Bioadhesive mannosamine-loaded nanoparticles for an effective ocular vaccination against animal brucellosis

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Introduction: Animal brucellosis is one of the major bacteriological diseases worldwide, and constitutes an important socioeconomic and sanitary problem. The current commercial vaccine used against this zoonosis is the *Brucella melitensis* Rev 1 vaccine, which, due to its live attenuated characteristics, displays a large number of drawbacks. Subcellular vaccines exhibit important advantages to face these handicaps. In this context, the hot saline (HS) subcellular antigenic extract from *B. ovis* has been proved to be highly immunogenic. However, due to its non-replicant nature, adequate adjuvants have to be associated. The need of an adequate adjuvant capable of increasing the mucosal immune response and protection, lead us to suggest the use of poly(anhydride) nanoparticles. To exploit the potential of these systems, nanoparticles were surface decorated with mannosamine, in order to specificly target mannose receptors highly expressed on the immune system cells.
Our purpose was to study the biopharmaceutical properties of HS loaded poly(anhydride) mannosylated (MAN-NP-HS) and conventional (NP-HS) nanoparticles, and evaluate their *in vivo* protective efficacy and biodistribution after ocular immunization.

Methods: Nanoparticles were prepared by the solvent displacement method, freeze-dried and characterized by PCS, ELDA, TEM, SEM, SDS-PAGE, Western-Blot and BCA^{TM} assay. The *in vitro* release and the stability in mucosal fluids were also evaluated. For the TLR's activation study, free HS, nanoparticles and adequate controls, were tested in duplicate on recombinant HEK-293 cell lines. An ocular immunization was performed in mice and, during this time, blood, faecal samples and spleens were recovered for IgG1, IgG2a, IgA, IL-2, Il-4, IFN-γ quantification. Eight weeks after vaccination animals were challenged against *B. ovis*, and 3 weeks later were slaughtered for bacteriological examinations. Moreover, ^{99m}technetium radiolabelled nanoparticles were ocular administered, and, at 2 and 24 hours, animals were slaughtered for *in vivo* biodistribution examinations.

Results: All freeze-dried formulations displayed a size of around 200-300 nm, with negative surface charge and low polydispersion (PDI<0.2), with a loading of about 30 µg HS per mg of nanoparticle with high entrapment efficiency (see Table 1). The mannosamine content, for MAN-NP-HS, was 32.1 ± 4.7 µg/mg nanoparticle. SEM analysis demonstrated the spherical and highly homogeneous nature of the nanoparticles (Figure 1). Figure 2 indicates that the protein profile, structural integrity and antigenicity of the entrapped HS in both nanosystems were maintained after preparation. Concerning to HS release, Figure 3 shows that both NP-HS and MAN-NP-HS showed a biphasic release pattern characterized by a burst effect followed by a continuous release of the antigen for at least 30 days. Furthermore, both systems were also highly stable in the mucosal environment, since after 2 h of incubation in lachrymal fluid, the remaining nanoparticles were of about 80%. The TLR's activation study demonstrated that both nanoparticles and free HS, clearly induced hTLR2, hTLR4 and hTLR5 expressing cell lines (Figure 4).
Interestingly, the elicited specific levels of IgG1, IgG2a, IgA, IL-2, IL-4 and IFN-γ levels showed a correlation with the bacteriological results. The degree of infection, expressed by the log mean ± SD CFU/spleen, was: i) MAN-NP-HS: 3.7 ± 0.1; ii) NP-HS: 4.9 ± 0.4; iii) Rev 1: 4.2 ± 0.3 and iv) control unvaccinated animals: 6.7 ± 0.3 (expressed by the log mean ± SD CFU/spleen, n=6). The *in vivo* biodistribution revealed that ^{99m}Tc-MAN-NP-HS were mainly located in the gastrointestinal tract, nasal and ocular mucosa and lymph nodes, probably due to their specific target and strong bioadhesive performance, thus enhancing the antigen delivery to the mucosal associated lymphoid tissue (MALT).

Conclusions: MAN-NP-HS revealed excellent characteristics as antigenic delivery systems throughout the ocular mucosa, by improving mucosal delivery and enhancing immune response. Their effective protection and intrisinc avirulence, make them a suitable anti-*Brucella* vaccine candidate.

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References

[1] Da Costa Martins R, Gamazo C, Irache, JM. Eur J Pharm Sci, **37** (2009) 563–572.

Figures and Tables

Table 1. Physicochemical characteristics of nanoparticles. NP-HS: HS loaded conventional nanoparticles; MAN-NP-HS: HS loaded mannosylated nanoparticles (data expressed as mean ±SD, n=10).

Vaccine formulation	Size (nm)	Zeta potential (mV)	Man content (µg/mg NP)	HS loading (µg/mg NP)
NP-HS	186±2	-37.7±0.7	-	33.5±0.4
MAN-NP-HS	306±11	-34.6±1.3	32.1±4.7	27.9±0.2

Figure 1. SEM microfotographs of NP-HS (A) and MAN-NP-HS (B).

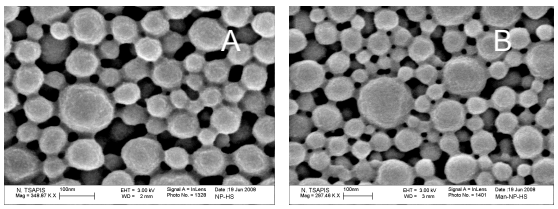

Figure 2. SDS-PAGE and Coomassie blue stain profiles of free and entrapped HS and Western blot against a pool of sera from *B. ovis* experimentally infected rabbits. A: Free HS (40 µg); B: NP-HS; C: MAN-NP-HS.

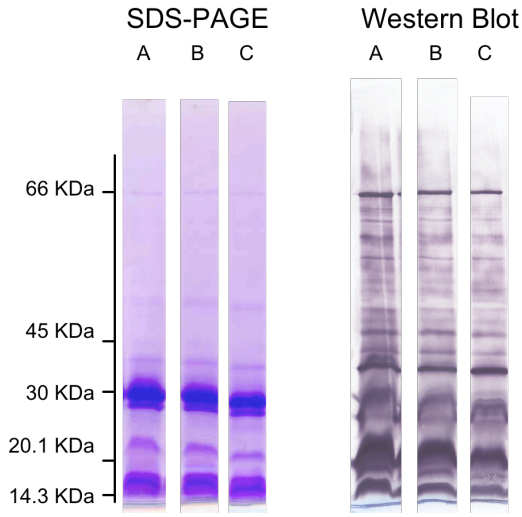

Figure 3. Antigenic release properties from HS containing nanoparticles. These graphs express in percentage the cumulative HS release from the formulations tested (BCA™ protein assay): NP-HS (●) and MAN-NP-HS (■). Data express the mean ± SD, n=3.

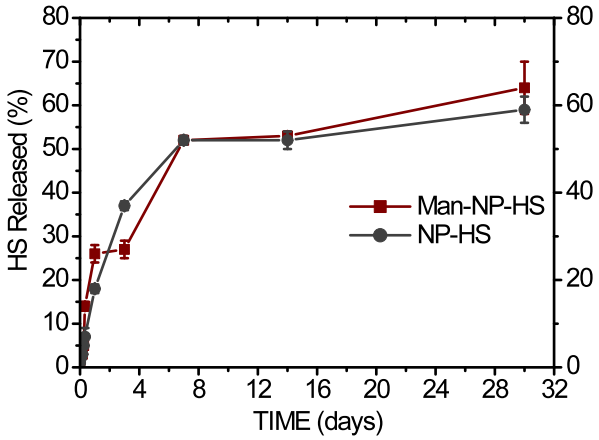

Figure 4. Effects of nanoparticles on the activation of TLR signaling. Bars represent engagement to TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 after incubation with positive controls (T+) and poly(anhydride) nanoparticles (NP). TLR non expressing recombinant cell line also included (TLR-). HEK-293 cells stably co-transfected with TLR and NF-κB-inducible, and secreted the alkaline phosphatase reporter gene. Results are given in optical density values (O.D.).