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PLGA nanoparticles loaded with the antileishmanial saponin β-aescin: Factor influence study and *in vitro* efficacy evaluation

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ABSTRACT

Colloidal carriers are known to improve the therapeutic index of the conventional drugs in the treatment of visceral leishmaniasis (VL) by decreasing their toxicity whilst maintaining or increasing therapeutic efficacy. This paper describes the development of poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles (NPs) for the antileishmanial saponin β-aescin. NPs were prepared by the W/O/W emulsification solvent evaporation technique and the influence of five preparation parameters on the NPs' size (Z_{ave}), zeta potential and entrapment efficiency (EE%) was investigated using a 2^{5-2} fractional factorial design. Cytotoxicity of aescin, aescin-loaded and blank PLGA NPs was evaluated in J774 macrophages and non-phagocytic MRC-5 cells, whereas antileishmanial activity was determined in the *Leishmania infantum ex vivo* model. The developed PLGA NPs were monodispersed with Z_{ave} < 500 nm and exhibited negative zeta potentials. The process variables 'surfactant primary emulsion', 'concentration aescin' and 'solvent evaporation rate' had a positive effect on EE%. Addition of Tween® 80 to the inner aqueous phase rendered the primary emulsion more stable, which in its turn led to better saponin entrapment. The selectivity index (SI) towards the supporting host macrophages increased from 4 to 18 by treating the cells with aescin-loaded NPs instead of free β-aescin. In conclusion, the *in vitro* results confirmed our hypothesis.

1. Introduction

Leishmaniasis is a protozoan infection that is transmitted by means of sandfly bite to vertebrate hosts including man. The potentially fatal visceral form, *i.e.* visceral leishmaniasis (VL), is caused by *Leishmania donovani* in the Indian subcontinent, Asia and Africa, *Leishmania infantum* in the Mediterranean basin and *Leishmania chagasi* in South America. According to the World Health Organization (WHO) about 500,000 new cases of VL are considered to occur every year globally. Further, the incidence of VL is thought to increase and its endemic areas are thought to expand due to environmental changes, migration and HIV-VL co-infection. Control of VL relies almost exclusively on chemotherapy, which consists of a handful of drugs with serious limitations such as drug unresponsiveness, difficult route of administration, high cost and toxicity (Chappuis et al., 2007; Kedzierski et al., 2009; Maltezou, 2010). Although miltefosine and paromomycin were registered as clinical agents against VL in the last decade, the antileishmanial drug arsenal still requires improvement (Richard and Werbovetz, 2010). For instance, miltefosine monotherapy has failed to cure relapsing VL in HIV-infected patients and thus its role against the increasing problem of HIV-associated VL remains unclear (Ezra et al., 2010). In light of these limitations, WHO strongly recommends and supports research into new drugs including natural plant products (DNDi annual report, 2008; Frézard and Demicheli, 2010).

Saponins are widely distributed in higher plants and have a wide range of biological and pharmacological properties such as antimicrobial, anti-inflammatory and antitumor activities. These surface-active glycosides can be classified into three groups based on the nature of their aglycone skeleton: (i) triterpenoid saponins, (ii) steroidal saponins and (iii) steroidal alkaloids (Sparg et al., 2004). The former group is most common and triterpenoid saponin extracts from *Maesa balansae*, *Careya arborea* and *Astragalus oleifolius* were reported to possess antileishmanial activity (Dinda et al., 2010). Recently our research group added β-aescin, the main saponin from the seeds of *Aesculus hippocastanum* (horse chestnut), to this list (Van de Ven et al., in press). β-aescin

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appeared to be moderately active on the intracellular amastigote stage of *L. infantum* with an average IC_{50} of 1.55 ± 0.32 μg/ml and inactive on the extracellular promastigote stage ($IC_{50} > 16$ μg/ml), suggesting a macrophage-mediated mechanism as is the case for the pentavalent antimonials (Kedzierski et al., 2009). The exact mechanism of action of β-aescin remains unknown and deserves further research, but saponins were previously shown to exert their antileishmanial activity through the induction of apoptosis or programmed cell death in the parasite (Delmas et al., 2000; Dutta et al., 2007). Saponins, however, also have undesirable properties such as high cytotoxicity, rendering them less suitable candidates for drug development (Kedzierski et al., 2009). In this study, we emphasise the strategy of using polymeric nanoparticles (NPs) composed of poly(D,L-lactide-co-glycolide) (PLGA) as delivery vehicle for β-aescin to circumvent the saponin's toxicity. Colloidal carriers are known to improve the therapeutic index of the conventional drugs in the treatment of VL (Gupta et al., 2010). The mechanism is presumed to be a facilitated delivery of the drugs to macrophages of liver, spleen and bone marrow either through natural affinity of these carriers (passive targeting) or through macrophage-associated receptors (active targeting). Passive targeting is enabled by the inherent capacity of phagocytic cells to ingest carrier systems, which are recognised as substances foreign to the organism (Briones et al., 2008).

The aescin-loaded PLGA NPs were prepared using the W/O/W emulsification solvent evaporation technique and the influence of five preparation parameters on the NPs' physicochemical characteristics was investigated using a 2^{5-2} fractional factorial design. The five factors were: (i) presence of surfactant to stabilise the primary emulsion, (ii) presence of viscosifying agent in the inner aqueous phase, (iii) aescin concentration, (iv) PLGA concentration and (v) solvent evaporation rate. The fractional design allowed us to identify the major factors influencing the entrapment of the amphiphilic saponin in the PLGA NPs and to select the optimal aescin-loaded PLGA NP formulation with the highest entrapment efficiency (EE%). The latter was withheld for the *in vitro* cytotoxicity and drug sensitivity assays. We determined the selectivity index (SI) for both β-aescin and the selected NP formulation and used it as a tool to test our hypothesis.

2. Materials and methods

2.1. Materials

The PLGA polymer was Resomer® RG 503 (Boehringer Ingelheim, Germany) with a molecular weight of 40 kDa, D,L-lactide:glycolide 52:48 molar ratio and inherent viscosity of 0.32–0.44 dl/g. Aescin was obtained from Fluka (Sigma, Belgium). Poly(vinylalcohol) (PVA), with an average molecular weight between 30 and 70 kDa, Tween® and Span® sorbitan surfactants, *p*-anisaldehyde, $NaHCO_3$, potato starch, Giemsa stain and resazurin were purchased from Sigma (Belgium), whereas Dulbecco's Phosphate-Buffered Saline (D-PBS), Minimal Essential Medium (MEM), RPMI-1640 medium, Glutamax®, L-glutamine and foetal calf serum (FCS) were supplied by Invitrogen (Merelbeke, Belgium). Poloxamer 188 or Lutrol® F68 with a molecular weight of 7680–9510 Da was purchased from BASF (Germany). The solid phase extraction (SPE) C_{18ec} cartridges were obtained from Macherey-Nagel Filter Service (Eupen, Belgium), the silica gel 60 F_{254} HPTLC-plates were from Merck (Belgium). Milli-Q water was prepared with a Millipore water purification system (Millipore Co., Bedford, USA). The organic solvents dichloromethane (DCM) and methanol (MeOH) were of analytical grade and purchased from Sigma (Belgium).

2.2. Parasites, animals and cell cultures

Leishmania infantum MHOM/MA(BE)/67 was kindly provided by the Institute of Tropical Medicine in Antwerp (Belgium) and was maintained in the laboratory by serial passage in golden hamsters (*Mesocricetus auratus*). Fresh *ex vivo* amastigotes were obtained from the spleen of an infected donor hamster. *L. infantum ex vivo* amastigotes were allowed to transform to extracellular promastigotes as described previously (Vermeersch et al., 2009). Primary mouse macrophages (PMM) were collected from Swiss CD-1 mice (Elevage Janvier, France) 2 days after intraperitoneal stimulation with one millilitre of a 2% (w/v) aqueous potato starch suspension. Cells were collected and grown in RPMI-1640 medium supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$ and 5% (v/v) inactivated FCS at 37 °C under 5% CO_2. The MRC-5_{SV2} cell line, an SV40 immortalised human fibroblast cell line, was obtained from the European Cell Culture Collection and cultured in MEM with Earle's salts supplemented with 0.029% (w/v) L-glutamine, 0.16% (w/v) $NaHCO_3$ and 5% (v/v) inactivated FCS at 37 °C under 5% CO_2. The murine macrophage-like cell line J774A.1 was kindly provided by the Laboratory of Physiopharmacology of the University of Antwerp (Belgium) and was grown in RPMI-1640 enriched with Glutamax® and 10% (v/v) FCS at 37 °C under 5% CO_2. All animal experiments were approved by the Ethical Committee of the University of Antwerp (Belgium).

2.3. Preparation of water-in-DCM (W/O) emulsions

Two millilitres of Milli-Q water, containing the hydrophilic surfactants under investigation in the concentration range 1–10% (w/v), were emulsified in DCM by means of ice-cooled sonication for 1 min at ±20 W (amplitude set at 60%) (Branson Sonifier® Model S-450D, Branson, UK) at a 1:5 (W:O) ratio. Appropriate volumes of stock solutions of the hydrophobic Span® surfactants in DCM were added to the organic phase. The stability of the obtained W/O emulsion was investigated at room temperature. The time required for the initial formation of water globules (*i.e.* creaming) was determined (Leo et al., 1998; Mohamed and van der Walle, 2006).

2.4. Preparation of PLGA NPs

The NPs were prepared by means of a W/O/W emulsification solvent evaporation method. Two millilitres of an aqueous aescin solution (5%, w/v or 2.5%, w/v), in some cases also containing polysorbate 80 as surfactant and/or poloxamer 188 as viscosifying agent, were emulsified by means of ice-cooled sonication for 1 min at 23W (amplitude set at 60%) in an organic phase that consisted of 0.50 or 1.00 g of PLGA dissolved in 10 ml of DCM. The resulting W/O emulsion was dispersed in 25 ml 1% (w/v) PVA stabiliser solution and sonicated for 1 min at 20 W (amplitude set at 50%) on ice to obtain a multiple W/O/W emulsion. The multiple emulsion was then diluted in 120 ml 0.3% (w/v) PVA stabiliser solution. The organic solvent was allowed to evaporate at room temperature during 4 h under agitation (700 rpm) with a magnetic stirrer or during 2 h at reduced pressure (Rotavapor R-200 and Vacuum Controller V-800, Büchi, Switzerland). In case of the latter a gradient method starting from 600 mbar to 50 mbar was used for the first hour, pressure was subsequently maintained at 50 mbar. Each sample was prepared in duplicate. The obtained PLGA NPs were purified by cross-flow filtration to remove non-incorporated aescin and excess stabiliser, however, a fraction of the non-purified NPs was withheld for the indirect determination of aescin entrapment efficiency. The NPs were filtered two times over regenerated cellulose membranes with a MWCO of 100 kDa (Vivaflow® 50, Sartorius) using a Masterflex® L/S pump (model 7518-00) and tubing (Sartorius, Germany). After the addition of mannitol (5%, w/v) as

cryoprotectant, the purified and non-purified NP formulations were cooled down to −18 °C and subsequently freeze-dried (Leybold-Heraeus D8B, GT-2A, Germany). The freeze-dried NPs were stored at +4 °C.

2.5. Viscosity measurements

The dynamic viscosity of a 5% (w/v) Lutrol® F68 solution was measured at 25 °C using a Brookfield DV-II+ Viscometer (Massachusetts, USA).

2.6. Surface tension measurements

Surface tension (σ) measurements of Tween® 80 and aescin solutions in Milli-Q water and D-PBS were conducted using the ring of Lecomte de Nouy and a torsion dynamometer (range 0–10 mN and accuracy 0.1 mN, Phywe Systeme, Germany). The ring was cleaned thoroughly with ethanol and Milli-Q water between measurements. Each measurement was performed in triplicate at 24 ± 1 °C and each solution was left to equilibrate for at least 30 min before measuring. The critical micelle concentration (cmc) was determined by measuring the surface tension as a function of Tween® 80 and aescin concentration. The cmc is found from a break in the plot of surface tension versus the natural logarithm of the surfactant concentration, and thus was obtained as the intersection of two straight lines.

2.7. Physical characterisation of PLGA NPs

The mean particle size and the size distribution of the NPs was determined by Photon Correlation Spectroscopy (PCS) with a Zetasizer 3000 (Malvern Instruments, UK). Freshly prepared and purified PLGA NPs were diluted with Milli-Q water, freeze-dried NPs were reconstituted in Milli-Q water using ice-cooled sonication. *Z*-average diameter (Z_{ave}) and polydispersity index (PI) were calculated with the Malvern software package by Cumulants method. All samples, before and after freeze-drying, were measured in triplicate. The average value of the measurements was calculated and used for statistical analysis.

The zeta potential of the NPs was determined with the Zetasizer 3000 (Malvern Instruments, UK) using Electrophoretic Light Scattering (ELS). Averages and standard deviations were obtained from ten consecutive measurements on the same sample.

2.8. Determination of entrapment efficiency

The efficiency at which aescin was entrapped in or adsorbed to the PLGA NPs was determined indirectly: the amount of free aescin present in the supernatant obtained after centrifugation of the non-purified PLGA NPs was determined using a validated thin-layer chromatographic method (HPTLC). The entrapment efficiency (EE%) was calculated using Eq. (1).

$$\text{EE\%} = ((\text{initial amount added to formulation} - \text{amount of free drug determined in supernatant})/\text{initial amount}) \times 100\% \quad (1)$$

Briefly, 200 mg of the freeze-dried NPs was resuspended in water, filled up to 10.0 ml with the same solvent and centrifuged at 4000 rpm for 4 h at 4 °C. In order to eliminate the interference caused by polysorbate 80 and mannitol a sample preparation step using C_{18ec} SPE cartridges was included. Five millilitres of the supernatant were transferred on a 500 mg cartridge previously conditioned with MeOH and water. After washing with water, aescin was eluted from the SPE cartridge with 18 ml MeOH. The eluate was diluted to 20.0 ml with MeOH and analysed for aescin content. The sample solutions were spotted in duplicate using a Camag automatic TLC sampler 4 (Camag, Switzerland) onto silica gel HPTLC-plates. The plate was developed using the upper layer of a mixture acetic acid–water–butanol (10:40:50) as mobile phase, subsequently dried, dipped in *p*-anisaldehyde detection reagent EP 1007301 and heated for 5 min at 100–105 °C. The remission absorption of the coloured product was measured at 535 nm by a Camag TLC scanner 3 (Apers et al., 2006). Validation of the method according to ICH guidelines showed that it was precise ($RSD_{between\ days}$ 1.5%) and accurate (mean recovery 97.7%).

2.9. Determination of drug loading

The saponin drug loading of the PLGA NPs was determined directly by measuring the amount of aescin entrapped in the NPs. With this purpose, the NPs were redissolved in DCM followed by the extraction of aescin in water. However, the saponin extraction is incomplete because of the surface-active properties of these compounds. In order to circumvent this problem, DCM was allowed to evaporate during sample preparation. Briefly, 200 mg of purified and freeze-dried PLGA NPs was accurately weighed and dispersed in 10 ml DCM, to which subsequently 10 ml of water was added. The mixture was stirred on a magnetic stirring plate (400 rpm) during 30 min. During this period the beaker was covered in order to prevent DCM from evaporating, so that the PLGA polymer could dissolve quantitatively. After 30 min the DCM was allowed to evaporate, inducing precipitation of PLGA. The dispersion was centrifuged at 4000 rpm for 1 h at 4 °C. The supernatant was subsequently quantitatively transferred on a 500 mg SPE C_{18ec} cartridge previously conditioned with MeOH and water. From this point onwards, a SPE purification step was introduced to eliminate the mannitol that interferes in the HPTLC determination of aescin. Sample preparation and analysis was performed as described in 2.8. Validation of the method according to ICH guidelines showed that it was precise ($RSD_{between\ days}$ 2.9%) and accurate (mean recovery 98.2%).

2.10. In vitro assays

2.10.1. Compounds

A stock solution of the reference saponin aescin was prepared by dissolving the compound in DMSO, further dilution in Milli-Q water was performed so that the final in-test concentration of DMSO was below 0.6% (v/v). The aescin-loaded PLGA NPs were reconstituted in sterile water in aseptic conditions by means of the Branson Sonifier®. Serial two-fold dilutions of the compounds and NPs were performed in sterile Milli-Q water in sterile 96-well microtiter plates and were stored, if necessary, at 4 °C. The in-test concentration range was 16 μg/ml to 0.03 μg/ml aescin equivalents, taking the drug loading of the aescin-loaded PLGA NP formulation into account, for the antileishmania assay and 64 μg/ml to 0.125 μg/ml for the cytotoxicity assay.

2.10.2. Cytotoxicity assay

The influence of blank and aescin-loaded PLGA NPs on *in vitro* cell viability was determined using a resazurin-based assay (Crouch and Slater, 2001; O'Brien et al., 2000; Van de Ven et al., 2009). The MRC-5_{SV2} and J774A.1 cells were seeded in sterile 96-well microtiter plates at 3×10^4 cells/well, 7.5×10^3 cells/well respectively. For this assay the cell culture medium was additionally supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). After 72 h of incubation with the test compounds at 37 °C and 5% CO_2, 50 μl of a resazurin stock solution was added and cell viability was measured fluorometrically after incubation for another 4 h (excitation λ, 550 nm; emission λ, 590 nm). The results were expressed as the percentage of reduction in cell

Table 1
Correspondence between orthogonal (−1 and +1) and real values of the five factors investigated in the factorial design.

Factor	Lower level (−**1**)	Upper level (+1)
Surfactant primary emulsion (x_1)	None	8.0% (w/v) Tween® 80
Concentration aescin (x_2)	2.5% (w/v)	5.0% (w/v)
Viscosifying agent in inner water phase (x_3)	None	5.0% (w/v) Lutrol® F68
Concentration PLGA (x_4)	5% (w/v)	10% (w/v)
Solvent evaporation conditions (x_5)	Atmospheric (AP)	Reduced pressure (RP)

growth/viability compared to untreated control wells (McMillian et al., 2002). All tests were performed in triplicate. The cytotoxic concentration causing 50% cell death (CC_{50}) was determined from the concentration-response curves using linear regression analysis.

2.10.3. Intracellular drug sensitivity assay

The collected PMM were seeded in sterile 96-well microtiter plates at 3×10^5 cells/well and were left for adhesion and differentiation. After 48 h of incubation at 37 °C and 5% CO_2, the cells were infected with *L. infantum ex vivo* amastigotes at a multiplicity of 15 amastigotes per cell. Two hours post infection, cells were washed and the compound dilutions were added. After incubation for another 120 h, the cells were fixed with MeOH and stained with 10% (v/v) Giemsa solution for microscopic reading. The total intracellular amastigote burden (=average number of amastigotes per cell) in treated wells was compared to that of control wells (Vermeersch et al., 2009). All tests were performed in duplicate in two independent repeats. The 50% inhibitory concentration (IC_{50}) was determined from the concentration-response curves using linear regression analysis. A compound is classified as active when the IC_{50} is lower than 1 μg/ml, a compound with IC_{50} between 1 and 16 μg/ml is classified as moderately active and a compound is classified as inactive when the IC_{50} is higher than 16 μg/ml (Cos et al., 2006). The antileishmania reference drug miltefosin (IC_{50}, 5.2 ± 0.8 μM) was included in each test run.

2.10.4. Axenic drug sensitivity assay

Assays were performed in sterile 96-well microtiter plates, with each well containing 10 μl of the compound dilution and 200 μl of parasite inoculum (1×10^5 log-phase promastigotes/well), at 25 °C under normal atmospheric conditions. Parasite multiplication was compared to that for untreated controls (100% growth) and uninfected controls (0% growth). After 72 h of incubation, resazurin was added and cell viability was measured fluorometrically after incubation for another 8 h (excitation λ, 550 nm; emission λ, 590 nm). The results were expressed as the percentage of reduction in the parasite burden compared to that in untreated control wells (Vermeersch et al., 2009).

2.11. Experimental design and statistical analysis

By means of factorial design the influence of the preparation parameters (presence of surfactant to stabilise the primary emulsion, presence of viscosifying agent in the inner aqueous phase, aescin concentration, PLGA concentration and solvent evaporation rate) on the NPs' physicochemical properties was investigated. The five factors were set at two levels, which could be represented as orthogonal values (Table 1). The factor x_6 was added to evaluate the effect of freeze-drying on NPs' size and zeta potential, but was excluded from the fractionation since its influence on drug EE% is out of scope. A full factorial design with five factors at two levels would take 2^5 or 32 experiments (see Table in Supplementary data). Additionally, a minimum of one replica for each experiment needs to be taken into account to estimate the experimental error. In the case of large numbers of experiments, *i.e.* 64 in our case, fractional factorial designs are preferred to full factorial designs in order to estimate the influence of the main effects without looking at higher order interactions. When a 2^5 full factorial design is split into a 1/4 fractional factorial design, the number of experiments is reduced from 2^5 to 2^{5-2} or 8. In order to fractionate we identified two independent generators, *i.e.* 134 and 1235. We divide the model matrix, as depicted in Table Supplementary data with main effects, first-order interactions, second-order interactions and higher order interactions in half by selecting those experiments for which the element in column 1235 is +1. We then further partition by taking the experiments for which the column 134 is equal to +1. The obtained quarter fraction of the initial 2^5 design consists of 8 experiments (Table 2) and is of resolution III. It allows main effects to be estimated independently of one another, but they are each confounded with first-order interactions. This fact needs to be taken into account in the interpretation of the results. The five factor interaction 12345 cannot be chosen as generator since this would result in a fractional design of resolution IV in which first-order, or two factor, interaction effects are confounded with one another (Lewis et al., 1999). The synergistic model for five factors contains 32 coefficients, of which only 8 can be calculated by performing 8 independent experiments. In the case of a 2^{5-2} fractional factorial design a model (Eq. (2)) containing only first degree terms estimating the main effects of the investigated factors on the response y, *i.e.* size, zeta potential and aescin EE% can be postulated.

Table 2
Generated combinations in orthogonal values for a 2^{5-2} fractional factorial design.

Run	Formula	X_1	X_2	X_3	X_4	X_5
4	I	+1	+1	−1	−1	−1
7	II	−1	+1	+1	−1	−1
9	III	−1	−1	−1	+1	−1
14	IV	+1	−1	+1	+1	−1
18	V	+1	−1	−1	−1	+1
21	VI	−1	−1	+1	−1	+1
27	VII	−1	+1	−1	+1	+1
32	VIII	+1	+1	+1	+1	+1

Run numbers refer to the table in Supplementary Data.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 \quad (2)$$

The design was set up using Statistica® software (Statsoft, Tulsa, USA) and multiple linear regression analysis and ANOVA were employed to model the data and develop the mathematical expression (Eq. (2)). Independent Samples *T*-test or One-way ANOVA followed by post-hoc analysis (Tukey HSD test) were used to determine whether data groups differed significantly from each other. A *p*-value lower than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Preparation and characterisation of PLGA NPs

One of the major drawbacks of polymeric NPs is their low drug-loading capacity (Breunig et al., 2008). In order to exert their *in vitro* and *in vivo* biological activity the NPs should exhibit high drug content and good drug retention (Ishihara and Mizushima, 2010). Various techniques for the encapsulation of both hydrophobic and hydrophilic drugs in PLGA NPs are available, however, an overall approach to entrap amphiphilic drugs is lacking. A screening of preparation conditions is thus compulsory in the development of the aescin-loaded PLGA NP formulation. Drug leakage into the

Table 3
Overview showing the different surfactants, surfactant blends and their corresponding Hydrophilic–Lipophilic Balance (HLB value).

Surfactant	HLB value
Span® 85	1.80
Span® 80	4.30
0.50 Span® 80 – 0.50 Tween® 80	9.65
0.25 Span® 80 – 0.75 Tween® 80	12.33
Tween® 80	15.00
Tween® 20	16.70
Lutrol® F68	29.00

external aqueous phase during the emulsification and solvent evaporation phase of the W/O/W emulsification solvent evaporation technique reduces considerably the entrapment efficiency (EE%) of water-soluble drugs (Reis et al., 2006). Proposals in literature to overcome this obstacle include: (i) increasing the viscosity of the organic phase (Cui et al., 2005), (ii) increasing the viscosity of the internal aqueous phase (Ubrich et al., 2004), (iii) increasing the rate of solvent removal (Ubrich et al., 2004) and (iv) improving the stability of the W/O emulsion (Mohamed and van der Walle, 2006). The latter is considered key point for the entrapment of hydrophilic compounds.

3.1.1. Stability of the primary emulsion

Double emulsions suffer from two types of instabilities: (i) coalescence of the small inner droplets with the globule interface or (ii) coalescence between the small inner droplets within the oil globule (Ficheux et al., 1998). The former leads to a complete delivery of the small inner droplets and their content towards the external phase, whereas the second one leads to hollow or capsular microspheres (Ficheux et al., 1998; Mohamed and van der Walle, 2006). The use of combinations of sorbitan esters and polysorbates as stabilisers of the primary emulsion has already been reported (Freitas et al., 2005; Mohamed and van der Walle, 2006). In our small-scale study the sorbitan surfactants or their blends with different Hydrophilic-Lipophilic Balance values (HLB values) (Table 3) were added to the water-in-DCM emulsions in the concentration range 1–10% (w/v). Coalescence and the formation of water globules occurred almost instantaneously (<30 s) and irrespective of the concentration for Span® 85, Span® 80 and Tween® 20 (Fig. 1). Phase separation was observed after 30 min. In contrast, Tween® 80 and the blends Span® 80/Tween® 80 yielded more stable emulsions in a concentration-dependent manner (Fig. 1). Mohamed and van der Walle (2006) observed emulsion stability (W/O ratio of 1:5) for at least 50 s when surfactant blends with an HLB value between 8 and 13 were used at

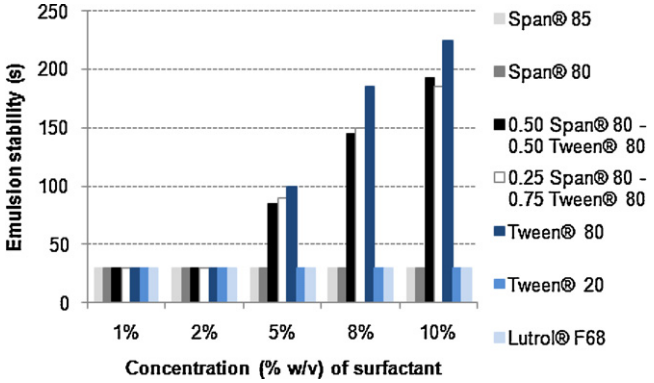

Fig. 1. Influence of surfactants on the stability of water-in-DCM (1:5 ratio) emulsions, measured as the time required for the initial formation of water globules (*i.e.* creaming).

a 1.0% (v/v) concentration. We preferred withholding Tween® 80 as stabiliser of the primary emulsion since this surfactant is approved by the FDA for intravenous administration (up to a concentration of 10%, w/v), whereas Span® 80 is not (Croy and Kwon, 2005; Date et al., 2007). Lutrol® F68 was shown to be a very poor stabiliser of the water-in-DCM emulsions (Fig. 1).

3.1.2. Statistical analysis of particle size

By means of a 2^{5-2} fractional factorial design, the influence of the preparation parameters (presence of surfactant to stabilise the primary emulsion, presence of viscosifying agent in the inner aqueous phase, aescin concentration, PLGA concentration and solvent evaporation rate) on the NPs' physicochemical properties was investigated. The five factors were set each at two levels, which could be represented as orthogonal values (Table 1). Poloxamer 188 or Lutrol® F68 was used as viscosifying agent in the inner water phase and for this purpose its concentration was fixed at 5.0% (w/v) (Table 1). The dynamic viscosity of the internal water phase containing 5.0% (w/v) Lutrol® F68 and 8.0% Tween® 80 amounted to 4.2 ± 0.1 mPa s and, as demonstrated in 3.1.1., Lutrol® F68 did not affect the primary emulsion stability. The eight aescin-loaded PLGA NP formulations, each with a different set of preparation parameters (Table 2), were prepared in duplicate. Experiments were randomised completely to reduce systematic errors. Particle size (Z_{ave}) and zeta potential of the PLGA NPs were measured both before and after freeze-drying, offering the possibility to evaluate statistically the influence of lyophilisation (factor x_6) on these two

Table 4
Experimental results expressed as mean ± S.D. for Z_{ave} ($n = 3$), zeta potential ($n = 10$) and EE% ($n = 3$) of the different aescin-loaded PLGA NP formulations prepared in duplicate.

Formulation	Before freeze-drying			After freeze-drying			EE%[a]
	Z_{ave} (nm)	PI (–)	Zeta potential (mV)	Z_{ave} (nm)	PI (–)	Zeta potential (mV)	
Ia	275.6 ± 2.6	0.18	−26.8 ± 0.8	293.7 ± 2.8	0.02	−25.2 ± 0.3	19.0 ± 0.6
Ib	265.2 ± 1.7	0.04	−25.8 ± 1.0	276.1 ± 2.3	0.11	−26.3 ± 0.3	17.6 ± 1.1
IIa	287.8 ± 1.9	0.10	−22.0 ± 0.4	306.2 ± 2.9	0.08	−24.1 ± 0.3	10.6 ± 2.6
IIb	296.0 ± 2.2	0.10	−25.4 ± 0.3	311.4 ± 1.2	0.08	−23.9 ± 0.4	8.2 ± 0.8
IIIa	427.9 ± 4.1	0.05	−27.1 ± 0.2	442.0 ± 4.8	0.35	−24.4 ± 1.5	13.9 ± 1.3
IIIb	447.1 ± 5.4	0.12	−25.2 ± 0.4	480.2 ± 5.7	0.39	−23.0 ± 0.8	7.7 ± 2.1
IVa	323.1 ± 2.4	0.29	−25.3 ± 0.6	401.6 ± 3.0	0.13	−25.8 ± 0.2	5.8 ± 2.6
IVb	331.6 ± 3.0	0.07	−29.2 ± 0.4	427.2 ± 3.8	0.23	−20.5 ± 0.4	2.8 ± 2.0
Va	270.5 ± 1.8	0.13	−23.1 ± 1.8	286.9 ± 1.8	0.12	−22.7 ± 1.2	13.1 ± 0.1
Vb	290.2 ± 2.0	0.24	−22.8 ± 1.5	304.8 ± 4.3	0.24	−21.7 ± 0.8	17.7 ± 0.2
VIa	313.3 ± 1.6	0.06	−20.4 ± 0.8	312.6 ± 3.2	0.12	−26.0 ± 0.3	6.0 ± 1.8
VIb	299.3 ± 1.1	0.04	−18.4 ± 0.7	300.0 ± 1.1	0.24	−23.3 ± 0.4	4.6 ± 2.2
VIIa	391.5 ± 1.8	0.10	−21.5 ± 1.1	439.7 ± 7.2	0.56	−26.7 ± 0.2	20.6 ± 2.5
VIIb	350.6 ± 1.3	0.22	−18.9 ± 0.8	367.5 ± 6.3	0.37	−25.3 ± 0.4	12.5 ± 1.6
VIIIa	269.6 ± 1.1	0.07	−26.5 ± 3.0	339.3 ± 3.1	0.05	−15.0 ± 0.3	31.8 ± 1.2
VIIIb	261.4 ± 1.6	0.12	−24.7 ± 1.0	339.9 ± 4.5	0.15	−18.1 ± 0.7	31.9 ± 2.5

[a] Determined indirectly.

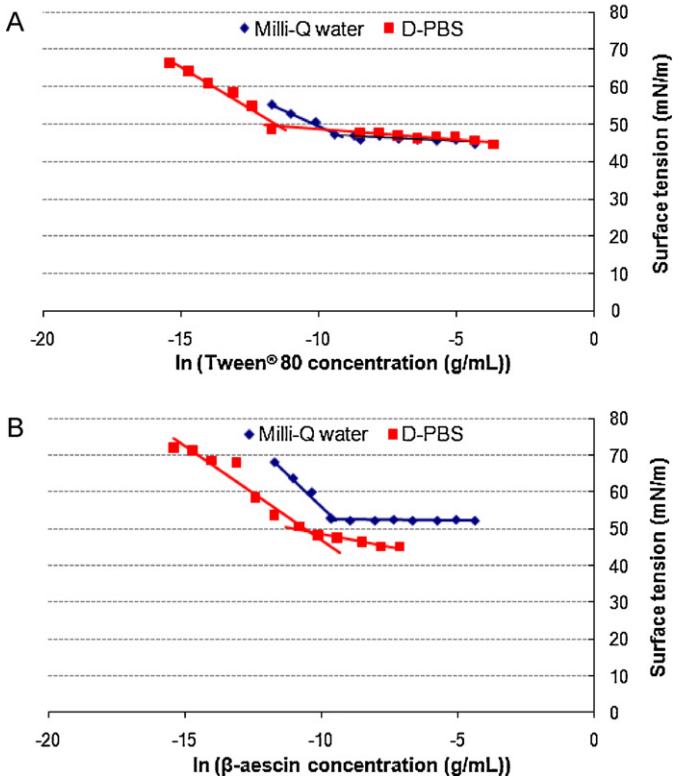

Fig. 2. Surface tension versus the natural logarithm of the concentration of (A) Tween® 80 and (B) β-aescin.

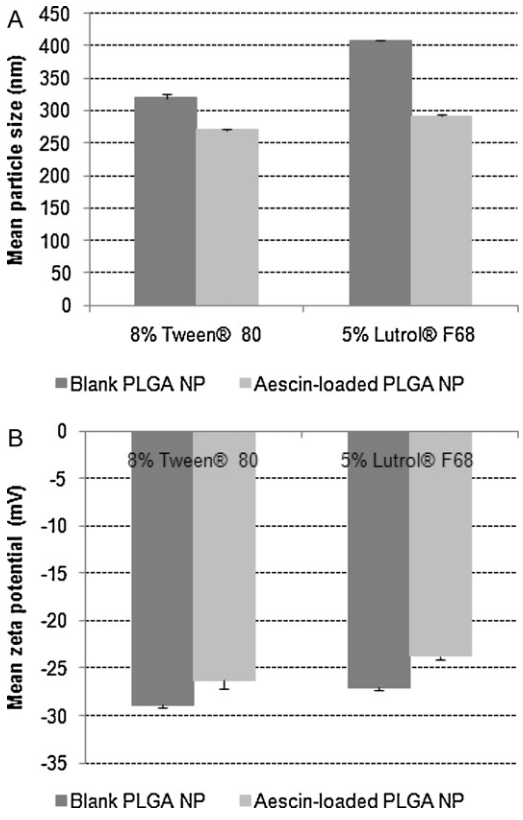

Fig. 3. Effect of drug loading on (A) particle size and (B) zeta potential. Blank and aescin-loaded PLGA NPs were prepared using the same formulation parameters, PLGA NPs were loaded with aescin at 20% (w/w) to PLGA (data represented as mean ± S.D.$_{pooled}$; $n = 6$ in case of size, $n = 20$ for zeta potential).

responses as well. Table 4 shows the results of the size determinations along with their standard deviations (S.D.) for all experiments. The obtained (non-lyophilised) aescin-loaded PLGA NPs, except formulation IVa, all had acceptable PI values (PI ≤ 0.25) (Table 4). Z_{ave} of the NPs was significantly influenced by the concentration of PLGA in the organic phase ($p \leq 0.001$), the presence of Tween® 80 in the formulation ($p < 0.05$) and freeze-drying ($p < 0.05$). The size of the effects of the six investigated factors are summarised in Table 5.

3.1.2.1. Effect of PLGA concentration. When the PLGA concentration in the organic phase was increased from 5% to 10% (w/v), the mean particle size increased with 93.2 nm (Table 5). The polymer concentration is a critical parameter influencing NPs' size since it determines the dynamic viscosity of the PLGA solution and thus its dispersability into the aqueous phase (Mainardes and Evangelista, 2005). The more viscous the organic phase, the lower the net shear stress at fixed energy input during emulsification and thus the larger the emulsion droplets and resulting NPs.

3.1.2.2. Effect of viscosifying agent. In contrast to the aforementioned findings (Section 3.1.2.1), the higher dynamic viscosity of the inner aqueous phase due to the presence of a viscosifying agent did not have a statistically significant effect on Z_{ave} ($p = 0.51$, Table 5).

3.1.2.3. Effect of primary emulsion stabiliser. The addition of Tween® 80 to the preparation resulted in a decrease in Z_{ave} of 27.7 nm or 8% (Table 5). Surfactants such as Tween® 80 decrease interfacial tension, which results in a significant reduction in the energy required during emulsification and thus the formation of smaller emulsion droplets. Generally, it is assumed that one NP originates from one (or only a few) emulsion droplet after its shrinkage and that the O/W emulsion droplet size directly determines the NP's size (Desgouilles et al., 2003; Galindo-Rodriguez et al., 2004).

3.1.2.4. Effect of aescin concentration. Saponins also possess surface-active properties and consequently the addition of aescin to the precursor emulsion was expected to lead to a decrease in the NPs' Z_{ave} as observed for Tween® 80 (Section 3.1.2.3). In order to check this hypothesis, we first measured the surface tension of Tween® 80 and aescin aqueous solutions as a function of surfactant concentration. Aescin was able to lower the surface tension of water (70.5 ± 1.2 mN/m), but to a lesser extent than Tween® 80 (Fig. 2). A surface tension value of 52.2 ± 0.8 mN/m was obtained for 1.3×10^{-2} g/ml aescin and 44.9 ± 0.01 mN/m respectively for the Tween® 80 aqueous solution with the same concentration (Fig. 2).

Table 5
Effects of investigated preparation parameters on the responses particle size (Z_{ave}), zeta potential and EE% of aescin-loaded PLGA NPs.

Response	Mean value	Main effects					
		Surfactant (X_1)	Conc. aescin (X_2)	Viscolyser (X_3)	Conc. PLGA (X_4)	Evaporation rate (X_5)	Freeze-drying (X_6)
Particle size (Z_{ave}) (nm)	**347.0**	**−27.7**	−13.4	−7.3	**93.2**	−5.2	**33.0**
Zeta potential (mV)	**−23.6**	−0.3	0.1	1.0	−0.4	**2.7**	0.7
EE% (%)	**14.0**	**7.0**	**10.1**	−2.5	0.2	6.6	–

Statistically significant ($p < 0.05$) effects are in bold.

The reduction of surface tension upon addition of the saponin to the internal water phase explains why aescin-loaded PLGA NPs were significantly smaller ($p < 0.05$) than their blank counterparts (Fig. 3A). The effect of drug loading on the NPs' size was more pronounced in the case of NPs prepared with the viscosifying agent than for those prepared with Tween® 80 (Fig. 3A). Furthermore, the concentration of the aqueous drug solution, *i.e.* factor x_2, did not significantly affect particle size ($p = 0.23$). Indeed, there is no significant difference in surface tension between the two aescin solutions used for the preparation of the different PLGA NPs. The concentration of the saponin in the nano-emulsion is for both the +1 level (*i.e.* 0.27%, w/v) and −1 level (*i.e.* 0.14%) above the cmc (cmc of aescin in Milli-Q water, 0.008%, w/v) (Fig. 2). Likewise we determined the cmc of the saponin in D-PBS, for which a value of 0.003% (w/v) was obtained. The observed decrease in the cmc of the saponin micelles with increasing salt concentrations is consistent with the literature (Mitra and Dungan, 2000).

3.1.2.5. Effect of freeze-drying. The mean particle size of the preparations increased by 33.0 nm due to the lyophilisation process. This apparent increase in Z_{ave} could be attributed to the formation of small populations of aggregated particles (Holzer et al., 2009). Formulations III and VII contained very polydispersed particles after freeze-drying and reconstitution, as indicated by the high PI values (PI > 0.25) (Table 4). It is well established that during the freezing of a sample there is a phase separation into ice and cryo-concentrated phase. The latter contains the particulates at high concentration, which may induce aggregation and in some cases irreversible fusion of NPs (Abdelwahed et al., 2006). The NP formulation III and VII were both prepared using the 10% (w/v) PLGA solution as organic phase in the absence of Tween® 80 (Table 2). The combination of a high PLGA concentration and the absence of a surfactant mixture that could facilitate redispersion led to particles with a high degree of aggregation after lyophilisation and reconstitution.

3.1.2.6. Response surface plot. Three-dimensional response surface plots are valuable tools in illustrating the complete picture of the effect of two independent variables simultaneously on the third variable (dependent variable). Fig. 4A shows the response surface plot of mean particle size in relation to the concentration of the surfactant of the primary emulsion, *i.e.* 0% (no surfactant) or 8% Tween® 80, and the concentration of the PLGA. In the case of fractional designs the response surface is an inclined plane since the mathematical model (Eq. (2), see Section 2.11) contains only first order terms estimating the main effects of the investigated factors on the responses. Concerning the NPs' size, the response surface slopes downwards towards a less concentrated PLGA organic solution and, to a lesser extent, towards 8% Tween® 80 (Fig. 4A). Control and optimisation of particle size is a critical step in the development of any drug delivery system (DDS). Indeed, size is an important determinant for both the cellular uptake mechanism (Yue et al., 2010) and biodistribution of polymeric NPs. It is well known that NPs with a size >150 nm and hydrophobic surface are cleared from the bloodstream within minutes upon intravenous injection (Chellat et al., 2005; Sheng et al., 2009; Wong et al., 2008), suggesting that the prepared PLGA NPs are in the specific size range for passive targeting to the macrophage-rich tissues of the reticuloendothelial system (RES).

3.1.3. Statistical analysis of zeta potential

A high surface charge (positive or negative) is generally preferred since it not only assures the colloidal stability of the NPs upon storage, but also promotes the NPs' cellular uptake. Nano- and microspheres with a zeta potential close to zero were found to be less phagocytised *in vitro* in comparison with charged spheres (Chellat et al., 2005; Roser et al., 1998). All aescin-loaded PLGA

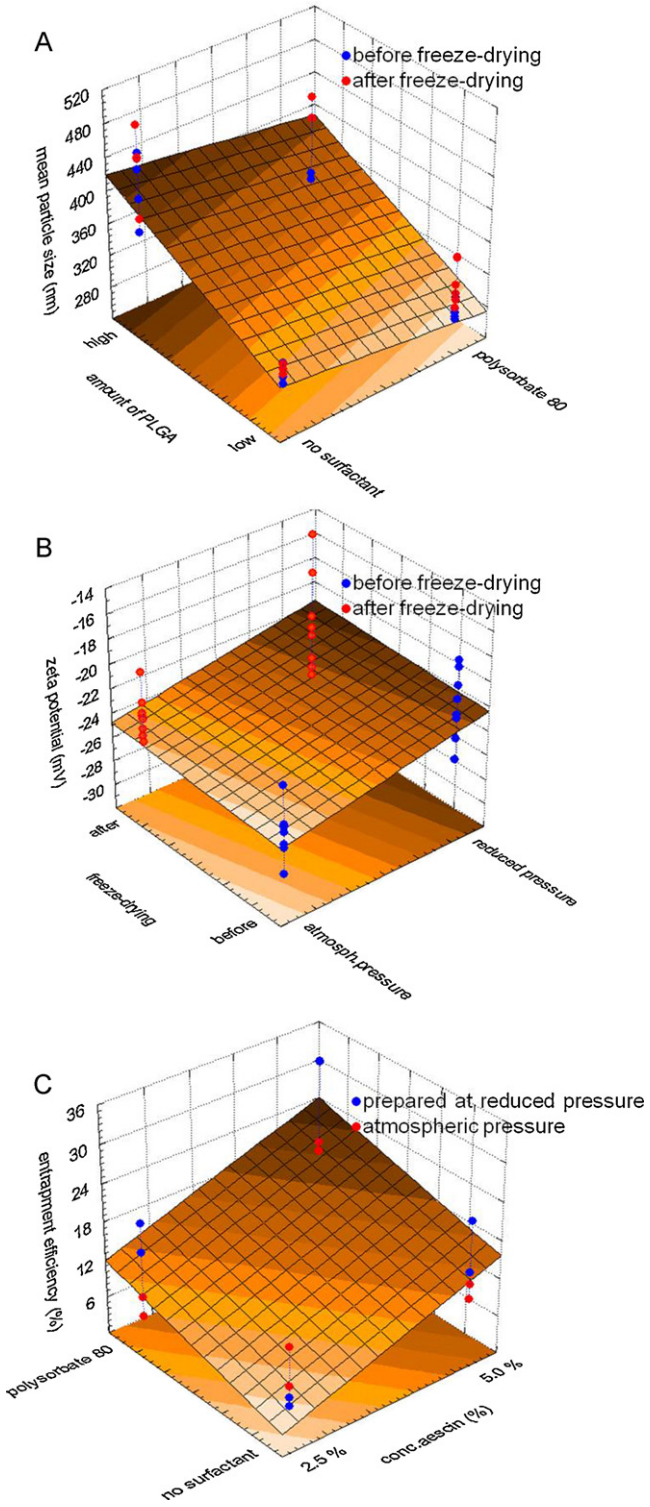

Fig. 4. Response surface plot of (A) mean particle size, (B) zeta potential and (C) EE% of aescin-loaded PLGA NPs.

NPs had negative surface potential values, ranging from −18.4 to −29.2 mV prior to lyophilisation (Table 4).

3.1.3.1. Effect of solvent evaporation rate. The zeta potential of NPs prepared at atmospheric pressure (AP) was significantly ($p < 0.05$) lower in comparison with NPs prepared at reduced pressure (RP), as shown in Fig. 4 B. The size of the effect of solvent evaporation rate amounted to 2.7 mV (Table 5). There are no studies, to the best

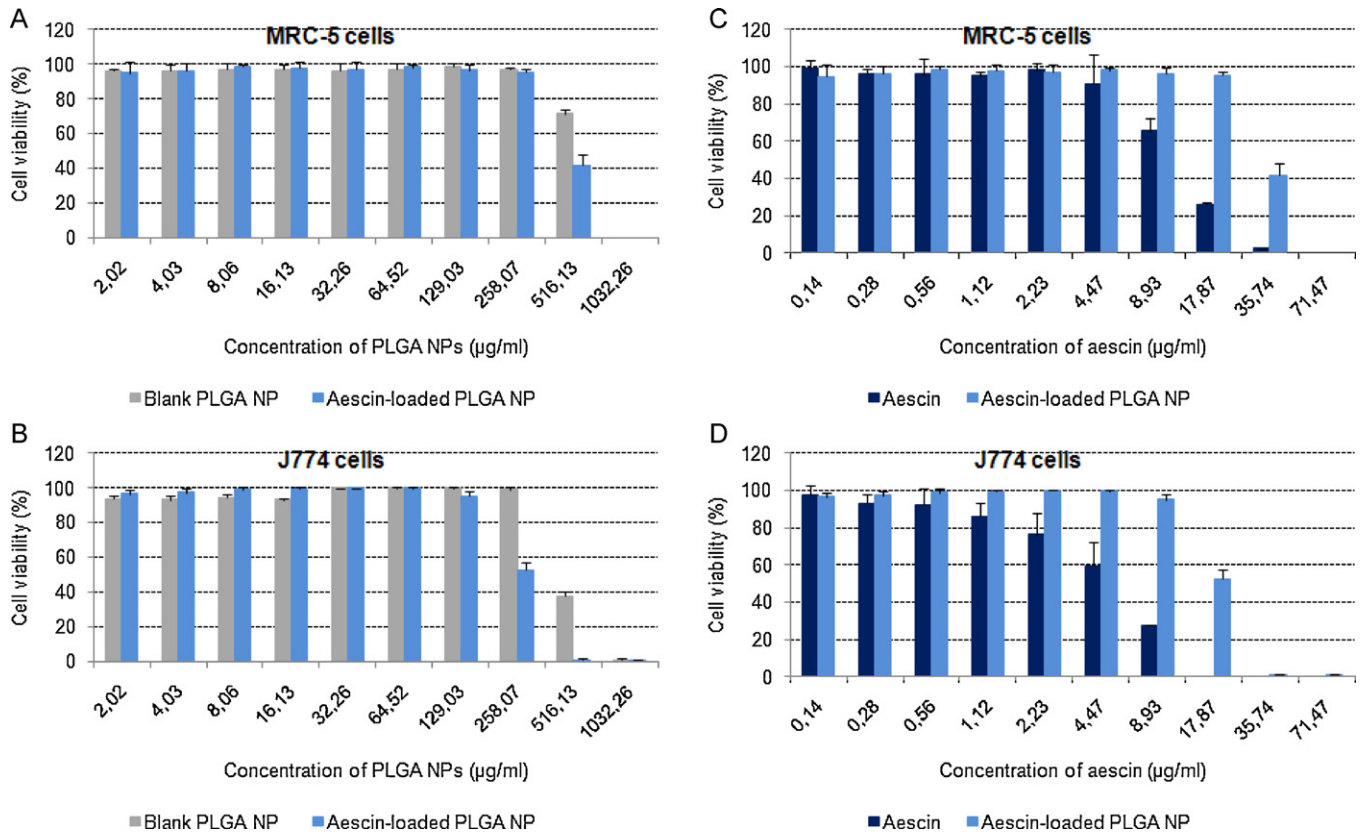

Fig. 5. Cytotoxicity of aescin, aescin-loaded and blank PLGA NPs on MRC-5 and J774 cells. Concentration-response curves of %cell viability of (A) MRC-5 cells as a function of PLGA concentration, (B) J774 cells, (C) MRC-5 cells as a function of aescin concentration and (D) J774 cells (data represented as mean ± S.D.; $n = 3$).

of our knowledge, describing a link between solvent removal rate and zeta potential of the obtained polymeric NPs. The review of Li et al. (2008) on microencapsulation reports that poly(L-lactide) microspheres (MPs) prepared using the AP method had a porous and rough surface with large surface area, whereas MPs prepared with the RP method exhibited smooth surfaces with smaller surface areas. The differences in surface morphology could be related to the crystalline nature of the MPs (Li et al., 2008). Moreover, Chung et al. (2001) made similar observations when comparing the surface morphology of MPs formulated with poly(L-lactide) or poly(D,L-lactide) respectively. If the solvent evaporation rate can influence the crystalline state of the polymer, then it is most likely that the interaction between the stabiliser PVA and the polymer is affected as well. PVA stabilises the emulsion droplet interface through penetration into the organic solvent and subsequent interaction with PLA/PLGA (Boury et al., 1995), this mechanism of interpenetration leads to an irreversible adsorption of PVA onto the NP surface (Galindo-Rodriguez et al., 2004; Shakesheff et al., 1997). A more rapid solvent removal, accelerating particle solidification, could lead to a higher degree of PVA (non-ionic) adsorption and thus NPs with less negative surface potentials.

3.1.3.2. Effect of aescin loading. The zeta potential of the PLGA NPs increased (*i.e.* became less negative) significantly ($p < 0.05$) upon loading with the drug (Fig. 3B), indicating that part of the saponin is present onto the surface.

3.1.3.3. Effect of freeze-drying. The NPs were less negatively charged after freeze-drying and reconstitution than before (Fig. 4B), however, the observed effect of 0.7 mV was not statistically significant ($p = 0.51$).

3.1.4. Statistical analysis of entrapment efficiency (EE%)

The EE% of aescin in the PLGA NPs varied between the extreme values of 2.8% and 31.9% (Table 4). The process variables surfactant primary emulsion, concentration aescin and solvent evaporation rate had a positive effect on EE% (*i.e.* EE% increased), whereas the presence of a viscosifying agent in the inner aqueous phase had a negative effect (*i.e.* EE% decreased). The size of the measured effects was in the ranking order *concentration aescin* > *surfactant primary emulsion* ≥ *evaporation rate* » *viscosifying agent* > *concentration PLGA* (Table 5).

3.1.4.1. Effect of aescin concentration. The average EE% of formulations prepared with a 5% (w/v) aqueous aescin solution (19.1%) was significantly ($p < 0.05$) higher than that of formulations prepared with a less concentrated solution (9.0%) (Fig. 4C). This observation is in line with the publication of Ubrich et al. (2004), who prepared PLGA NPs loaded with various amounts of propranolol hydrochloride. The authors assumed that the drug preferentially localised at the O/W interfaces, of both the primary W/O and external O/W emulsion, due to its surface-active properties. Saturation of the outer interface would take place with increasing propranolol hydrochloride concentration, favouring the entrapment of the drug in the inner water phase. Fractional factorial designs are especially useful when there are five or more variables, as the full factorial designs comprise large numbers of experiments with frequently many non-significant higher order interaction effects. The fractional design that we proposed was an important first step in the screening of as many process variables as possible and allowed us to identify the major factors influencing the drug entrapment in the PLGA NPs. One needs to keep in mind, however, that the estimated main effects are each confounded with first-order interactions. This may mean that certain estimates of the main effects

have to be interpreted with some caution. It is the defining relation of the design matrix (Eq. (3)) that determines which effects are aliased with one another (Lewis et al., 1999).

$$I \equiv 134 \equiv 245 \equiv 1235 \tag{3}$$

Each main effect is confounded with a number of interactions of different degrees (Eq. (4)), of which all second (*i.e.* three factor) and higher order interactions can be neglected.

$$\begin{aligned} E(b_0) &= \beta_0 + \beta_{134} + \beta_{245} + \beta_{1235} \\ E(b_1) &= \beta_1 + \beta_{34} + \beta_{1245} + \beta_{235} \\ E(b_2) &= \beta_2 + \beta_{1234} + \beta_{45} + \beta_{135} \\ E(b_3) &= \beta_3 + \beta_{14} + \beta_{2345} + \beta_{125} \\ E(b_4) &= \beta_4 + \beta_{13} + \beta_{25} + \beta_{12345} \\ E(b_5) &= \beta_5 + \beta_{1345} + \beta_{24} + \beta_{123} \end{aligned} \tag{4}$$

This implies that, for instance, the effect of the concentration of aescin (β_2) is aliased with the interaction between the factors concentration of PLGA and evaporation rate (β_{45}). Due to the fact that the effects of interactions are usually smaller than the effect of the factors themselves, the obtained estimate is to be attributed mainly to β_2. Nevertheless, it is reasonable to consider synergism between the quantity of polymer that needs to precipitate and the solvent removal rate, which is inversely proportional to the particle solidification time.

3.1.4.2. Effect of solvent evaporation rate. Solvent evaporation at reduced pressure induces rapid NP solidification, which is preferred if the drug easily partitions into the continuous phase. The effect of 6.6% was just not statistically significant ($p = 0.06$) (Table 5).

3.1.4.3. Effect of primary emulsion stabiliser. As expected, aescin EE% were significantly ($p < 0.05$) higher in the case of more stable primary emulsions (Fig. 4C). Addition of Tween® 80 to the water-in-DCM emulsion resulted in sufficient stabilisation, as demonstrated previously in Section 3.1.1, which in its turn led to better entrapment of the saponin. PLGA NPs prepared with the surfactant entrapped, on average, 17.5% aescin compared to 10.5% for formulations omitting the surfactant. The PLGA NP formulation VIII had the highest EE% of about 32% (Table 4) and was considered best candidate for further *in vitro* and *ex vivo* testing. The aescin drug loading of this preparation was determined by means of the DCM extraction method and amounted to 6.72 ± 0.24% (w/w) PLGA, corresponding with an EE% of 33.6 ± 1.2%. The latter agrees well with the result obtained previously using the indirect method.

3.2. In vitro cytotoxicity and efficacy evaluation

DDS allow for the selective delivery of antibiotics, antifungal and antiparasitic agents to phagocytic cells and, by doing so, they offer a strategy to improve antibiotic chemotherapy against intracellular infections. They must be able to increase the therapeutic index of the drug, *i.e.* by decreasing its toxicity and maintaining its therapeutic efficacy (Briones et al., 2008). In *in vitro* test settings this can be translated into the determination of a selectivity index (SI) towards the supporting host cell. Cytotoxicity on host cells is indeed an important criterion for assessing the selectivity of the observed pharmacological activities (Cos et al., 2006). In present study we used MRC-5 cells and macrophage-like J774 cells for that purpose. The former are commonly used in high-throughput cytotoxicity screening (Cos et al., 2006), whereas the latter are known to be a representative model of resident macrophages of the reticuloendothelial system (RES) (Jones and Grainger, 2009; Nakano et al., 2008).

3.2.1. Cytotoxicity of blank PLGA NPs

The blank PLGA NPs exhibited cytotoxic effects on both MRC-5 and J774 cells. Cell viability after 72 h of incubation with the NPs was comparable to the control (100%) in the concentration range 2–258 μg/ml, but decreased at a concentration of 516 μg/ml to ~70% in the case of MRC-5 cells and ~40% in the case of J774 cells respectively (Fig. 5Aand B). The difference between the cell types was statistically significant ($p < 0.05$). It is logic to assume that the phagocytic capacity of the J774 cells is involved in the cytotoxicity of the biodegradable polymeric NPs (Lemarchand et al., 2006; Müller et al., 1996). Our previous *in vitro* studies (Van de Ven et al., in press) showed that PLGA NPs in the size range 200–400 nm were efficiently taken up by J774 cells and trafficked towards the lysosomes within 2 h of incubation. Taking these findings into account, the internalisation and subsequent intracellular accumulation of polymer degradation products could be regarded as underlying mechanism of the observed cytotoxicity (Müller et al., 1996). Furthermore, Müller et al. (1996) reported on the significant correlation between the cytotoxicity of PLA-lw (low molecular weight), PLA-hw and PLGA NPs on human granulocytes and the degradation velocity of the respective polymer. Cell viability of the granulocytes after 2 h of incubation with the NPs was in the ranking order PLA-hw NPs > PLA-lw NPs > PLGA NPs, reaching ~0% for the latter at a concentration of 7 mg/ml. Thus, highest *in vitro* toxicity was observed for the faster degrading polymer (Müller et al., 1996). In comparison, we obtained remarkably low cell viabilities at concentrations as low as 516–1032 μg/ml PLGA NPs (Fig. 5B). This is probably due to the extended incubation period (72 h). Testing prolonged toxicity is as interesting as short-term toxicity, especially since PLGA NPs are known to be relatively slowly biodegraded. In addition, it is important that the incubation time covers the exponential growth phase of the cells in order to ascertain any toxicity due to inhibition of proliferation and/or cell death in the assay (Nafee et al., 2009). The cytotoxic effects of the blank PLGA NPs on the non-phagocytising MRC-5 cells (Fig. 5A) can be explained by the same mechanism of particle uptake. Virtually all nucleated eukaryotic cells are indeed able to carry out receptor-mediated endocytosis and/or fluid-phase endocytosis (*i.e.* nonspecific adsorptive endocytosis), whereas phagocytosis is restricted to specialised cells such as macrophages, neutrophils and dendritic cells for particles with size >0.5 μm (Bareford and Swaan, 2007; Faraji and Wipf, 2009; Xiang et al., 2006). Nafee et al. (2009) also observed a significant decline in survival rates of non-phagocytising (fibroblast-like) COS-1 cells upon incubation with 900 μg/ml PLGA NPs.

3.2.2. Cytotoxicity of β-aescin and aescin-loaded PLGA NPs

Saponins were shown to directly affect cell membrane integrity: the aglycone portion of the saponin molecule inserts into the phospholipid bilayer and then binds to membrane sterols, leading to the formation of transmembrane pores, loss of membrane integrity and cell necrosis (Armah et al., 1999; Hu et al., 2010). In our study the lytic effects of β-aescin on the investigated cells were partially blocked by encapsulating the saponin in PLGA NPs (Fig. 5C and D). Survival rates of both cell types were comparable to the control (100%) when incubated with the aescin-loaded PLGA NPs in the concentration range 0.14–8.93 μg/ml. The free saponin, on the contrary, exhibited cytotoxic effects in the same concentration range with cell viability decreased to ~65% and ~30% in the case of MRC-5 cells, J774 cells respectively at 8.93 μg/ml β-aescin (Fig. 5 Cand D). Recently Hu et al. (2010) also reported that cell membrane damage and necrotic effects caused by saponin fractions of *Quillaja saponaria Molina* (QS) on both normal and cancer cells were abolished by converting QS fractions into stable NPs.

Table 6
In vitro cytotoxic and antileishmanial activity, selectivity index (SI) of β-aescin and β-aescin-loaded PLGA NPs.

PLGA NP formulation or reference saponin	*In vitro* activity, $IC_{50} \pm$ S.D. (ug/ml)[a] on				SI CC_{50} (J774)/ IC_{50} (*L. infantum*)
	MRC-5_{SV2} cells (CC_{50})	J774A.1 cells (CC_{50})	*L. infantum* intracellular amastigotes	*L. infantum* extracellular promastigotes	
β-Aescin	10.95 ± 0.82	6.48 ± 2.81	1.55 ± 0.32	>16	4
β-Aescin-loaded PLGA NP VIII	25.22 ± 7.23[b,†]	18.73 ± 1.33[b,†]	1.04 ± 0.23[b,†]	>16[b]	18

[a] $n = 3$ (minimum).
[b] CC_{50} and IC_{50} expressed as equivalent concentration of aescin entrapped in PLGA NP.
[†] Significant difference with free drug aescin, $p < 0.05$.

3.2.3. Determination of the selectivity index (SI)

The CC_{50} of aescin-loaded NPs on both MRC-5 and J774 cells was significantly ($p < 0.05$) increased in comparison with the CC_{50} of the free drug (Table 6). In addition, the efficient macrophage uptake of the particulate form of β-aescin results in significantly ($p < 0.05$) improved antileishmanial activity in the *ex vivo* macrophage model (IC_{50}, 1.04 ± 0.23 μg/ml *vs.* 1.55 ± 0.32 μg/ml for the free saponin). Taken together, the SI towards the supporting host cell (*i.e.* J774 macrophages) increased from 4 to 18 by treating the cells with aescin-loaded NPs instead of free β-aescin (Table 6). The SI of the saponin is not high enough for the compound itself to be of any clinical value, however, a SI > 10 for the aescin-loaded PLGA NPs suggests that the latter can be considered a promising antileishmanial formulation (Delmas et al., 2000).

4. Conclusion

In conclusion, β-aescin formulated into NPs represents a promising antileishmanial formulation with respect to its physicochemical characteristics and *in vitro* activity profile. The selected aescin-loaded PLGA NPs (formulation VIII) exhibited a SI of 18 towards the supporting host macrophage, indicating that the drug carrier was able to reduce the cytotoxicity of β-aescin whilst maintaining its antileishmanial efficacy. Further optimisation of the NPs could be done in the future in order to obtain EE% > 32% for the saponin. Also surface modifications of the carriers by the binding of ligands with affinity for macrophage receptors, *e.g.* scavenger receptors, are possible future research strategies. Nevertheless, the more logic following step in the development of a carrier to treat VL involves *in vivo* assays to provide us with the *in vivo* proof-of-concept. *In vivo* efficacy testing of the aescin-loaded PLGA NPs in the hamster model of chronic leishmaniasis with *L. infantum* is planned in the laboratory in the near future.

Declaration of interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.08.016.

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