Journal of Microbiological Methods 162 (2019) 1–7

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

# Encapsulation of proteins from *Leishmania panamensis* into PLGA particles by a single emulsion-solvent evaporation method

Juan David Ospina-Villa[a], Catalina Gómez-Hoyos[b], Robin Zuluaga-Gallego[c], Omar Triana-Chávez[a,*]

[a] Grupo Biología y Control de Enfermedades Infecciosas (BCEI), Sede de Investigación Universitaria, Universidad de Antioquia, Medellín, Colombia
[b] Programa de Ingeniería en Nanotecnología, Universidad Pontificia Bolivariana, Circular 1° N° 70-01, Medellín 050031, Colombia
[c] Facultad de Ingeniería Agroindustrial, Universidad Pontificia Bolivariana, Circular 1° N° 70-01, Medellín 050031, Colombia

ARTICLE INFO

*Keywords:*
Leishmania
Hypothetical proteins
Biodegradable polymers
Encapsulated microparticles
Vaccine

ABSTRACT

The current therapy for the treatment of leishmaniasis is unsatisfactory because it has multiple side effects, and resistance has been reported among the parasites that cause these diseases. Numerous efforts have been made to develop new candidates for vaccines. In recent years, particles of biodegradable polymers have been proposed as vehicles to transport and protect antigens, proteins, drugs and vaccines. In this work, the oil/water (o/w) single emulsion-solvent evaporation technique was used to prepare PLGA biodegradable particles. The encapsulation of two hypothetical proteins from *Leishmania panamensis* was performed to validate the proposed method. For this validation, different concentrations (50, 100, 150, 200, 250, 500, and 750 μg/ml) of both proteins were encapsulated into PLGA particles, and the particle sizes and shapes were evaluated by optical microscopy and scanning electron microscopy (SEM), respectively. The release of proteins was confirmed by SDS-PAGE and Western blot analyses. The integrity of both proteins was conserved, and they were released from day one until day 15, with a maximum amount of 46 ± 4.25% for the LpanUA.27.1260 protein and 26.19 ± 3.41% for LpanUA.22.1860. Additionally, the protective efficacy of one of these encapsulated proteins was evaluated *in vivo* using BALB/c mice infected with *L. panamensis*. Therefore, the encapsulation of proteins is presented here as an excellent alternative to evaluate the antigenicity of proteins from parasites of medical importance such as *L. panamensis*.

## 1. Introduction

Leishmaniasis is a group of vector-borne diseases caused by protozoan parasites of the genus Leishmania (Srivastava et al., 2016), which is widely distributed throughout five of the continents (Alvar et al., 2012). The most common treatments of choice for Leishmaniasis are based on the use of pentavalent antimonials, oral miltefosine, amphotericin B, liposomal amphotericin B, and paromomycin (Aronson et al., 2017). However, the high cost, duration of treatment, route of administration, side effects, and presence of resistant parasites are a persistent problem (Chakravarty and Sundar, 2010). The development of an effective vaccine for Leishmaniasis has been a constant challenge (Müller et al., 2017; Osman et al., 2017; Ghorbani and Farhoudi, 2018; CDC, 2018; https://www.cdc.gov/parasites/leishmaniasis/prevent.html). In recent years, efforts have focused on searching for potential antigen candidates for vaccines using next-generation sequencing techniques (Llanos-Cuentas et al., 2010; Chakravarty et al., 2011; Martin et al., 2014; Brito et al., 2018). The recombinant proteins identified show great potential but present some limitations and disadvantages, such as short half-life, degradation by proteases, immunological reactions, dissemination, and tissue accumulation.

To reduce those drawbacks, one effective approach is the use of microparticles as vehicles to transport, protect and control the release of proteins. Microparticles offer greater effectiveness, lower toxicity, and better stability than conventional dosage forms (Giri et al., 2013). Moreover, they provide many advantages, such as enhancing antigenic presentation, creating a *"vaccine deposit"* at the site of the injection without dissemination, protecting proteins from degradation, decreasing dose and toxicity and extending storage time (Specht et al., 2013; Gupta, 2016; De Jong and Borm, 2008; Mudshinge et al., 2011). A wide variety of synthetic and natural biodegradable polymers have increased in pharmaceutical and biomedical fields, and such polymers

*Abbreviations:* PLGA, poly(lactic-*co*-glycolic acid); SEM, scanning electron microscope

* Corresponding author at: Calle 70 52-21, Universidad de Antioquia, Medellin, Colombia.
*E-mail address:* omar.triana@udea.edu.co (O. Triana-Chávez).

https://doi.org/10.1016/j.mimet.2019.05.004
Received 20 February 2019; Received in revised form 24 April 2019; Accepted 8 May 2019
Available online 10 May 2019
0167-7012/ © 2019 Elsevier B.V. All rights reserved.

have been used to improve active compound efficacy and bioavailability (Marin et al., 2013). Poly(lactic-*co*-glycolic acid) (PLGA) is a biodegradable polymer approved by The United States Food and Drug Administration (FDA) for use in drug and protein delivery systems, diagnostics, and other applications of clinical and basic science research (Lü et al., 2009). Single emulsion solvent evaporation is one method by which PLGA can be used to encapsulate hydrophobic and hydrophilic drugs on the micro- or nanoscale (Derman, 2015; Song et al., 2008; Allahyari and Mohit, 2016).

In this work, single emulsion solvent evaporation was used as a method for encapsulating proteins from *L. panamensis* into PLGA particles. For the encapsulated proteins, one insoluble protein highly overexpressed in Leishmania promastigotes, LpanUA.22.1260, and one soluble protein, LpanUA.27.1860, which presented similar expression in the amastigote and promastigote stages, was used. We evaluated the size distribution, morphology, encapsulation efficiency and release capacity of these microparticles and their relevance as a vehicle to transport, protect and control the release of Leishmania recombinant proteins. Moreover, the efficacy of one of these encapsulated proteins was evaluated *in vivo* using BALB/c mice infected with *L. panamensis*.

## 2. Materials and methods

### 2.1. Ethics statement

Mice were maintained in a SPF animal facility at the *Sede de Investigación Universitaria (SIU), Universidad de Antioquia*. Ethical approval for all *in vivo* procedures was obtained by the Animal Ethics Committee of the Universidad de Antioquia, Colombia. Animals were treated in strict accordance with the good animal practice procedures declared by the Colombian National Statute for the Protection of Animals (Bill n°84 of 1989).

### 2.2. Parasites

*Leishmania panamensis* strain UA-946, kindly provided by the *Programa de estudio y control de enfermedades tropicales, PECET (Universidad de Antioquia)*, was used in all experiments. The promastigotes were cultivated in liquid Schneider's (Caisson) medium supplemented with 10% fetal bovine serum (FBS) inactivated by heat, penicillin 10,000 U/ml and streptomycin 10,000 µg/ml (P/S).

### 2.3. Reagents

The reagents used in this study were as follows: Poly(D,L-lactide-*co*-glycolide) (PLGA) lactide:glycolide 50:50 with an inherent viscosity of 0.45–0.60 dl/g and Mw ~ 38–54 kDa (Sigma Aldrich, Seelze, Germany); dichloromethane ACS reagent (DCM) (HONEYWELL) (PanReacAppliChem ITW Reagents); poly(vinyl alcohol) PVA (Merck, Darmstadt, Germany); acetone (Merck); acetonitrile (Merck); ultrapure water, which was obtained from a Millipore MilliQ Gradient system; and phosphate-buffered saline (1 × PBS) at pH 7.4, which was prepared in the laboratory.

### 2.4. Cloning of hypothetical proteins

The full-length LpanUA.22.1260 (804 bp) and LpanUA.27.1860 (1017 bp) genes were amplified by PCR from genomic DNA of *L. panamensis* promastigotes and ligated into the *Hin*dIII/*Eco*RI sites of the pET-28a(+) plasmid (Novagen, Madison, WI). Specific oligonucleotides (F-5'-CCATTGAATTCATGAACCACGATGATGCCAT-3′ and R-5′-ACGTTAAGCTTCTACAGGTCTTCTTCAGAGATCAGTTTCTGTTCCCACT CGAGGTCCTCCAG −3′) and (F-5'-CCATTGAATTCATGCCACTCTGCG CCAGCGT-3′ and R- 5'-ACGTTAAGCTTCTACAGGTCTTCTTCAGAGAT CAGTTTCTGTTCCTCGTGGTCGTTCACCTT-3′) were used for the PCR amplification. PCR amplification was performed with an initial denaturation at 98 °C for 30″, followed by 30 cycles of 98 °C for 10″, 60 °C for 30″, and 72 °C for 50″, and a final cycle at 72 °C for 10′. The cloned fragments were confirmed by sequencing (Macrogen Inc., Seoul, Republic of Korea; www.dna.macrogen.com).

### 2.5. Expression and purification of recombinant proteins (rLpanUA.22.1260 and rLpanUA.27.1860)

The pET28-LpanUA.22.1260 and pET28-LpanUA.22.1860 plasmids were transformed into competent *E. coli* BL21 (DE3) pLysS bacteria and grown in LB medium at 37 °C containing kanamycin 100 mg/ml and chloramphenicol 34 mg/ml. Recombinant proteins were induced by adding 1 mM IPTG (isopropyl beta-D-thiogalactopyranoside) and incubating the cultures at 37 °C for four h. Bacteria were collected by centrifugation at 5000*g* for 15 min, suspended in lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, and 20 mM imidazole, pH 8.0), and sonicated with 30 pulses at a duration of 60 s (10 s interval among pulses). Recombinant proteins were isolated from total protein extracts by affinity chromatography Ni2 + NTA under nondenaturing conditions (Qiagen). The identity and integrity of the recombinant proteins were confirmed by 10% SDS-PAGE and Western blot using 1:1000 anti-6 × -His primary antibody and 1:5000 IRDye® 800CW goat anti-mouse secondary antibody and visualized with an Odyssey Imaging System (LI-COR Biosciences).

### 2.6. Western blot assays

The recombinant proteins rLpanUA.22.1260 and rLpanUA.27.1860 were subjected to 10% SDS-PAGE and electrotransferred to a nitrocellulose membrane. Subsequently, membranes were incubated with anti-6xHis tag monoclonal antibodies (1:1000 dilution) and then with an IRDye-800-linked anti-mouse secondary antibody (diluted 1:50000; Abcam, Cambridge, United Kingdom). Fluorescent band detection was achieved *via* the Odyssey system (LI-COR Biosciences, Lincoln, NE). The recombinant nitroreductase (rNTR) protein, which was previously purified in the laboratory, was used as a positive control protein with a 6xHis-tag.

### 2.7. Protein quantification and dialysis

Protein quantification was carried out using a commercial Pierce BCA Protein Assay kit (Thermo Scientific), measuring absorbance at 562 nm, and using BSA protein as a protein standard. To remove excess imidazole, PD-10 Desalting Columns (GE Healthcare) were used. The proteins were recovered in 1 × PBS pH 7.4, and the concentration was determined using a BCA Protein Assay Kit (ThermoScientific), as described above.

### 2.8. Microparticle synthesis and protein encapsulation

The proteins LpanUA.27.1860 and LpanUA.22.1260 were encapsulated into PLGA polymer using a single emulsion-solvent evaporation method. Briefly, 100 mg of PLGA was dissolved in two ml of dichloromethane. Then, the PLGA solution was mixed with a known amount of protein (50, 100, 150, 200, 250, 500, or 750 µg/ml) in 1 × PBS and stirred to ensure that all materials were dissolved. Four milliliters of PVA (3%) was added dropwise under constant agitation, and the solution obtained was emulsified by sonication (Fq 37 Hz, Power 30%, 8 min). Immediately, the solvent was evaporated by rotoevaporation (Heidolph Rotary Evaporator, Laborota 4010) at 60 rpm and 40 °C with the following profile: 820 mbar for 10 min, 700 mbar for 5 min, 650 mbar for 5 min, 550 mbar for 5 min, 350 mbar for 5 min, and 30 mbar for 10 min. Microparticles were suspended with 15 ml of ultrapure water and centrifuged at 4 °C and 9000 rpm for 5 min several times. The samples were freeze-dried in a Labconco for 48 h at −80 °C and 0.0014 mbar for further analyses (Fig. 1).

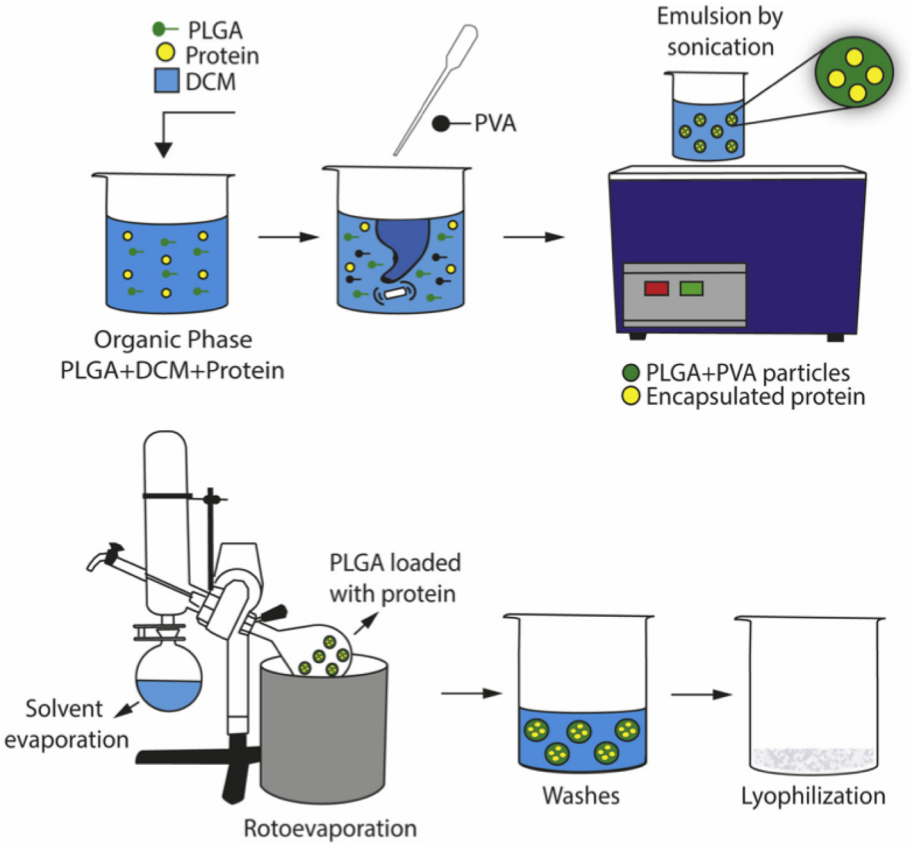


**Fig. 1.** Graphic representation of the single emulsion-solvent evaporation method used to obtain empty and L. *panamensis* protein-loaded PLGA microparticles. The organic phase consisted of PLGA + protein + DCM, which was mixed, and then PVA was added dropwise. Next, an emulsion was obtained by sonication. DCM was evaporated by rotoevaporation, and after several washes with ultrapure water, the microparticles were lyophilized.

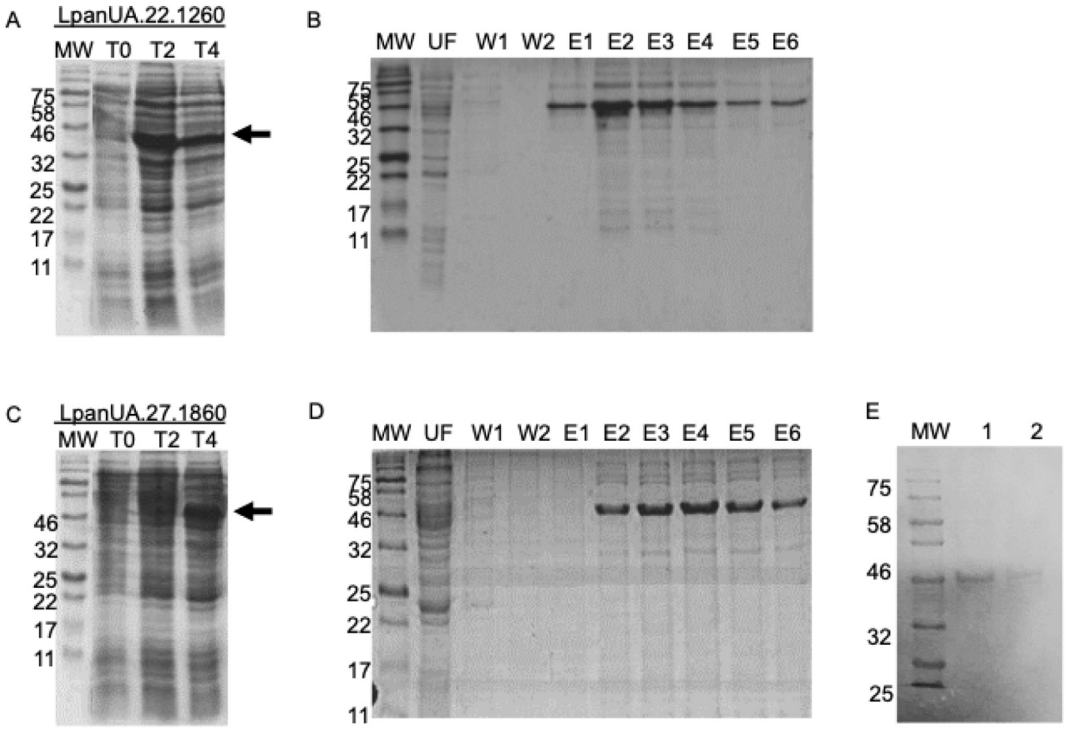


**Fig. 2.** Expression and purification of recombinant proteins. Expression and purification of the LpanUA.22.1260 (A and B) and LpanUA.27.1860 proteins (C and D). (E) Western blot assay using anti-6×-His tag primary antibody against the LpanUA.22.1260 (1) and LpanUA.27.1860 (2) proteins. MW: Molecular Weight, T0: time 0, T2: time 2 (2 h), T4: time 4 (4 h), UF: Unbound Fraction, W1, W2: Washes, and E1 to E6: Elution.

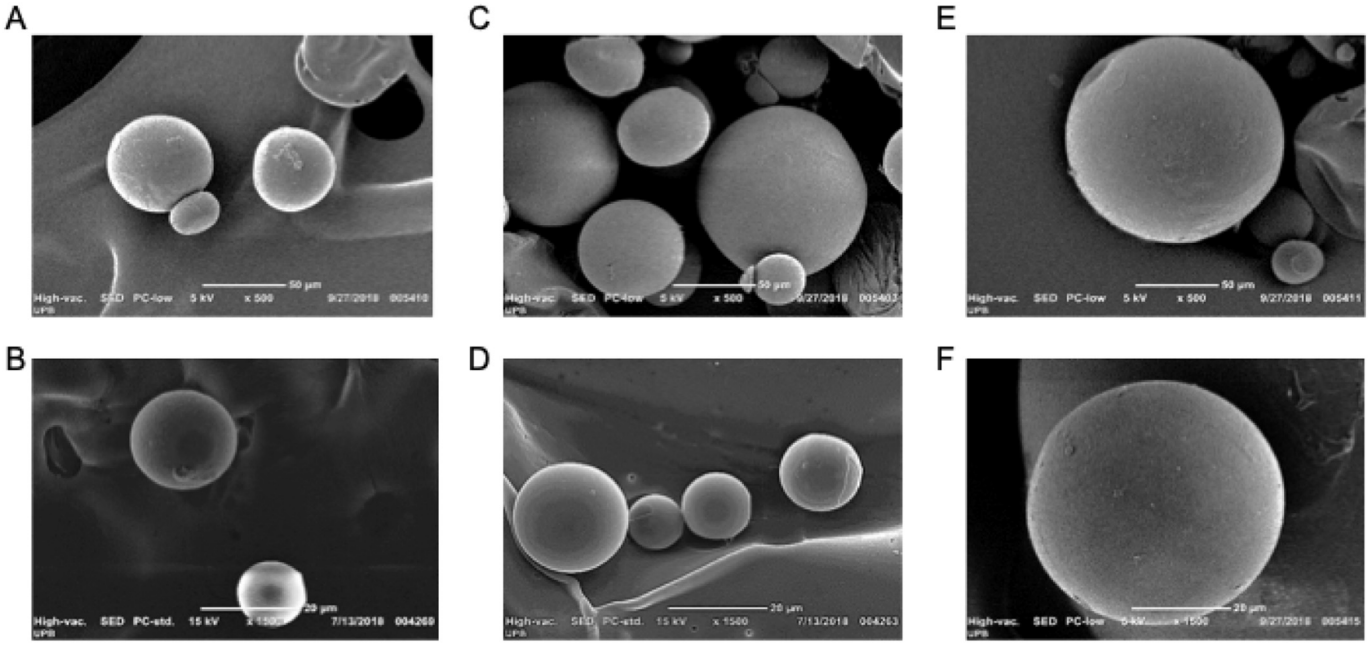


**Fig. 3.** PLGA microparticle scanning electron microscopy analysis. Empty microparticles (A, B), microparticles loaded with the LpanUA.22.1260 protein (C, D), and microparticles loaded with the LpanUA.27.1860 protein (E, F).

**Table 1**
Size distribution by optical microscopy of empty particles and particles loaded with the proteins LpanUA.22.1260 and LpanUA.27.1860.

| Particles | Protein concentration (μg/ml) | Min. value (μm) | Max. value (μm) | Mean size (μm) |
|---|---|---|---|---|
| Empty | – | 1.20 | 25.70 | 9.03 |
| LpanUA.22.1260 | 50 | 0.43 | 84.71 | 22.58 |
| | 100 | 0.40 | 102.61 | 18.64 |
| | 150 | 0.84 | 143.22 | 27.75 |
| | 200 | 0.42 | 129.36 | 26.99 |
| | 250 | 0.42 | 48.88 | 15.35 |
| | 500 | 6.61 | 112.245 | 39.95 |
| | 750 | 2.07 | 133.41 | 44.97 |
| LpanUA.27.1860 | 50 | 2.04 | 96.48 | 21.17 |
| | 100 | 0.67 | 90.93 | 21.80 |
| | 150 | 1.43 | 176.12 | 20.31 |
| | 200 | 2.05 | 95.76 | 20.66 |
| | 250 | 2.05 | 87.53 | 26.73 |
| | 500 | 2.06 | 140.47 | 43.29 |
| | 750 | 2.05 | 126.39 | 37.56 |

**Table 2**
Encapsulation efficiency (EE %) of protein in PLGA particles.

| Protein | Concentration (μg/ml) | EE% (mean/SD) |
|---|---|---|
| LpanUA.22.1260 | 50 | 32.67 ± 4.15 |
| | 100 | 33.62 ± 4.22 |
| | 150 | 47.40 ± 2.06 |
| | 200 | 57.82 ± 4.79 |
| | 250 | 58.267 ± 7.71 |
| | 500 | 62.54 ± 1.99 |
| | 750 | 94.66 ± 4.86 |
| LpanUA.27.1860 | 50 | 26.24 ± 4.12 |
| | 100 | 25.53 ± 0.86 |
| | 150 | 26.44 ± 0.14 |
| | 200 | 28.86 ± 2.12 |
| | 250 | 21.58 ± 6.62 |
| | 500 | 64.85 ± 9.84 |
| | 750 | 89.03 ± 4.91 |

### 2.9. Encapsulation efficiency

The encapsulation efficiency was calculated using the following equation:

EE (%) = ($W_f$/$W_i$) x 100 (%).

where $W_f$ is the amount of protein not encapsulated and $W_i$ is the amount of protein added initially during the preparation. $W_f$ and $W_i$ were determined using the Micro BCA Protein Assay Kit.

### 2.10. Scanning electron microscopy (SEM)

The surface morphology of microparticles was analyzed using SEM. Briefly, freeze-dried microparticles were placed on double-sided black carbon tape. Samples were coated with a gold layer under vacuum and examined with SEM (JEOL -JCM-6000 Plus) at 5 or 15 kV.

### 2.11. Optical microscopy

Five hundred naked and protein-loaded microparticles were analyzed by optical microscopy of a clear field. The images were analyzed using ImageJ software (Rueden et al., 2017). All experiments were repeated at least three times. GraphPad Prism 7 software was used to obtain the minimum, maximum and mean ± standard deviation values and to plot the cumulative percentage of release.

### 2.12. In vitro protein release

Fifty milligrams of protein-loaded PLGA microparticles was suspended in 1 ml of PBS at pH 7.4, and the suspension was incubated at 37 °C in an incubator (Innova40) shaking at 60 rpm. At selected time intervals (every 24 h for 15 days), the released medium was centrifuged at 12000 rpm for 10 min. The supernatant was collected, and the pellet was suspended with fresh PBS pH 7.4 buffer. The protein concentration in the supernatant was determined with the BCA method (Micro BCA Protein Assay Kit -Thermo Fisher Scientific). The results are presented as the cumulative percentage of release.

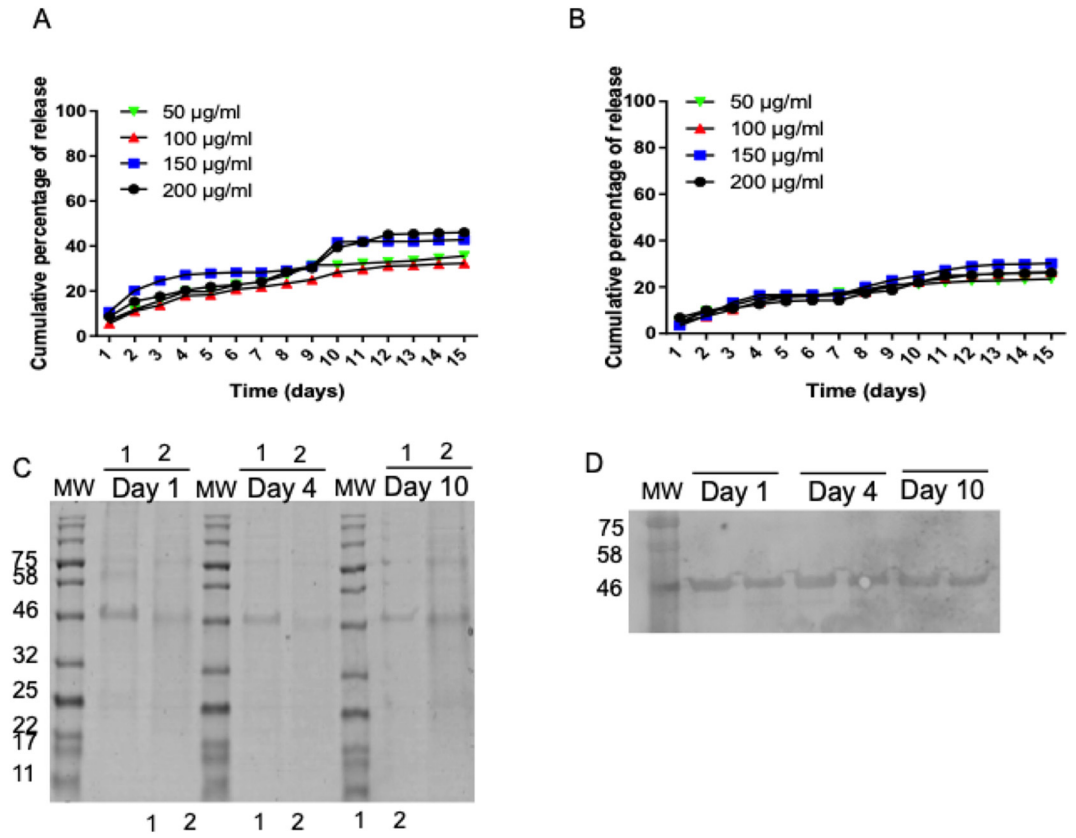


**Fig. 4.** Protein release from PLGA microparticles. Cumulative percentage released of the (A) LpanUA.22.1260 and (B) LpanUA.27.1860 proteins; (C) SDS-PAGE analysis of protein release from PLGA microparticles; 1-LpanUA.22.1260 and 2- LpanUA.27.1860. (D) Western blot assay using anti-6×-His tag primary antibody against the LpanUA.22.1260 (1) and LpanUA.27.1860 (2) proteins at 1, 4 and ten days postrelease.

### *2.13. In vivo assays*

Female BALB/c mice 8–12-weeks of age were injected in the caudal vein every two weeks with three 100-µl doses of the PLGp-rLpanUA.27.1860 preparation. Nine weeks after the first injection, mice were infected with $1 \times 10^5$ promastigotes in the ear dermis. After that, cutaneous lesion size and severity were assessed over eight weeks (Urrea et al., 2018). Mice inoculated with PBS and empty microparticles were used as a control.

## 3. Results

### *3.1. Protein purification and protein solubility in organic solvents*

Induction of the LpanUA.22.1260 protein was evident after 2 h of incubation (Fig. 2A), while the LpanUA.27.1860 protein was observed only after 4 h (Fig. 2C). Both proteins showed a clear band after being eluted six times in one ml of elution buffer (Fig. 2B, D). Western blotting with an anti-6×-His tag as the primary antibody showed a clear band corresponding to each protein (Fig. 2E).

### *3.2. Microparticle morphology and size distribution*

The SEM images showed well-defined and individual nonfused microparticles of PLGA, with a smooth surface morphology and a broad range of sizes distributed throughout the sample (Fig. 3).

To determine the microparticle size, measurements were taken directly from the images obtained with optical microscopy using the "Analyze particles" function in ImageJ (Table 1). Five hundred microparticles were analyzed for each group (empty and loaded with the proteins LpanUA.22.1260 and LpanUA.27.1860). The empty microparticles had a mean size of 9.03 µm. In contrast, microparticles loaded with the LpanUA.22.1260 and LpanUA.27.1860 proteins had a mean size of 15.35 ± 44.97 and 20.31 ± 43.29 µm, respectively. There was no statistically significant difference between size distribution and concentration of both encapsulated proteins.

### *3.3. Encapsulation efficiency and protein release*

The results obtained showed that the concentration of 750 µg/ml presented the best encapsulation efficiency in both the LpanUA.22.1260 (94.66% ± 4.86) and LpanUA.27.1860 (89.03% ± 4.91) proteins, respectively (Table 2). Encapsulation efficiency showed a correlation with protein concentration, in which encapsulation efficiency increased as the protein concentration increased. This result is in accordance with previously observed results in other studies (Derman, 2015). These results together show that between 100 and 250 µg/ml, an excellent microparticle size and good encapsulation efficiency were obtained.

The amount of protein obtained every 24 h was added to obtain a cumulative release, which was plotted as the cumulative percentage of release (Fig. 4A, B). The LpanUA.27.1860 protein presented a cumulative release between 35.50 and 46.00%, while the LpanUA.22.1260 protein was between 23.49 and 30.22%.

The molecular weight of the release proteins was verified by SDS-PAGE gels at approximately 46 kDa, and the identity was confirmed by Western blot using an anti-6×-His tag. SDS-PAGE showed that the LpanUA.27.1860 and LpanUA.22.1260 proteins continued to be released at days 1, 4 and 10, and they were not degraded (Fig. 4C, D).

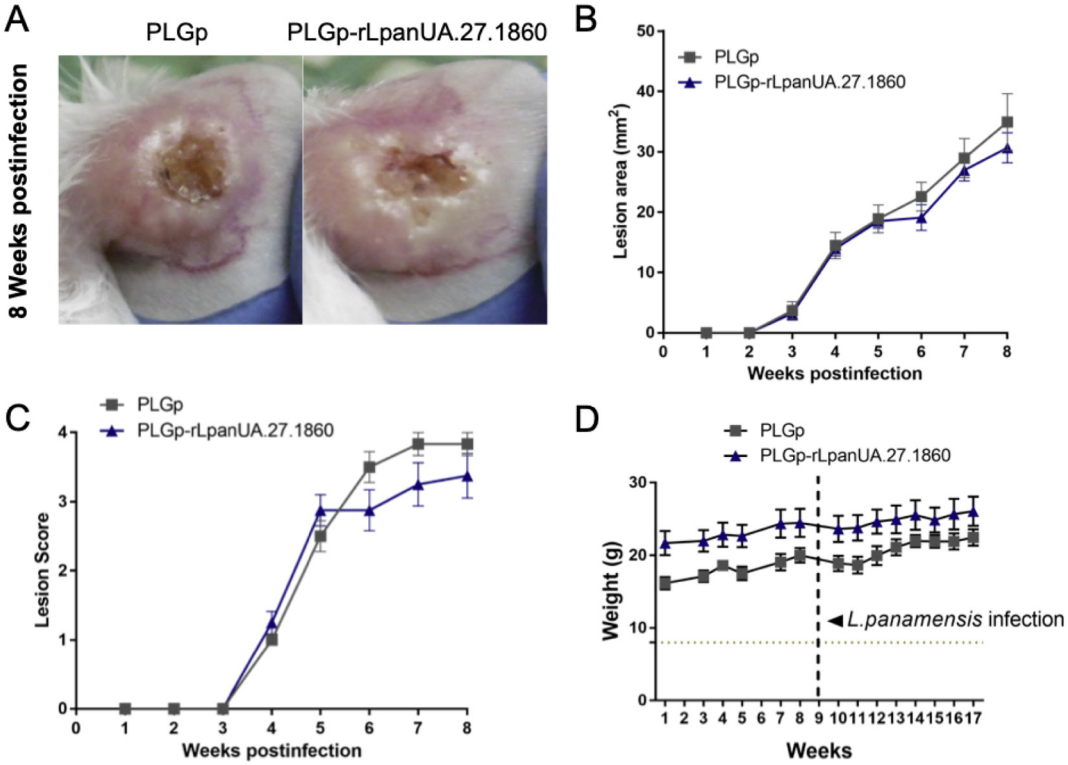


**Fig. 5. Validation of** PLGp-rLpanUA.27.1860 microparticles **in mice infected with** *L. **panamensis***. BALB/c mice were inoculated with empty PLGp ($n = 6$) or PLGp-rLpanUA.27.1860 ($n = 8$) prior to intradermal challenge. **(A)** Representative images of mouse ears eight weeks postinfection. Average lesion size **(B)** and severity **(C)** were assessed over an eight-week period postinfection. **(D)** Measurement of body mass during the course of the experiment. Error bars depict the SEM; *, $p < .05$; **, $p < .01$; and ***, $p < .001$.

### *3.4. Immunization with microparticles did not induce damage in mice infected with L. panamensis*

To evaluate the protective efficacy of microparticles in mice, the protein expressed similarly in both parasite stages was selected for *in vivo* studies. For these studies, mice were inoculated with three doses of the PLGp-rLpanUA.27.1860 preparation and subsequently infected with *L. panamensis* promastigotes. Eight weeks postinfection (Fig. 5A), we found that PLGp-rLpanUA.27.1860-treated mice did not have lesions (Fig. 5A-C). In addition, treatments did not cause adverse effects on body mass or overall health (Fig. 5D). These findings highlight the potential use of PLGA microparticles in vaccine development.

## 4. Discussion

In this work, the preparation of biodegradable particles and the encapsulation of two hypothetical proteins from *L. panamensis* into PLGA microparticles were standardized. The proposed method showed that the morphology and size of these microparticles were the most adequate when a protein concentration between 100 and 150 μg/ml was used. Although there were no significant differences in the microparticle size when a higher concentration of protein was used, the recommendation is to use between 100 and 150 μg/ml because of the lower cost and adequate release percentage for *in vivo* experiments. Similar to this result, other studies have reported that increasing the protein concentration did not change the size of the particles significantly (Taghipour et al., 2014).

In contrast, the encapsulation efficiency achieved here is similar to that obtained in different studies that used the same method of encapsulation and the same polymer (PLGA) (Derman, 2015; Morales-Cruz et al., 2012). It has been reported that the encapsulation efficiency can be influenced by (i) the partition coefficient of the target molecule in the solvents used in the preparation of the formulation, (ii) the method used for the encapsulation process (*e.g.*, temperature, pH, and mechanical stress), and (iii) the size distribution of the capsules (Jyothi et al., 2010). Likewise, it has been proposed that the concentration of protein to be released is another factor to take into account. In this sense, it is well known that the amount of protein that must be released in the murine model to evaluate the protective effect of a candidate vaccine protein should not exceed 100 μg because an increased amount causes a toxic effect in mice (Brito et al., 2018). Thus, the tested concentrations of 100 μg/ml and 150 μg/ml, which have an encapsulation efficiency of 33.62 ± 4.22 and 47.40 ± 2.06, respectively, for the microparticles loaded with LpanUA.22.1260 and 25.53 ± 0.86 and 26,44 ± 0.14, respectively, for the microparticles loaded with LpanUA.27.1860, would be encapsulating between 30 and 50 μg/ml of protein, which is a safe quantity for injection in the murine model.

Once the encapsulated proteins come into contact with an aqueous medium, proteins are released from PLGA microparticles by two principal mechanisms: protein is carried out by diffusion from the polymer during the initial release phase, and during the later stage, the delivery of the protein occurs by erosion of the polymeric matrix. Initial protein release is related to protein type, protein concentration and polymer hydrophobicity (Makadia and Siegel, 2011). The water inside the matrix hydrolyzes the polymer into soluble oligomeric and monomeric products, which creates a way for proteins to be released by diffusion and erosion until complete polymer solubilization (Gentile et al., 2014). In our results, we observed two phases of protein release: the first four days, where a large part of the protein is released (30% for the LpanUA.27.1860 protein and 18% for the LpanUA.22.1260 protein) and approximately day 8 or 9, where 10%–15% additional protein was released (Fig. 4). The concentrations of 100 and 150 μg/ml of both microparticles could be used to perform tests in the murine model. First, at lower protein concentrations, lower side effects would be expected, and second, the smaller size of the microparticles would allow them to be phagocytosed more efficiently by immunological cells. In

fact, the results show that the concentrations used are not toxic to the mice.

Regarding PLGA microparticles and their possible application as a vehicle for a Leishmania vaccine, it has been reported that J774 macrophages cannot phagocytose PLGA microparticles with the same avidity demonstrated for nanoparticles. Instead, they attach to the cell membrane and constitute a more potent inflammatory stimulus after the uptake process (Nicolete et al., 2011). Thus, our results provide a new possibility for the use microparticles as vaccine vehicles.

Finally, the results shown in this work present the induction and purification of two hypothetical proteins that could be candidates for evaluation as vaccines in the murine model of *L. panamensis*. Our model could be applied to other Leishmania proteins, but every protein must be tested individually. Subsequently, the encapsulation of these proteins using the biodegradable polymer PLGA and the simple emulsion-solvent evaporation method allowed us to obtain microparticles with adequate size and shape that would enable the release of the proteins in a sustained way for up to 10 days without completely degrading the protein. The next step should be to test these microparticles in the murine model to evaluate whether they have a protective effect in cutaneous Leishmaniasis.

## Acknowledgements

We are grateful to Grupo de Inmunomodulación, Universidad de Antioquia, Colombia for help with the *in vivo* experiments.

## Competing interests

The authors declare that they have no competing interests.

## Author's contributions

OTC conceived the project, and JDOV and CGH performed the experiments. JDOV, CGH, RZG and OTC wrote the paper. All authors read and approved the final manuscript.

## Funding

This work was supported by Colciencias project Grant No. 111556933438 and Universidad de Antioquia, UdeA.

## References

Allahyari, M., Mohit, E., 2016. Peptide/protein vaccine delivery system based on PLGA particles. Hum. Vaccin. Immunother. 12, 806–828. https://doi.org/10.1080/21645515.2015.1102804.

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., Leishmaniasis Control Team, W.H.O., 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7 (5), e35671. https://doi.org/10.1371/journal.pone.0035671.

Aronson, N., Herwaldt, B.L., Libman, M., Pearson, R., Lopez-velez, R., Weina, P., Carvalho, E., Ephros, M., Jeronimo, S., Magill, A., 2017. Guidelines Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH) * 96, 24–45. https://doi.org/10.4269/ajtmh.16-84256.

Brito, R.C.F. De, Cardoso, J.M.D.O., Reis, L.E.S., Vieira, J.F., Reis, A.B., 2018. Peptide Vaccines for Leishmaniasis. pp. 9. https://doi.org/10.3389/fimmu.2018.01043.

CDC, 2018. Leishmaniasis. Prev. Control. https://www.cdc.gov/parasites/leishmaniasis/prevent.html Accessed:2019-02-14.

Chakravarty, J., Sundar, S., 2010. Drug resistance in Leishmaniasis. J. Glob. Infect. Dis. 2 (2), 167–176. https://doi.org/10.4103/0974-777X.62887. May.

Chakravarty, J., Kumar, S., Trivedi, S., Rai, V.K., Singh, A., Ashman, J.A., Laughlin, E.M., Coler, R.N., Kahn, S.J., Marie, A., Cowgill, K.D., Reed, S.G., Sundar, S., Piazza, F.M., 2011. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1 + MPL-SE vaccine for use in the prevention of visceral leishmaniasis. Vaccine 29 (19), 3531–3537. https://doi.org/10.1016/j.vaccine.2011.02.096. 2011 Apr 27.

De Jong, W.H., Borm, P.J., 2008. Drug delivery and nanoparticles:applications and hazards. Int. J. Nanomedicine 3 (2), 133–149.

Derman, S., 2015. Caffeic acid phenethyl ester loaded PLGA nanoparticles: effect of various process parameters on reaction yield, encapsulation efficiency, and particle size. J. Nanomater. 2015, 1–12. https://doi.org/10.1155/2015/341848.

Gentile, P., Chiono, V., Carmagnola, I., Hatton, P.V., 2014. An Overview of Poly (lactic-co-glycolic) Acid (PLGA) -Based Biomaterials for Bone Tissue Engineering. pp. 3640–3659. https://doi.org/10.3390/ijms15033640.

Ghorbani, M., Farhoudi, R., 2018. Leishmaniasis in humans: drug or vaccine therapy? Drug Des. Dev. Ther. 12, 25–40. https://doi.org/10.2147/DDDT.S146521. 2018.

Giri, T.K., Choudhary, C., Alexander, A., Badwaik, H., Tripathi, D.K., 2013. Prospects of pharmaceuticals and biopharmaceuticals loaded microparticles prepared by double emulsion technique for controlled delivery. Saudi Pharm. J. 21, 125–141. https://doi.org/10.1016/j.jsps.2012.05.009.

Gupta, A. Sen, 2016. Role of Particle Size, Shape, and Stiffness in Design of Intravascular Drug Delivery Systems: Insights from Computations, Experiments. 8. pp. 255–270. https://doi.org/10.1002/wnan.1362.

Jyothi, N.V.N., Prasanna, P.M., Sakarkar, S.N., Prabha, K.S., Ramaiah, P.S., Srawan, G.Y., 2010. Microencapsulation techniques, factors influencing encapsulation efficiency. J. Microencapsul. 27, 187–197. https://doi.org/10.3109/02652040903131301.

Llanos-Cuentas, A., Calderón, W., Cruz, M., Ashman, J.A., Alves, F.P., Coler, R.N., Bogatzki, L.Y., Bertholet, S., Laughlin, E.M., Kahn, S.J., Beckmann, A.M., Cowgill, K.D., Reed, S.G., Piazza, F.M., 2010. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1 + MPL-SE vaccine when used in combination with sodium stibogluconate for the treatment of mucosal leishmaniasis. Vaccine 28 (46), 7427–7435. https://doi.org/10.1016/j.vaccine.2010.08.092. 2010 Oct 28.

Lü, J.M., Wang, X., Marin-Muller, C., Wang, H., Lin, P.H., Yao, Q., Chen, C., 2009. Current advances in research and clinical applications of PLGA-based nanotechnology. Expert. Rev. Mol. Diagn. 9 (4), 325–341. https://doi.org/10.1586/erm.09.15. 2009 May.

Makadia, H.K., Siegel, S.J., 2011. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. Polymers (Basel) 3, 1377–1397. https://doi.org/10.3390/polym3031377.

Marin, E., Briceño, M.I., Caballero-George, C., 2013. Critical evaluation of biodegradable polymers used in nanodrugs. Int. J. Nanomedicine 8, 3071–3090. https://doi.org/10.2147/IJN.S47186.

Martin, V., Vouldoukis, I., Moreno, J., Mcgahie, D., Gueguen, S., Cuisinier, A., 2014. The Protective Immune Response Produced in Dogs after Primary Vaccination with the LiESP / QA-21 Vaccine (CaniLeish ®) Remains Effective against an Experimental Challenge One Year Later 45, 1–15. https://doi.org/10.1186/1297-9716-45-69.

Morales-cruz, M., Flores-fern, G.M., Morales-cruz, M., Orellano, E.A., Ruiz, M., Griebenow, K., 2012. Results in pharma sciences two-step nanoprecipitation for the production of protein-loaded plga nanospheres. 2, 79–85. https://doi.org/10.1016/j.rinphs.2012.11.001.

Mudshinge, S.R., Deore, A.B., Patil, S., Bhalgat, C.M., 2011. Nanoparticles: emerging carriers for drug delivery. Saudi Pharm. J. 19, 129–141. https://doi.org/10.1016/j.jsps.2011.04.001.

Müller, K.E., Solberg, C.T., Aoki, J.I., Floeter-Winter, L.M., Nerland, A.H., 2017. Developing a vaccine for leishmaniasis: how biology shapes policy. Tidsskr. Nor. Laegeforen. 137 (3). https://doi.org/10.4045/tidsskr.17.0620. Dec 25.

Nicolete, R., Santos, D.F. dos, Faccioli, L.H., 2011. The uptake of PLGA micro or nanoparticles by macrophages provokes distinct in vitro inflammatory response. Int. Immunopharmacol. 11, 1557–1563. https://doi.org/10.1016/j.intimp.2011.05.014.

Osman, M., Mistry, A., Keding, A., Gabe, R., Cook, E., Forrester, S., Wiggins, R., Marco, S. Di, Colloca, S., Siani, L., Cortese, R., Smith, D.F., Aebischer, T., Kaye, P.M., Lacey, C.J., 2017. RESEARCH ARTICLE a third generation vaccine for human visceral leishmaniasis and post kala azar dermal leishmaniasis: first-in-human trial of ChAd63- KH 1–24. PLoS Negl. Trop. Dis. 11 (5). https://doi.org/10.1371/journal.pntd.0005527. 2017 May 12. e0005527.

Rueden, C.T., Schindelin, J., Hiner, M.C., Dezonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the Next Generation of Scientific Image Data. pp. 1–26. https://doi.org/10.1186/s12859-017-1934-z.

Song, X., Zhao, Y., Hou, S., Xu, F., Zhao, R., 2008. Dual agents loaded PLGA nanoparticles: systematic study of particle size and drug entrapment efficiency. 69, 445–453. https://doi.org/10.1016/j.ejpb.2008.01.013.

Specht, C.A., Lee, C.K., Huang, H., Hester, M.M., Liu, J., Luckie, B.A., Torres-Santana, M.A., Mirza, Z., Khoshkenar, P., Abraham, A., Shen, Z.T., Lodge, J.K., Akalin, A., Homan, J., Ostroff, G.R., Levitz, S.M., 2013. Vaccination with recombinant cryptococcus proteins in glucan particles protects mice against cryptococcosis in a manner dependent upon mouse strain and cryptococcal species. MBio 8 (6). https://doi.org/10.1128/mBio.01872-17. 2017 Nov 28. pii: e01872–17.

Srivastava, S., Shankar, P., Mishra, J., Singh, S., 2016. Possibilities and challenges for developing a successful vaccine for leishmaniasis. Parasit. Vectors 1–15. https://doi.org/10.1186/s13071-016-1553-y.

Taghipour, B., Yakhchali, M., Haririan, I., Tamaddon, A.M., Samani, S.M., 2014. The effects of technical and compositional variables on the size and release profile of bovine serum albumin from PLGA based particulate systems. Res. Pharm. Sci. 9, 407–420.

Urrea, D.A., Duitama, J., Imamura, H., Alzate, J.F., Gil, J., Muñoz, N., Villa, J.A., Dujardin, J.C., Ramirez-Pineda, J.R., Triana-Chavez, O., 2018. Genomic analysis of Colombian *Leishmania panamensis* strains with different level of virulence. Sci. Rep. 8 (1), 17336. https://doi.org/10.1038/s41598-018-35778-6.