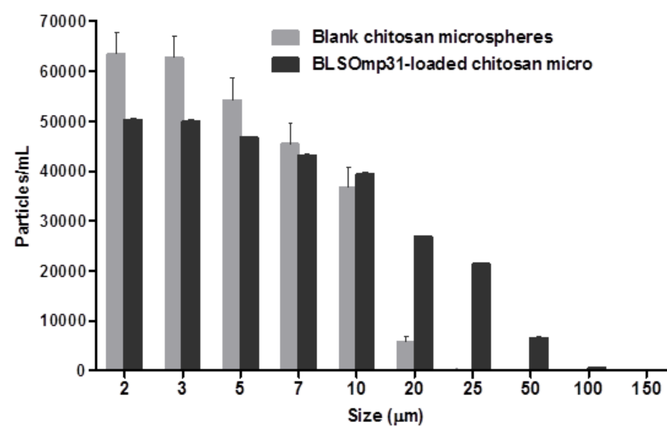

Fig. 1

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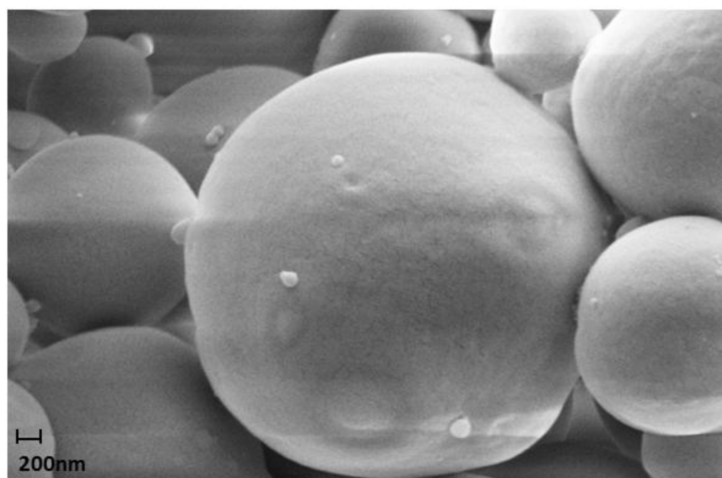

Fig. 2a

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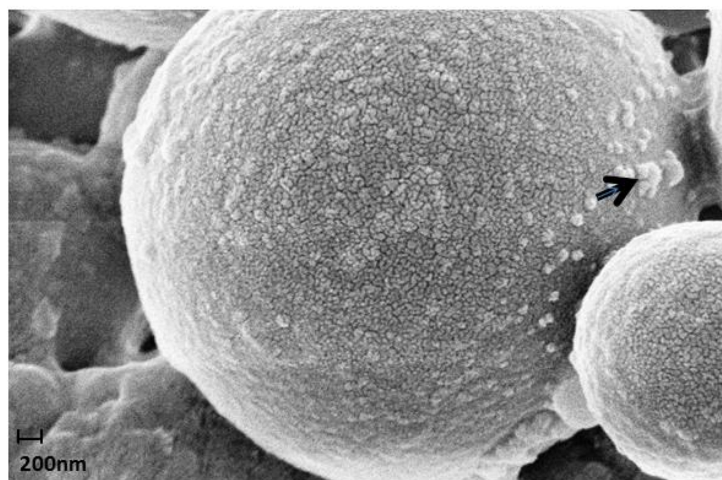

Fig. 2b

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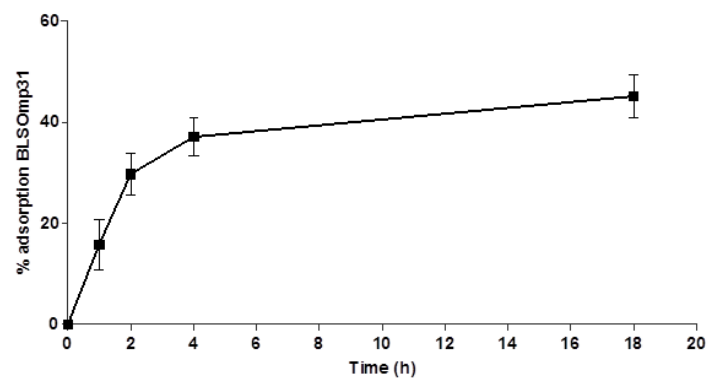

Fig. 3

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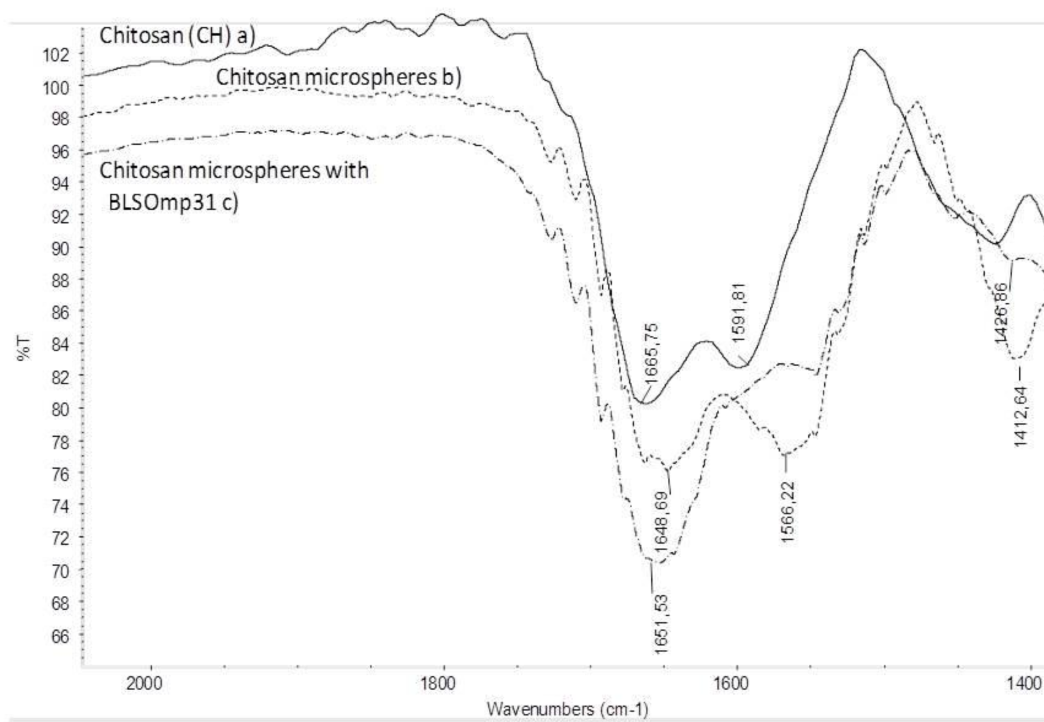

Fig. 4

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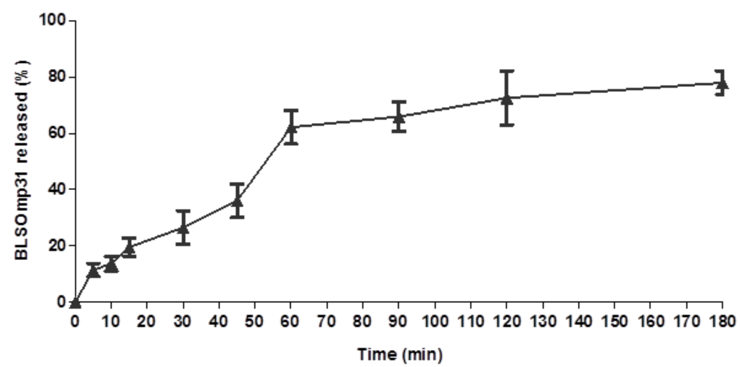

Fig. 5

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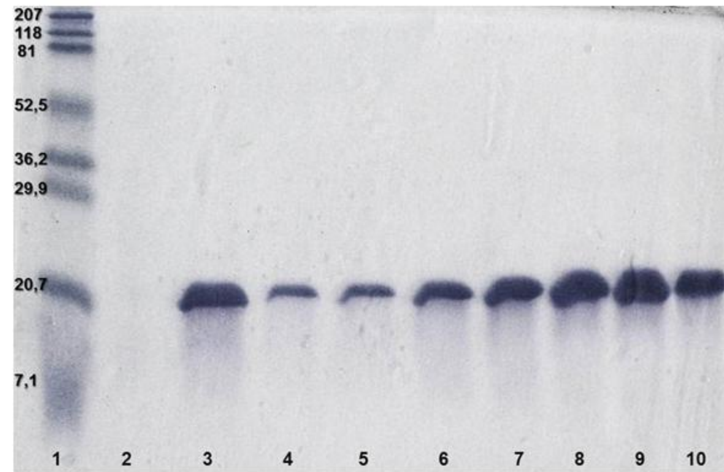

Fig. 6

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Fig. 7

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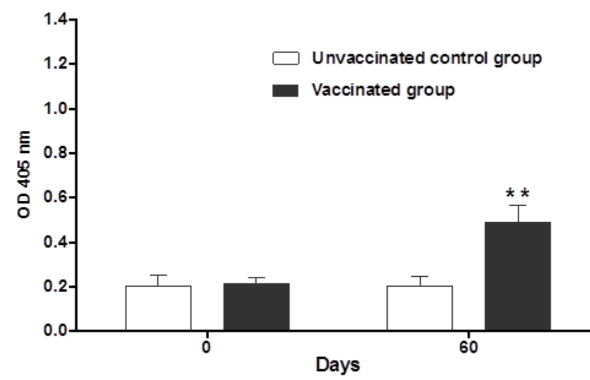

Fig. 8

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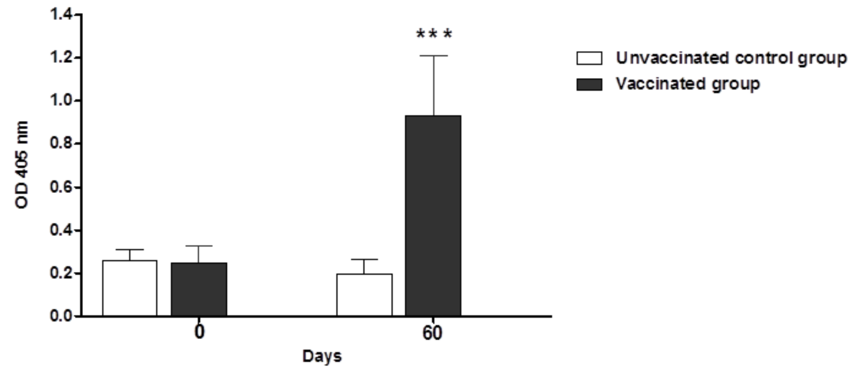

Fig. 9a

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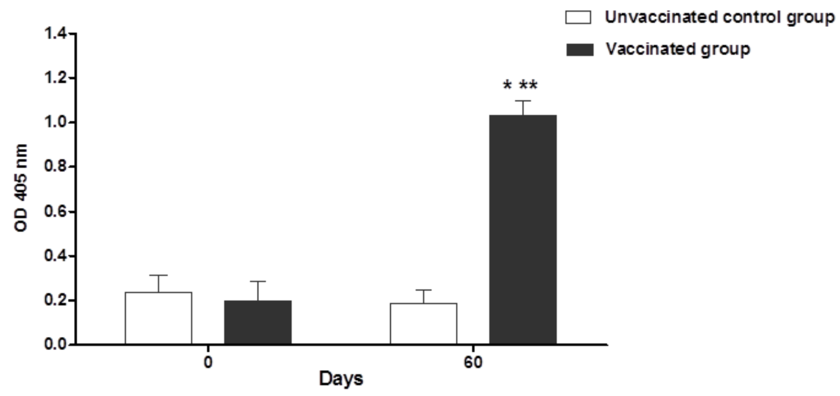

Fig. 9b

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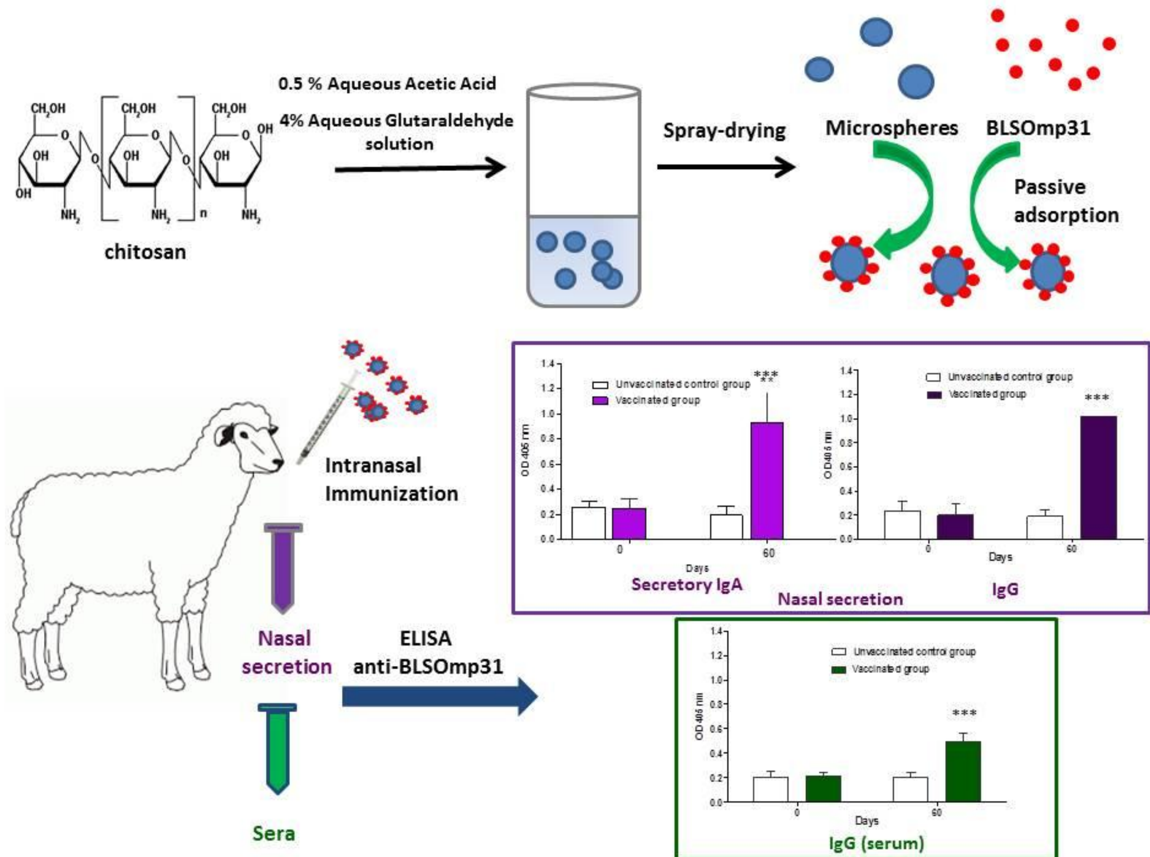

Graphical abstract

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Highlights

Spray dried chitosan microspheres as delivery system of BLSOmp31 against brucellosis

Chitosan microspheres had a good sphericity, particle size and a positive charge

The loading and release rate of BLSOmp31 was studied *in vitro*

Passive adsorption of BLSOmp31 onto microparticles did not affect its integrity

Intranasal immunization induced systemic and local antibodies in rams

BLSOmp31-loaded microspheres: new system for nasal immunization against brucellosis

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