When nanomedicines meet tropical diseases

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Nanostructured lipid carriers containing benznidazole: physicochemical, biopharmaceutical and cellular in vitro studies

Giuliana Muraca, María Esperanza Ruiz, Rocío C. Gambaro, Sebastián Scioli-Montoto, María Laura Sbaraglini, Gisela Padula, José Sebastián Cisneros, Cecilia Yamil Chain, Vera A. Álvarez, Cristián Huck-Iriart, Guillermo R. Castro, María Belén Piñero, Matías Ildebrando Marchetto, Catalina Alba Soto, Germán A. Islan* and Alan Talevi*

Abstract

Chagas disease is a neglected endemic disease prevalent in Latin American countries, affecting around 8 million people. The first-line treatment, benznidazole (BNZ), is effective in the acute stage of the disease but has limited efficacy in the chronic stage, possibly because current treatment regimens do not eradicate transiently dormant Trypanosoma cruzi amastigotes. Nanostructured lipid carriers (NLC) appear to be a promising approach for delivering pharmaceutical active ingredients as they can have a positive impact on bioavailability by modifying the absorption, distribution, and elimination of the drug. In this study, BNZ was successfully loaded into nanocarriers composed of myristyl myristate/Crodamol oil/poloxamer 188 prepared by ultrasonication. A stable NLC formulation was obtained, with ≈80% encapsulation efficiency (%EE) and a biphasic drug release profile with an initial burst release followed by a prolonged phase. The hydrodynamic average diameter and zeta potential of NLC obtained by dynamic light scattering were approximately 150 nm and ~13 mV, respectively, while spherical and well-distributed nanoparticles were observed by transmission electron microscopy. Fourier-transform infrared spectroscopy, differential scanning calorimetry, thermogravimetric analysis, and small-angle X-ray scattering analyses of the nanoparticles indicated that BNZ might be dispersed in the nanoparticle matrix in an amorphous state. The mean size, zeta potential, polydispersity index, and %EE of the formulation remained stable for at least six months. The hemolytic effect of the nanoparticles was insignificant compared to that of the positive lysis control. The nanoparticle formulation exhibited similar performance in vitro against T. cruzi compared to free BNZ. No formulation-related cytotoxic effects were observed on either Vero or CHO cells. Moreover, BNZ showed a 50% reduction in CHO cell
viability at 125 µg/mL, whereas NLC-BNZ and non-loaded NLC did not exert a significant effect on cell viability at the same concentration. These results show potential for the development of new nanomedicines against T. cruzi.

Introduction

Chagas disease is a neglected disease endemic to Latin America, affecting around 8 million people and causing 2000 deaths per year, according to the World Health Organization [1]. Currently, this health problem is not restricted to Latin American countries, as it has spread to non-endemic regions such as the United States and Europe [2,3]. It is caused by the hemoflagellate protozoan Trypanosoma cruzi, whose life cycle involves transitioning from non-flagellated multiplicative intra-cellular forms (amastigotes) to blood-circulating non-multiplicative forms (trypomastigotes). It is primarily transmitted by an insect vector of the Triatominae subfamily, although other modes of transmission (blood transfusion, organ transplant, and congenital transmission) have gained importance over the last decades. It is characterized by two stages: acute, and chronic. During the acute stage, which lasts up to two months after infection, the patients might present or mild, nonspecific, or no symptoms. This phase is followed by a chronic stage where parasites can be primarily found inside specific tissues. Decades after infection, signs and symptoms of damage to target organs, mainly the heart, gastrointestinal tract, and brain appear in 20–30% of chronically infected individuals [1,4].

Currently, two drugs have been approved for the treatment of Chagas disease: benznidazole (BNZ) and nifurtimox. The first-line treatment, BNZ, is a nitroimidazole that generates radical intermediates via the reduction of its nitro group, which covalently bind to macromolecules under aerobic and anaerobic conditions [5]. Cure rates are high when BNZ is administered during the acute phase [6]; however, in the chronic stage the cure rate is estimated to be less than 10% [7]. Some authors differ about this percentage owing to the variability in sensitivity of the tests that are used to establish cure criteria [8,9]. BNZ is associated with a variety of adverse reactions including allergic dermatitis, hypersensitivity syndrome, gastric pain, anorexia, insomnia, vomiting, which ultimately lead to withdrawal in 12–18% of the patients [10]. Additionally, the BENEFIT (Benznidazole Evaluation for Interrupting Trypanosomiasis) trial could not prove that the standard treatment with BNZ can prevent disease progression [11].

BNZ has been classified as a class IV drug (low solubility, low permeability) in the Biopharmaceutics Classification System (BCS) [12]. It has an apparent volume of distribution (Vd) of 0.56 L/kg, and reactive products of its metabolism [13]. Such Vd and low permeability values across biological barriers could result in difficulties for BNZ to reach intracellular amastigotes. The encapsulation of BNZ within nanoscale pharmaceutical carriers has been proposed as a strategy to reduce toxicity and improve efficacy [13]. Incorporation of drugs into nanoscale vehicles could result in changes in its absorption, distribution, metabolism, and excretion, which in turn could translate into improved efficacy and diminished BNZ toxicity. For example, BNZ-loaded nanoparticles could accumulate in the site of inflammation delivering the drug in the surroundings of their molecular target. In addition, nanocarriers may pass through the cell membrane via endocytosis to avoid BNZ efflux via the P-glycoprotein efflux pump [14-16], thus delivering the drug more efficiently. Many developments have been made in the past years resulting in lipid formulations such as liposomes, solid lipid nanoparticles (SLNs), and nanoemulsions, which increased the apparent solubility of BNZ and its efficacy against parasites [17]. Remarkably, oil-in-water nanoemulsions improved the trypanocidal activity against trypomastigotes compared to that of the free drug [18]. Among the aforementioned nanosystems, SLNs have recently gained special attention owing to their biocompatibility properties, biodegradability, relatively easy surface and composition modification, and efficacy in loading and delivering active principles [19]. SLNs comprise a lipid core, solid at 25 °C, stabilized by steric effects with a surfactant. The addition of small amounts of a liquid lipid at 25 °C leads to the improvement of SLNs in terms of sustained drug release and encapsulation efficiency (EE%), enabling the development of nanostructured lipid carriers (NLC) [20].

Here, we resort to NLC encapsulating BNZ, describing the preparation, physicochemical and biopharmaceutical characterization, and in vitro evaluation against T. cruzi intracellular and blood circulating forms. Interestingly, our formulation achieves a higher cumulative release and considerable higher activity against amastigotes compared to previously reported BNZ-loaded NLCs. Moreover, we report the dose-response intrinsic activity of myristyl myristate, a relatively common constituent of NLCs, against T. cruzi, which might be of future interest to other researchers working in the field.

Results and Discussion

Formulation and physicochemical characterization of NLC-BNZ

Nanoparticle formulations were prepared by the ultrasonication method and named as NLC-BNZ or NLC-VEHICLE, in that
order, depending on whether they contained BNZ or not. Stable homogeneous formulations were prepared. The encapsulation efficiency of NLC-BNZ was considerably high for the lipid formulations, reaching approx. 80%. The theoretical drug loading was 2.5%. Our results were in concordance with the encapsulation results of a previous study by Vinuesa et al., involving different types of nanoparticles and BNZ, including SLN and NLC [21]. The NLC-BNZ formulation was analyzed using transmission electron microscopy (TEM) to confirm the presence of nanoparticles showing a spherical morphology and a narrow distribution of sizes (Figure 1). Image analysis through ImageJ [22] software showed a mean particle size of 150 ± 13 nm.

Accordingly, the hydrodynamic diameter of the nanoparticles measured by dynamic light scattering (DLS) was in the 100–200 nm range (≈150 nm), with a moderate distribution of sizes as indicated by a polydispersity index (PDI) of 0.204. The zeta potential (ζ) was measured by Doppler anemometry, and it was found to be around −13 mV.

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were performed to determine the thermal stability and melting/recrystallization processes of the components after drug encapsulation. Overlaid DSC thermograms are shown in Figure 2, whereas the melting temperature ($T_m$), the enthalpy of fusion ($ΔH_f$), and crystallinity index (CI) are presented in Table 1. Whereas BNZ showed an endothermic peak at its melting point (191.2 °C) [23], the formulation showed two endothermic peaks in the range of 40–50 °C, which could be referred to the melting points of the lipid and the surfactant, respectively. This suggests that no other endothermic changes occur to the formulation constituents or its load during the high-energy sonication procedure. A peak matching the phase transition peak of BNZ did not appear in the nanoparticle thermogram, indicating that BNZ was dispersed within the lipid matrix [24]. Correlating with the lower enthalpy of fusion, the CI (%) value of the nanoparticles was lower than that of the bulk myristyl myristate. Lipid molecules could be less ordered in the nanoparticles than in the bulk material, considering the disarrangement caused by the incorporation of the drug and the surfactant. For that reason, it might require less energy to melt in comparison to the pure crystalline substance [25].

Thermogravimetric curves of myristyl myristate, poloxamer 188, and BNZ showed one thermal degradation process, whereas NLC-BNZ and NLC-VEHICLE presented two events (Figure 3). That was also observed in the derivative curves. The weight loss process for the lipid started at 180 °C and finished at 320 °C. The poloxamer 188 thermogram showed a decomposition process starting at 300 °C and ending at 410 °C, and BNZ degradation occurred in the 190–300 °C range. Considering these processes, nanoparticle thermal behavior might be attributed first to lipid degradation, and second to poloxamer weight loss.

The attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) technique was used to analyze the nano-
Figure 2: DSC thermograms of BNZ, myristyl myristate, poloxamer 188, NLC VEHICLE, and NLC-BNZ.

Figure 3: Thermogravimetric (A) and derivative thermogravimetric (B) curves of BNZ, myristyl myristate, poloxamer 188, NLC-VEHICLE, and NLC-BNZ.

Figure 4: ATR-FTIR spectra of BNZ, myristyl myristate, poloxamer 188, NLC VEHICLE, and NLC-BNZ.

Table 1: Thermal properties of benznidazole (BNZ), myristyl myristate, poloxamer 188, and nanoparticles (NLC-VEHICLE and NLC-BNZ). Abbreviations: $T_m$, melting temperature; $\Delta H_f$, fusion enthalpy; CI (%), crystallinity index.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_f$ (J/g)</th>
<th>CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNZ</td>
<td>191.2</td>
<td>142.3</td>
<td>100</td>
</tr>
<tr>
<td>myristyl myristate</td>
<td>42.9</td>
<td>239.4</td>
<td>100</td>
</tr>
<tr>
<td>poloxamer 188</td>
<td>54.2</td>
<td>148.8</td>
<td>100</td>
</tr>
<tr>
<td>NLC-VEHICLE</td>
<td>41.4–51.7</td>
<td>61.1</td>
<td>12.7</td>
</tr>
<tr>
<td>NLC-BNZ</td>
<td>40.9–50.0</td>
<td>59.4</td>
<td>12.4</td>
</tr>
</tbody>
</table>

particle surface composition and determine the possible interactions among the formulation components (Figure 4). The BNZ spectrum presented its characteristic peaks at 3264 cm$^{-1}$ corresponding to N–H in the secondary amide bond, 1652 cm$^{-1}$ to C=O in the amide (1500–1400 cm$^{-1}$ is also the absorption range of the C=C in the benzyl group), 1357 cm$^{-1}$ to the symmetric vibration of R–NO$_2$, and 1141 cm$^{-1}$ to C–N in the imidazole ring [26]. Myristyl myristate displayed peaks at 2913 and 2848 cm$^{-1}$ corresponding to C–H of alkane, 1731–1184 cm$^{-1}$ to C=O and C–O stretching of ester groups, respectively. The peak at 1467 cm$^{-1}$ was associated with the deformations of the C–H of alkanes [27]. The characteristic peaks of poloxamer 188 were at 3600 cm$^{-1}$ relative to the O–H stretching, the intense peak at 2873 cm$^{-1}$ corresponding to C–H stretching of alkanes, another intense peak at 1105 cm$^{-1}$ to the symmetric stretching of C–O–C, and 964–833 cm$^{-1}$ to asymmetrical and symmetrical stretching of C–O–C, respectively [28]. The NLC-BNZ spectra showed myristyl myristate and poloxamer characteristic peaks (overlapping of the most intense peaks in the 3000 cm$^{-1}$ region – 2910 cm$^{-1}$, 2883 cm$^{-1}$, and 2854 cm$^{-1}$ – due to the presence of the lipid and surfactant). In contrast, the spectra did
not show peaks that could be linked to BNZ, suggesting that drug molecules were not on the nanoparticle surface but rather dispersed into the lipid matrix [24].

Structural analysis was performed by selecting different angular regions from the small-angle X-ray scattering (SAXS) and wide-angle X-ray scattering (WAXS) patterns. The WAXS patterns (Figure 5) showed contributions of diffraction peaks from BNZ, myristyl myristate, and NLC. The nanostructured lipid carriers showed contributions from both the isolated myristyl myristate and additional Bragg peaks at 19.1° and 23.3° corresponding to the copolymer. This indicates that there was phase segregation, most likely a core–shell structure with the lipidic phase inside and the hydrophilic part of the copolymer in the outer part of the NLC. Myristyl myristate major peak positions expressed in terms of \( d \) spacing were 4.1 and 3.8 Å, corresponding to a family of the \( \beta' \) polymorph [29] and did not change after NLC synthesis or BNZ addition. Furthermore, there were no contributions from the crystalline phase of BNZ within NLC because of its small quantity or to dissolution inside the NLC.

The long period Bragg diffraction peaks for Myristyl myristate could be observed in SAXS patterns at the \( q \) range between 0.15 and 0.2 Å\(^{-1}\) (Figure 6). Bare myristyl myristate confirmed the presence of a \( \beta' \) polymorph with long period \( d \) spacing of 3.99 (001) and 3.47 nm (002), while in the NLC only the 3.47 nm of \( d \) spacing peak remains. Also, in the NLC systems, the main Bragg peak was wider, attributed to a nanosized crystal effect where the estimated crystallite average sizes were 94 ± 5 nm and 101 ± 5 nm for NLC-VEHICLE and NLC-BNZ, respectively, using the Scherrer approximation. However, a broadening of the lower part of the main peak in the NLC-BNZ samples suggests defects in the structure, probably due to the inclusion of BNZ in the formulation. At smaller angles, the copolymer on the surface exhibited a lamellar-like structure [30]. The Lorentz/Kratky plot (\( q^2 I(q) vs q \)) is shown in Figure 7, where the peaks of NLC and NLC-BNZ remained at the same position, independently of the presence of the BNZ load. The linear correlation function was obtained by using the following transformation (Equation 1) [31,32]:

\[
K(z) = \frac{\int_0^\infty I_{\text{norm}}(q)q^2 \cos(qz) dq}{\int_0^\infty I_{\text{norm}}(q)q^2 dq}
\]

where \( I_{\text{norm}} \) is the normalized intensity after removing the myristyl myristate (i.e., MM) contribution: \( I_{\text{norm}} = I_{\text{NLC}}(q) - I_{\text{background}} - c(I_{\text{MM}}(q) - I_{\text{background}}) \), \( c \) being a constant or weighted proportionality between phases. From this transformation the lamellar period obtained from the first maximum of the oscillation was 12.6 nm for both systems (Figure 7). In contrast with amphiphilic low-weight loading, BNZ is a lipophilic molecule that did not change the structure of the copolymer. Thus, it is proposed to be dissolved in the core of the lipidic nanoparticle.

Drug release and physical stability

The release profiles (Figure 8) showed that 78% of the free drug was dissolved in the first 15 min of the experiment. In contrast, during the first 15 min only about 12% of the drug was released from the NLC formulation. An initial burst release was observed, followed by a sustained release. This phenomenon could be explained in part by considering the presence of free drug molecules in the formulation (around 20% of the initial drug load) and in part by the release of drug molecules located...
near the surface of the NLC, which rapidly diffuse out of the vehicle. The slow increase of the drug concentration in the release medium observed after the initial stage could be attributed to the gradual release of drug molecules from the matrix core, where the drug is mainly located according to X-ray diffraction (XRD) results [33]. Remarkably, although our NLC possess a comparatively lower drug load, the maximal accumulated drug release is higher than that of similar systems previously reported [21].

The in vitro release data were fitted to different mathematical models. The model that best adjusted the data was the Korsmeyer–Peppas model followed by a first-order model (Supporting Information File 1, Table S1). The Korsmeyer–Peppas model, also called power law, was initially used to describe drug release in polymeric systems where the two predominant mechanisms were relaxation of the polymer chains and diffusion. In this model (Equation 2), $M_t/M_\infty$ is the fraction dissolved, $K$ is a constant that incorporates structural and geometrical information, and the exponent $n$ is the diffusional or transport exponent, that provides information about the release mechanism. However, it can also be viewed as a generalization for the explanation of two different drug release mechanisms that could coexist [34]. The mechanism that dominates the release can be inferred through the value of the release exponent $n$. For spherical systems, $n$ will take a value of 0.43 for drug release governed by Fickian diffusion, a value of 1 for zero-order release, and intermediate values for intermediate behavior, often regarded as anomalous transport. In our case, the estimated value of $n$ was 0.56, suggesting mixed release mechanisms at play with a strong contribution of diffusion. As in our case there is no polymer relaxation involved, it may be hypothesized that the burst effect could be slightly affecting the global kinetics of the process [35]. Although this description has its limitations, it has been widely used to describe drug release from similar lipidic formulations [35-38].

$$\frac{M_t}{M_\infty} = Kt^n$$  \hspace{1cm} (2)

The mean particle size, PdI, zeta potential, and encapsulation efficiency were selected as parameters to follow the physical stability of the nanoparticle dispersion for six months under the selected storage conditions (refrigerator at 4 °C) (Figure 9). Based on these results, the formulation could be stored at 4 °C for at least three months without losing its initial properties in terms of size; polydispersity and encapsulation efficiency values remained unaltered during the storage period, and the zeta potential parameter started at $-10$ mV and ended up at $-15$ mV after six months. Dynamic light scattering analysis of the formulations revealed nanoparticles with a hydrodynamic diameter in the 100–200 nm range ($\approx 50$ nm) starting at 146 nm and slightly increasing in the third month up to 155 nm. The $Z$-average parameter was chosen to report the nanoparticles size. The size values were consistent with the TEM image analysis. The surface charges required to achieve a good dispersion of nanoparticles stabilized by electrostatic repulsion are around ±30 mV [39]. The $\zeta$ value of our formulation was $\approx 14$ mV. Although this value is not optimal for stabilization by electrostatic repulsion, it still contributes with a positive aspect, as high-negative $\zeta$ values may impede cellular uptake [40]. On the other hand, it was observed that the nanoparticulated systems remained stable after six months with no precipitation. This suggests that in this case the stabilization is not achieved...
by means of surface charge alone, but also by the steric repulsion after adding a non-ionic surfactant [41].

Cytotoxicity and hemolytic activity
Cytotoxicity assays using the tetrazolium 3-[4,5-dimethylthiazol-2-yi]-2,5-diphenyltetrazolium bromide salt method (MTT) showed that Chinese hamster ovary cells (CHO) viability was affected by BNZ concentration in a dose-dependent manner (Figure 10). Interestingly, the cell viability for NLC-VEHICLE or NLC-BNZ at the same tested concentrations of free BNZ resulted in values above 80% in all cases, suggesting a decreased cytotoxic effect. That decrease in toxicity generated by NLC-BNZ, in comparison with free BNZ, could be attributed to the release profile of BNZ from NLC, exposing cells to lower doses of BNZ during the first stages of cellular division. This is a remarkable result, as toxic effects of BZN are a major cause of treatment discontinuation in the clinical setting [42]. Additionally, cytotoxicity was evaluated in the Vero cell line by flow cytometry, where the percentage of dead cells labeled with propidium iodide (PI, Supporting Information File 1, Figure S2) was measured. Neither the drug-loaded or unloaded NLCs elicited significant toxicity in Vero cells.

As it is common to parenterally administer nanoparticle formulations, it is of interest to study the potential toxicity of pharmaceutical nanocarriers in blood cells. Most of the published
papers evaluated the hemolytic activity (HA) of nanoparticles after 2, 3, or 5 h of incubation [43-45]. The standard methods to test hemolytic activity of nanoparticles (ISO/TR 7406 or ASTM E2524-08 standard) established that biomaterials that induce a critical hemolytic ratio of <5% can be considered safe for biological applications [46]. In our study, it was observed no hemolytic effects for BNZ, NLC-VEHICLE, and NLC-BNZ at different concentrations after 3 and 24 h of incubation (data not shown). However, some hemolytic activity was observed for NLC-VEHICLE and NLC-BNZ after 48 h of incubation (Figure 11). Despite NLC-BNZ showed 4.8% HA at the highest concentration, the formulation could still be considered safe according to the regulations. In fact, hemolytic activity could be caused by several reasons, including the ageing of the blood sample after 48 h of incubation with the concomitant release of hemoglobin, but also by the presence of surfactants that could destabilized the erythrocyte membrane [47]. On the other hand, the differences between NLC-VEHICLE and NLC-BNZ, the latter exhibiting a higher HA, could be explained by adding the HA of the free drug to the effect of the vehicle on erythrocytes. More studies would be necessary to investigate the effect of the composition, size, or porosity of these nanoparticles after a long term exposure to blood samples as was described for other type of nanoparticles [45]. Our results suggest that the reported NLC-BNZ formulations are hemocompatible [43].

In vitro antiparasitic activity
As shown in Figure 12A, free BNZ displayed a clear dose-dependent effect on *T. cruzi* trypomastigotes (with an EC₅₀ of 6.07 µM), whereas the NLC-BNZ and NLC-VEHICLE also exhibited a dose-response behavior despite comparatively large variability across replicates. While for free BNZ the estimated EC₅₀ value was 6.07 µM with similar reported values (6.04 µM [48]) for the same parasite classification (TcI), the NLC-BNZ presented a full trypanocidal effect at concentrations higher than 5 µM (10 µM). A similar observation was found for the empty particles (NLC-VEHICLE) suggesting that the formulation itself possesses intrinsic toxicity on *T. cruzi* trypomastigotes. A separate assay of the individual constituents of the formulations was thus performed, demonstrating that myristyl myristate, at a
relatively low concentration, has a negative effect on parasite viability (dose-response studies for myristyl myristate against amastigotes are shown in Supporting Information File 1). This may imply that myristyl myristate cannot be considered, in our case, as a pharmacologically inert constituent in our formulation. Instead, it should be considered as a pharmaceutical active ingredient based on its intrinsic effects against *T. cruzi*.

The dose-response effects of BNZ, NLC-VEHICLE, and NLC-BNZ on *T. cruzi* amastigotes were also evaluated (Figure 12B), and the corresponding EC$_{50}$ were calculated. Benznidazole and NLC-BNZ presented inhibition of the intracellular growth of the parasites even at the lowest concentration, with no significant differences observed between the treatments. Benznidazole and NLC-BNZ EC$_{50}$ values were 3.15 and 3.33 µM, respectively. In agreement with the in vitro trypanocidal assay, NLC-VEHICLE also displayed intrinsic anti-amastigote activity with an EC$_{50}$ value of 10.29 µM. This was unexpected, although not necessarily a negative outcome, having in mind that our formulation displayed reduced cytotoxicity against mammal cells. Isolated myristyl myristate lipid was also tested against amastigotes, showing a reduced amastigote density at effective concentrations (Supporting Information File 1, Figure S1). Neither P188 nor GTCC-LQ displayed any effect against trypomastigotes or amastigotes up to 50 µM. Of note, the more efficacious NLC encapsulating BNZ previously reported in the literature [21] had an EC$_{50}$ against amastigotes of 17.6 µM. The higher efficacy of our system may be explained by a higher maximal drug release and/or by the intrinsic activity of myristyl myristate, which adds to that of BNZ.

A hypothesis about the intrinsic toxicity of our nanoscale vehicle on *T. cruzi* may be linked to a modification of glycosylphosphatidylinositols (GPIs). Glycosylphosphatidylinositols are the main anchor complexes used by protozoans to bind to cell surface proteins. It covalently attaches to the C terminus of a protein connecting it to the outer leaflet of the lipid bilayer [49]. *Trypanosoma brucei* predominant membrane protein variant surface glycoprotein (VSG), which is involved in parasite host immune system evasion, is anchored by a GPI that requires myristate for its synthesis. Analogs of myristate have shown toxicity towards the parasite [50]. *T. cruzi* trypomastigotes connect mucin (a surface molecule implicated in parasite virulence) to the membrane through a GPI which is synthesized exclusively with a C16 fatty acid [51], though a C14 fatty acid incorporation could be toxic to the parasite. Experiments in *T. brucei* indicated that the specificity of fatty acid incorporation depends on chain length [52]. The lipid in our formulation is an ester of fatty acids that could hypothetically interrupt the anchoring of mucin to the lipid bilayer in *T. cruzi*, thus rendering the parasites non-viable. However, further studies are required to test this hypothesis.

**Conclusion**

Among the spectra of nanoformulations encapsulating BNZ that exist to date, the nanoparticles presented in this work might be considered a novelty in terms of the lipid and manufacturing technique of choice. We achieve physical stability for at least six months with acceptable particle size, PdI, and EE%. Complementary to these results, TEM images showed a spherical configuration. Thermal and crystallographic experiments indi-
icated that BNZ was dispersed into the lipid matrix. The formulations showed a sustained drug release profile for 24 h, achieving a maximal accumulated release above 74% during 24 h. The release profile was adequately fitted to the Korsmeyer–Peppas model with an estimated release exponent of 0.56, suggesting a mixed mechanism of release with a dominant Fickian behavior. In vitro experiments on *T. cruzi* trypomastigotes and amastigotes showed similar performances against the intracellular form of the parasite when comparing encapsulated and free BNZ. Surprisingly, the empty nanoparticles exhibited activity against the parasite, which was later attributed to one of the constituents of the formulation, myristyl myristate. This may explain why our formulations exhibited increased performance against *T. cruzi* compared with other previously reported BNZ-loaded NLC. It would be interesting to study the effect of other lipids on the parasite to optimize the efficacy of the formulations based on a potential additive or synergistic effect of BNZ and the formulation itself. Remarkably, the cytotoxicity effect on host cells was lower for the BNZ-loaded nanoparticles compared to that of the free drug, showing a possible benefit for the use of our formulation.

### Experimental

#### Materials

Benznidazole (Lot #MKCD5602, purity ≥ 97%) and Kolliphor®P188 (poloxamer 188) were purchased from Sigma-Aldrich. Myristyl myristate (Crodamol™ MM, melting range = 36–40 °C), and the oil (Crodamol™ GTCC-LQ, a mixture of fully saturated triglycerides, melting point = −5 °C) were kindly donated by Croda Argentina. All reagents used in the preparation and analysis of the formulations were of analytical grade and were obtained from different commercially available sources.

#### Formulation of benznidazole-loaded nanostructured lipid carriers

BNZ-loaded NLCs were obtained via ultrasonication as previously described in Scioli-Montoto et al. (2022) [53]. Solid myristyl myristate (2% w/v, 400 mg) was melted in a water bath at 60–70 °C. The oil (40 μL) was added to the melted lipid phase simultaneously with BNZ (10 mg). The aqueous phase was prepared by dissolving 600 mg (3% w/v) of poloxamer 188 (poly(ethylene glycol)-block-poly(propylene glycol)block-poly(ethylene glycol)) in 20 mL of ultrapure water (Milli-Q®, Millipore, Ma., USA) and was preheated at the same temperature as the melted lipid in the water bath. After 30 min of thermostatization, the aqueous solution was poured over the lipid phase, and ultrasonication was carried out for 20 min at an 80% amplitude using an ultrasonic processor (130 Watts, Cole-Parmer, USA) equipped with a 6 mm titanium tip. After the sonication process, NLC-BNZ were obtained by leaving the hot suspension to cool to room temperature. The remaining volume was then measured.

### Measurement of the encapsulation efficiency

Concentration of the free drug in the dispersion medium was measured to calculate the encapsulation efficiency (EE%). For this, 500 μL of the formulation was placed in Microcon® centrifugation filters (MWCO = 10000, Merck Millipore, Billerica, MA, USA) and centrifuged at 10000 rpm for 15 min. The amount of BNZ was estimated by performing a high-performance liquid chromatography (HPLC) analysis of the filtrate. Considering the initial amount of BNZ added to the formulation, the EE% was calculated as follows:

\[
EE(\%) = \frac{M_0 - (C_{\text{free}} \cdot V_f)}{M_0} \times 100
\]

where \(M_0\) is the initial amount of BNZ added to the formulation, \(C_{\text{free}}\) is the drug concentration of the filtrate (i.e., the free drug concentration) in μg/mL, and \(V_f\) is the volume after ultrasonication (mL).

The theoretical drug loading (DL%) was calculated as follows:

\[
DL(\%) = \frac{\text{Mass of drug incorporated (mg)}}{\text{Lipid mass (mg)}} \times 100
\]

### HPLC analysis of benznidazole

Chromatographic separation was achieved by HPLC (Gilson SAS, Villiers-Le-Bel, France) via UV detection. A Platinum EPS C8 (150 mm × 4.6 mm, 5 μm, Grace™, Columbia, MD, USA) column was used; the mobile phase consisted of a mixture of methanol and 0.02% phosphoric acid solution (60:40) for a final pH of 2.5. The system was operated isocratically at a 1.0 mL/min flow rate and the detection was performed at 324 nm. The volume of injection was 20 μL.

### In vitro benznidazole release assay

The release of BNZ from the nanoparticles was performed in a rotating paddle apparatus (Vision Classic 6, Hanson Research, Chatsworth, CA, USA) at 75 rpm using 500 mL of KH₂PO₄ buffer (pH 6.8) as the dissolution medium. The bath temperature was set at 37.0 ± 0.5 °C. A volume of 5 mL of each formulation was placed in a pre-hydrated dialysis membrane (MWCO 10 kDa) and submerged into the dissolution vessels. A solution of free BNZ at the same concentration was used as control. At 0, 5, 10, 15, and 30 min, and at 1, 2, 3, 4, 5, 6, 7, and 24 h, 1 mL
of the dissolution medium was taken from the vessel. Samples were analyzed by HPLC as described above. Experiments were performed in triplicate and the mean values were used for data analysis. The data were fitted to mathematical models of drug release (i.e., First order, Hopfenberg, Baker–Lonsdale, Korsmeyer–Peppas, and Hixon Crowell) via the DDSolver complement developed by Zhang et al. and available for Excel® [54]. The model that best fitted the data according to the goodness-of-fit measures (R², R²-adj, MSE, and AIC) was chosen.

Particle size, zeta potential and polydispersion index
Nano ZS Zetasizer (Malvern Instruments Corp, Worcestershire, UK) was used to measure particle size distribution and mean diameter by DLS at 25 °C in polystyrene cuvettes with a thickness of 10 mm. The zeta potential was determined by Doppler anemometry using the previously described equipment. As an estimation of the distribution of particle sizes, the polydispersion index was determined. All experiments were carried out in triplicate, except for the particle size estimation, which was measured six times.

Physical stability
The mean particle size, PdI, zeta potential, and encapsulation efficiency were measured to assess the physical stability of the nanoparticle dispersion during storage at 4 °C protected from light. Physical parameters (e.g., particle size, PdI, zeta potential) were measured by DLS and EE% was measured by HPLC, once a month, during a six-month period.

Differential scanning calorimetry analysis
Thermal analysis of BNZ, myristyl myristate, poloxamer 188, and NLC-BNZ was performed by differential scanning calorimetry (DSC Q2000, TA Instruments, New Castle, DE, USA) under an inert atmosphere of dry nitrogen (50 mL·min⁻¹). A standard aluminum pan containing approximately 5 mg of the sample after freeze drying the formulations was used. Scans were run in the range from 0 to 250 °C at a heating rate of 10 °C/min.

The degree of crystallinity (% crystallinity index, CI) was calculated using the following equation [55]:

\[
CI(\%) = \frac{\Delta H_{NLC \text{ aqueous dispersion}}}{\Delta H_{\text{bulk material}} \times \text{Concentration lipid phase}} \times 100
\]

where \(\Delta H_{NLC}\) and \(\Delta H_{\text{bulk material}}\) are the melting enthalpies (J·g⁻¹) of the NLC dispersion and the bulk lipid, respectively. The concentration of the lipid phase was 2%.

Thermogravimetric analysis
Thermogravimetric analysis was performed to assess the thermal stability of BNZ, myristyl myristate, poloxamer 188, and NLC-BNZ on a TGA Q500 apparatus (TA Instruments, New Castle, DE, USA). Freeze dried formulations of approximately 10 mg were accurately weighed in a platinum pan. Measurements were performed from room temperature to 600 °C at a heating rate of 10 °C/min under nitrogen atmosphere to avoid thermo-oxidative degradation.

Attenuated total reflection Fourier-transform infrared spectroscopy
Fourier-transform infrared spectroscopy spectra were obtained. The attenuated total reflection mode was used to record the spectra over the range of 400–4000 cm⁻¹ at a resolution of 2 cm⁻¹.

Transmission electron microscopy
Transmission electron microscopy images were captured using a Jeol-1200 EX II-TEM microscope (Jeol, MA, USA). A drop (10 µL) of the nanoparticle dispersion previously diluted (1:10) with ultrapure water was spread onto a collodion-coated Cu grid (400 mesh). Excess liquid was drained with filter paper. A drop of phosphotungstic acid was added to the dispersion for contrast enhancement.

X-ray diffraction structural analysis
Small angle X-ray scattering/wide angle X-ray scattering measurements were performed using a XEUSS 2.0 equipment (XENOCS, France). Patterns were registered with two synchronous 2D photon-counting pixel X-ray detectors for SAXS Pilatus 200k (DECTRIS, Switzerland), and a Pilatus 100k (DECTRIS, Switzerland) placed 159 mm from the sample with a tilted angle of 36 °C for WAXS. The SAXS measurements were performed using two samples to detect distances, 1194 and 337 mm, to obtain a wide angular range. The scattering intensity, \(I(q)\), was recorded by means of the scattering momentum transfer \(q\), where \(q = 4πλ \sin(θ)\), 20 is the scattering angle and \(λ = 0.15419 \text{ nm}\) is the weighted average of the X-ray wavelength of the Cu Kα₁,₂ emission lines. Owing to the small beam size pointed at the sample (< 1 mm × 1 mm) smearing effects were not considered. The NLC samples were placed under vacuum between Kapton® tapes. The measurements were done in transmission mode. The SAXS/WAXS patterns were taken for 10 min each.

Cell toxicity assay on CHO cells
The viability of CHO cells was analyzed by the reduction of the tetrazolium salt to a formazan product (i.e., the MTT method). A 96-well polystyrene microplate containing 1 × 10⁴ cells per well of CHO cells (obtained from the American Type Culture
Collection, Manassas, VA, USA) were cultured in Ham’s F12 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Notocor Laboratories, Cordoba, Argentina) and antibiotics (50 IU penicillin and 50 µg/mL streptomycin) (Bagó Laboratories, Buenos Aires, Argentina) in a humidified atmosphere with 5% CO₂. After 24 h, the cells were incubated with increasing concentrations of RPMI as control, BNZ, NLC-VEHICLE, and NLC-BNZ (0, 15, 31, 62, and 125 µg·mL⁻¹). The MTT reagent (5 mg·mL⁻¹ in phosphate-buffered saline (PBS)) was then added for 3 h. Dimethyl sulfoxide (DMSO,100 µL per well) was added under agitation for 10 min to dissolve the MTT. The color was measured in a microplate reader (MultiskanTM GO spectrophotometer, Thermo Fisher Scientific) at 550 nm. The assays were performed in triplicate.

**Cell toxicity assay on Vero cells**

Cell viability was analyzed by flow cytometry as described in the “In vitro anti-amastigote effect” section after adding PI to obtain the percentage of dead cells following the incubation with the formulation of nanoparticles or the free drug.

**Hemolytic effect**

Hemolysis was assessed on 3 mL of a freshly drawn heparinized suspension of fresh human blood placed on a 6-well cell culture plate. Increasing concentrations of freshly prepared dilutions of the free drug and NLC-BNZ (1, 5, and 50 µg·mL⁻¹) were added to each well and incubated for 3, 24, and 48 h at 37 °C. Samples were then centrifuged for 5 minutes at 2500 rpm, and the absorbance of the supernatant was determined at 540 nm in a microplate reader (MultiskanTM GO spectrophotometer, Thermo Fisher Scientific). Triton X 100 (10%), saline solution, NLC-VEHICLE, and BNZ were used as the positive, negative, vehicle, and reference drug controls, respectively. The hemolytic activity was calculated as [56]:

\[
	ext{HA(\%)} = \frac{A_{540\ nm\ sample} - A_{540\ nm\ saline}}{A_{540\ nm\ Triton} - A_{540\ nm\ saline}} \times 100
\]

where \( A_{540\ nm\ sample} \) represents the absorbance value of the sample, \( A_{540\ nm\ Triton} \) the absorbance value of the positive control, and \( A_{540\ nm\ saline} \) the absorbance value of the negative control.

The blood was obtained from the “Institute of Hemotherapy” in La Plata, Buenos Aires, Argentina as part of a formal agreement between the “Instituto de Genética Veterinaria (IGEVET, UNLP-CONICET La Plata)” and this institution. Also, this assay protocol was approved by the National University of La Plata Ethics Committee and it was developed in accordance with the principles proclaimed in the Universal Declaration of Human Rights of 1948, the ethical norms established by the Nuremberg Code of 1947, and the Declaration of Helsinki of 1964 and its successive amendments and clarifications. Special attention was paid to Patient Rights in their relationship with health professionals and institutions and the National Law 25326 on the Protection of Personal Data.

**Parasites**

The *T. cruzi* strain K98 (TcI, low virulence) was used. Tissue culture trypomastigotes were obtained from the supernatants of 2- to 3-day-old infected Vero cells (African green monkey kidney epithelial cells) maintained in RPMI-1640 medium supplemented with 10% FBS (Internegocios S.A. Argentina) at 37 °C in a 5% humidified CO₂ atmosphere. Amastigotes were obtained after infecting Vero cells at a multiplicity of infection (MOI) of 1:2.

**In vitro anti-trypomastigote effect**

A trypomastigote suspension (1 × 10⁵ trypomastigotes per well) was co-cultured in a 96 well-plate with dilutions of both a solution of the free drug and of the nanoparticle formulations (concentration range = 1, 2.5, 5, and 10 µM) in RPMI-1640 supplemented with 5% FBS at 37 °C in 5% CO₂ atmosphere. The NLC-VEHICLE sample was tested using the same dilutions as the NLC-BNZ formulation. After 24 h of incubation, motile parasites were counted in a hemocytometer chamber under a light microscope. Controls consisted of RPMI-1640 supplemented with 5% FBS as well as RPMI-1640 with 0.1% of DMSO.

Results were expressed as mean viability of trypomastigotes (%) (regarding to RPMI-1640 + DMSO control). Experiments were performed in triplicate. The half maximal effective concentration (EC₅₀) against the trypomastigote form was determined from concentration-response curves fitted through a non-linear regression on GraphPad Prism version 8.0.1 software (San Diego, CA, USA).

**In vitro anti-amastigote effect**

Vero cells were infected with the trypomastigote form of GFP-expressing *T. cruzi* (K98 strain) [57] at a multiplicity of infection (MOI) 1:2. After 24 h the cells were washed with PBS, trypsinized for 10 min, and seeded onto 96-well plates (5 × 10⁴ cells/well). After the cells attached to the microplate (i.e., 2–3 h), increasing concentrations of freshly prepared dilutions of the formulations (1, 5, 10, 25, and 50 µM) or the free drug were added. After 72 h of treatment, the cells were harvested with a trypsin/EDTA solution and processed for flow cytometry analysis using a BD Biosciences FACS CANTO II Flow Cytometer (Franklin Lakes, NJ, USA). Propidium iodide (Sigma, St. Louis, USA) was added to the cell suspensions and harvested with a trypsin/EDTA solution and processed for flow cytometry analysis using a BD Biosciences FACS CANTO II Flow Cytometer (Franklin Lakes, NJ, USA). Propidium iodide (Sigma, St. Louis, USA) was added to the cell suspensions.
(50 µg/mL) for 10 min, prior to analysis. In total, 20,000 events were acquired for each sample. Data analysis was performed using the FlowJoTM software (FlowJo, LCC). The EC50 values were determined from dose-response curves fitted through a non-linear regression using GraphPad Prism version 8.0.1 software (San Diego, CA, USA). The experiments were performed in duplicates.

Statistical analysis
The normality of the variable distribution was assessed using the Shapiro–Wilk normality test. Comparisons of the means were performed by analysis of variance (ANOVA) followed by Tukey or Dunnet comparison tests. Statistical significance was set at $p < 0.05$.

Supporting Information
This file includes a summary of the goodness-of-fit measures that indicate how different mathematical models of drug release fit our experimental data.

Supporting Information File 1
Supplementary material.
[https://www.beilstein-journals.org/bjnano/content/supplementary/2190-4286-14-66-S1.pdf]

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Authors
Giuliana Muraca1, María Esperanza Ruiz1, Rocío C. Gambaro2, Sebastián Scioli-Montoto3, María Laura Sbaragli1i, Gisel Padula2, José Sebastián Cisneros2, Cecilia Yamil Chain3, Vera Alvarez3, Cristián Huck-Iriart4, Guillermo Castro5, María Belén Piñero8, Matias Idelbrando Marchetto8, Catalina Alba Soto8, Germán A. Islan9* and Alan Talevi1*.

Addresses
1Laboratorio de Investigación y Desarrollo de Bioactivos (LIDeB), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina, 2Instituto de Genética Veterinaria (IGEVET, UNLP-CONICET La Plata), Facultad de Ciencias Veterinarias Universidad Nacional de La Plata (UNLP), La Plata, Argentina, 3Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (CONICET-UNLP), La Plata, Buenos Aires, Argentina, 4Grupo de Materiales Compuestos Termoplásticos (CoMP), Instituto de Investigaciones en Ciencia y Tecnología de Materiales (INTEMA), Facultad de Ingeniería, Universidad Nacional de Mar del Plata (UNMDP) – CONICET, Av. Colón 10850 (B7608FDQ), Mar del Plata, Buenos Aires, Argentina, 5Laboratorio de Cristalografía Aplicada, Escuela de Ciencia y Tecnología, CONICET, Universidad Nacional de San Martín (UNSAM), Campus Miguelete, 25 de Mayo y Francia, San Martín B1650KNA, Buenos Aires, Argentina, 6ALBA Synchrotron Light Source, Carrer de la Llum 2–26, Cerdanyola del Vallès, 08290 Barcelona, España, 7Nanomedicine Research Unit (Namomed), Federal University of ABC (UFABC), Santo André, SP, Brazil, 8Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPaM) - Consejo Nacional de Investigaciones Científicas y Técnicas - Universidad de Buenos Aires Departamento de Microbiología, Facultad de Medicina, Buenos Aires, Argentina and 9Centro de Investigación y Desarrollo en Fermentaciones Industriales (CINDEFI), Laboratorio de Nanobiomateriales, Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP)-CONICET (CCT La Plata), Calle 47 y 113, (B1900AJI), La Plata, Buenos Aires, Argentina.

ORCID® iDs
Giuliana Muraca - https://orcid.org/0000-0002-4785-8050
María Esperanza Ruiz - https://orcid.org/0000-0001-8617-667X
Sebastián Scioli-Montoto - https://orcid.org/0000-0003-3314-3406
María Laura Sbaragli - https://orcid.org/0000-0002-4671-2658
José Sebastián Cisneros - https://orcid.org/0000-0001-7563-978X
Cecilia Yamil Chain - https://orcid.org/0000-0002-8234-0556
Vera A. Álvarez - https://orcid.org/0000-0002-4909-4592
Guillermo R. Castro - https://orcid.org/0000-0002-6187-7805
María Belén Piñero - https://orcid.org/0000-0003-4431-368X
Catalina Alba Soto - https://orcid.org/0000-0002-2724-0421
Alan Talevi - https://orcid.org/0000-0003-3178-826X

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Green SPIONs as a novel highly selective treatment for leishmaniasis: an in vitro study against *Leishmania amazonensis* intracellular amastigotes

Brunno R. F. Verçoza¹, Robson R. Bernardo¹,², Luiz Augusto S. de Oliveira¹,² and Juliana C. F. Rodrigues*¹

**Abstract**

The main goal of this work was to evaluate the therapeutic potential of green superparamagnetic iron oxide nanoparticles (SPIONs) produced with coconut water for treating cutaneous leishmaniasis caused by *Leishmania amazonensis*. Optical and electron microscopy techniques were used to evaluate the effects on cell proliferation, infectivity percentage, and ultrastructure. SPIONs were internalized by both parasite stages, randomly distributed in the cytosol and located mainly in membrane-bound compartments. The selectivity index for intracellular amastigotes was more than 240 times higher compared to current drugs used to treat the disease. The synthesized SPIONs showed promising activity against *Leishmania* and can be considered a strong candidate for a new therapeutic approach for treating leishmaniases.

**Introduction**

Leishmaniasis is one of the most important neglected diseases of chronic nature and remains a serious global health problem. A worrying increase has been observed in the number of leishmaniasis cases worldwide in recent decades. It is estimated that about 600 million people live in risk areas, and 0.6–1.2 million new leishmaniasis cases appear annually [1]. The treatment for
this disease involves using pentavalent antimonials, miltefosine, amphotericin B, paromomycin, or pentamidine. However, side effects of these drugs and an increased number of drug-resistant parasites have been reported [2-5]. These facts demonstrate the need to develop new treatments or alternatives that are safer, more effective, and more accessible to patients.

In this context, nanomedicine is one of the most promising branches of contemporary medicine, currently concentrating a large part of the scientific effort on the search for new treatments for different diseases. Its main objective is to develop therapies with higher specificity, effectiveness, and safety, as well as less toxicity [6]. One interesting class of nanomaterials in medicine are superparamagnetic iron oxide nanoparticles (SPIONs). SPIONs exhibit theranostic properties, that is, they can be used simultaneously for diagnosis and therapy. Thus, SPIONs have emerged as one of the best options for the development of new therapeutic methods. SPIONs offer several features such as good biocompatibility, degradability under moderate acid conditions, the ability for magnetic manipulation, the possibility of being used in magnetic resonance imaging, and the ability to generate controlled heat non-invasively when exposed to an alternating magnetic field [7,8]. In 2019, our group published an article describing a low-cost green synthesis of SPIONs using coconut water [9]. In this article, the ability of macrophages to uptake these SPIONs was evaluated, together with some physical and chemical characterizations. The synthesized green SPIONs are around 4 nm in diameter, are composed of pure nonstoichiometric magnetite, exhibit superparamagnetic behavior at room temperature, and are taken up by macrophages without being toxic for these mammalian cells [9].

The application of SPIONs in treating leishmaniasis has been studied by different groups over the past few years, showing promising and satisfactory results [10-13]; thus, using SPIONs to develop new topical treatments can mean a revolution. SPIONs could be used for topical application, associated with drugs and combined or not with thermotherapy by magnetic hyperthermia. Furthermore, the treatment can be applied to the localized cutaneous lesion, making the treatment more specific and less toxic to the patient. Thus, the main goal of this study is to evaluate the effects of green SPIONs against *Leishmania amazonensis* (*L. amazonensis*) in vitro.

Results

**Uptake of SPIONs by *L. amazonensis***

*promastigotes and intracellular amastigotes*

Bright-field optical microscopy of *L. amazonensis* promastigotes and intracellular amastigotes incubated with Prussian blue revealed that both parasite stages can uptake the SPIONs (Figure 1). The arrows and arrowheads in Figure 1 show the characteristic blue stain that indicates the positive reaction between potassium ferrocyanide and ferrous compounds. In promastigotes (Figure 1A,B), the SPIONS are distributed throughout the cytosol. In contrast, in the intracellular amastigotes cultivated in macrophages, the SPIONs appear in the mammalian cytosol, inside the parasitophorous vacuole, and in the parasite cytosol (Figure 1C,D).

After the first microscopic analysis, scanning electron microscopy and chemical element mapping analysis were carried out to confirm the uptake of the SPIONs by *L. amazonensis* intracellular amastigotes after removing the plasma membrane to expose the cytoplasmic environment (Figure 2). Secondary electron imaging revealed intracellular amastigotes inside the parasitophorous vacuoles (Figure 2A). Backscattered electron imaging showed several small electron-lucent structures randomly distributed throughout the macrophage cytosol, inside the parasitophorous vacuoles (Figure 2B, arrows), and in the intracellular amastigotes (Figure 2B, arrowheads). The ferrous nature of the observed structures was assessed by chemical element mapping analysis using energy-dispersive X-ray spectroscopy (Figure 2C), confirming that the electron-lucent structures contain iron atoms (Figure 2D).

Transmission electron microscopy (TEM) was used to confirm the internalization of the SPIONs. First, promastigotes were treated with 100 µg/mL of SPIONs for 24 h (Figure 3A–C). TEM images confirmed the presence of SPION aggregates randomly distributed throughout the cytoplasm of the promastigotes (Figure 3A–C, arrowheads). The images suggest that these aggregates have different sizes. Furthermore, at high magnification, it is possible to observe that the SPIONS are frequently surrounded by membranes (Figure 3B, arrows). In addition, SPIONs were also observed inside the flagellar pocket (Figure 3C, arrowheads) and closely associated with the membrane.

The uptake of SPIONs was also observed in macrophages infected with *L. amazonensis* intracellular amastigotes after treatment with 100 µg/mL of SPIONs for 24 h (Figure 3D–F). The images confirmed the presence of SPION aggregates inside the macrophage cytosol, the parasitophorous vacuoles, and the intracellular amastigotes (Figure 3C,D, arrowheads). SPIONs were also observed inside the macrophages close to the parasitophorous vacuole membrane (Figure 3D, large arrow), sometimes appearing inside membrane-bound structures and exhibiting different sizes (Figure 3E, arrowheads). Some alterations in amastigote ultrastructure can also be observed, namely electron-lucent lipid bodies, a multivesicular body close to the Golgi

**Notes and intracellular amastigotes incubated with Prussian blue**
complex, and endoplasmic reticulum profiles very close to organelles such as mitochondrion and glycosome. Higher magnification revealed that the SPION aggregates are constituted of small nanoparticles that appear associated with tiny filaments (Figure 3F, thin arrow).

Antiproliferative effects of SPIONs in L. amazonensis promastigotes and intracellular amastigotes

The analysis of the antiproliferative effects of SPIONs in L. amazonensis promastigotes showed that they could not alter the growth for any of the concentrations evaluated (Figure 4A). In contrast, the SPIONs were very active against intracellular amastigotes (Figure 4B). Furthermore, analysis of the growth curve shows a statistically significant reduction in the percentage of infection for all tested concentrations of SPIONs (1, 5, 10, 25, and 50 µg/mL) and treatment times (24, 48, and 72 h) when compared with the control of infected macrophages.

After the first 24 h of treatment, it was possible to observe a reduction in the percentage of infection of about 50% for a concentration of 1 µg/mL and of about 90% for 50 µg/mL of SPIONs. The data revealed a concentration-dependent effect, which increased within 48 and 72 h of treatment. The percentage of infection significantly reduces over time, indicating a time-dependent effect. The IC$_{50}$ values were calculated for each treatment time and confirmed the results obtained (Figure 4B), that is, 1.206, 0.848, and 0.668 µg/mL for treatment times of 24, 48, and 72 h, respectively.

Evaluation of possible effects on the ultrastructure of L. amazonensis intracellular amastigotes

Transmission electron microscopy allowed us to analyze ultrastructural alterations induced by treating L. amazonensis intracellular amastigotes with 100 µg/mL of SPIONs for 24 h (Figure 5). The images revealed several alterations, namely
Figure 2: Scanning electron microscopy of macrophages infected with L. amazonensis intracellular amastigotes after treatment with 100 µg/mL SPIONs for 24 h. The plasma membrane was gently removed to observe the presence of nanoparticles inside the cells. Panel A shows infected macrophages with some amastigotes (arrowheads) inside the parasitophorous vacuoles (thin arrows). Panel B shows the same macrophage; however, the image was obtained by detecting backscattered electrons, revealing several electron-lucent aggregates (arrows). Digital magnification (highlighted rectangular area) showed electron-lucent aggregates even inside intracellular amastigotes (arrowheads). Panels C and D show the X-ray microanalysis mapping of infected macrophages, indicating the presence of iron in the cytosol (red color in Figure 2D).

Discussion
SPIONs represent a new approach to diagnosing and treating diseases, particularly when associated with magnetic hyperthermia, an emerging form of active treatment [14-18]. However, despite all their potential, the synthesis processes of the SPIONs are characterized by being expensive and toxic to humans and the environment [6]. In this scenario, our group demonstrated the therapeutic potential of low-cost biocompatible SPIONs produced by green synthesis [9]. The present study
Figure 3: Transmission electron microscopy of L. amazonensis promastigotes and intracellular amastigotes treated with 100 µg/mL of SPIONs for 24 h. Electron-dense aggregates of SPIONs (arrowheads) are randomly distributed in both developmental stages. (A) SPIONs (arrowheads) were observed in the promastigote cytosol, closely associated with endoplasmic reticulum profiles and lipid bodies. (B) High-magnification image with SPION aggregates (arrowheads) inside membrane-bound compartments (arrows). (C) SPIONs (arrowheads) are associated with thin filaments inside the flagellar pocket and in the cytosol closely associated with the flagellar pocket membrane. (D) In the macrophages infected with intracellular amastigotes, the SPIONs appear inside the parasitophorous vacuole and in the macrophage and parasite cytosol (arrowheads). In this image, it is also possible to observe the SPIONs surrounded by a membrane (arrows) and an aggregate close to the membrane of the parasitophorous vacuole (large arrow). (E, F) High-magnification images of intracellular amastigotes revealing SPIONs (arrowheads) inside membrane-bound compartments (arrows). The aggregates are formed by smaller individual nanoparticles (small arrow). Figure 3E also shows many lipid bodies, vacuoles, and a multivesicular structure, which are features typically found in treated parasites. F, flagellum; FP, flagellar pocket; LB, lipid body; M, mitochondrion; N, nucleus; and PV, parasitophorous vacuole.
aimed to evaluate in vitro the therapeutic potential of SPIONs produced with coconut water to treat cutaneous leishmaniasis caused by *L. amazonensis*.

Microscopy techniques efficiently revealed the uptake and distribution of SPIONs in *L. amazonensis* promastigotes and intracellular amastigotes. The first analysis confirmed the uptake of SPIONs by macrophages, which was published previously by our group [9]. Furthermore, in the article here, the images revealed SPIONs inside the parasitophorous vacuole and in the cytosol of intracellular amastigotes. In addition, SPIONs were also observed randomly distributed throughout the cytosol of promastigotes, in the flagellar pocket, and inside membrane-bound structures. It is the first time that superparamagnetic iron oxide nanoparticles SPIONs are observed inside the *Leishmania* spp and the parasitophorous vacuole. Chemical element mapping analysis by scanning electron microscopy confirmed the ferrous nature of the nanoparticle aggregates. These results prove the ability of both promastigotes and intracellular amastigotes to uptake SPIONs from the culture medium.

The acquisition of iron by *Leishmania* intracellular amastigotes that live inside mammalian host cells is important for cell differentiation and the pathogenesis of the disease [19-21]. Thus, it is possible to speculate that SPIONs use iron transport mechanisms to reach the parasitophorous vacuole and amastigote

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**Figure 4:** Analysis of the antiproliferative effect in *L. amazonensis* promastigotes and intracellular amastigotes treated with different concentrations of SPIONs. (A) Growth curve of *L. amazonensis* promastigotes; the SPIONs were added to the culture medium after 24 h of growth. (B) For intracellular amastigotes, infected macrophages were treated, and the percentage of infection was obtained for each treatment condition; the SPIONs were added to the infected macrophage culture after 24 h of infection. *P* values for panel B: **** *p* < 0.0001.

<table>
<thead>
<tr>
<th>Time</th>
<th>IC50 (µg/mL)</th>
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<tr>
<td>24 h</td>
<td>1.206 µg/mL</td>
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<tr>
<td>48 h</td>
<td>0.848 µg/mL</td>
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<tr>
<td>72 h</td>
<td>0.668 µg/mL</td>
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Figure 5: Transmission electron microscopy of *L. amazonensis* intracellular amastigotes treated with 100 µg/mL of SPIONs for 24 h. Different ultrastructural changes were observed in intracellular amastigotes: (1) many lipid bodies (A–C, thin arrows), (2) increased secretion of extracellular vesicles (A–C, broad arrows), (3) intracellular vacuolization (A–C, arrows), (4) myelin-like figures (A, arrowhead), (5) mitochondrial swelling (C, star), and (6) destroyed amastigotes (D, triangle). F, flagellum; k, kinetoplast; LB, lipid body; M, mitochondrion; and N, nucleus.

cytosol [21]. However, further studies need to be carried out to confirm this hypothesis and to elucidate the mechanisms of SPION uptake in promastigotes and amastigotes.

We evaluated the antiproliferative effects of SPIONs in *L. amazonensis* promastigotes and intracellular amastigotes. Despite being internalized by promastigotes, SPIONs did not affect the cell proliferation of the parasites (Figure 4A). A completely different result was observed for intracellular amastigotes, where the reduction in the percentage of infection was very significant already with the lowest concentration of SPIONs used [1 µg/mL] (Figure 4B). The IC<sub>50</sub> values found for intracellular amastigotes during the treatment were 1.206, 0.848, and 0.668 µg/mL for treatment times of 24, 48 and 72 h, respectively. In a previous study published by our group, we analyzed the cytotoxicity of SPIONs against macrophages [9]. The results revealed no toxic effects up to a concentration of 300 µg/mL, indicating that SPIONs are well tolerated by
macrophages. Because $CC_{50}$ values are difficult to calculate, we used GraphPad Prism software to estimate them. $CC_{50}$ values are essential to calculate the selective index (SI), and both quantities are important to understand how effective the nanoparticles are against the parasite while being less toxic for mammalian cells (Table 1).

The SI revealed that the SPIONs were highly selective for \textit{L. amazonensis} intracellular amastigotes (Table 1), presenting values significantly higher when compared with other compounds and drugs used to treat \textit{Leishmania sp}. (Table 2) [22-27]. These data indicate a high selectivity index for SPIONs compared with current treatments, different from most compounds, drugs, and nanomaterials developed in the last decades.

During TEM analyses, we observed that intracellular amastigotes were undergoing substantial ultrastructural alterations (Figure 5) when treated with SPIONs. These alterations include (1) accumulation of lipid bodies, (2) intense intracellular vacuolization, (3) mitochondrial swelling, (4) myelin-like figures, and (5) cell death. The observed ultrastructural effects corroborate the significant antiproliferative effect found and give indications of the possible mechanisms of action of these nanoparticles, which may be closely associated with intracellular iron homeostasis.

Iron homeostasis has been extensively studied because of its essential role in maintaining the cellular functions of several cell types. It is well established that, in mammalian cells, iron in its free state can participate in the Haber–Weiss reaction, catalyzing the formation of highly reactive hydroxyl radicals that lead to oxidative stress [28,29]. Thus, one of the possibilities for the observed antiproliferative effects could be the result of an imbalance in iron homeostasis with the consequent induction of oxidative stress and death of the parasites. However, further studies need to be carried out to confirm this hypothesis.

In \textit{Leishmania}, it is well known that available iron has an important influence on the homeostasis of reactive oxygen species [30]. Studies have already shown that iron excess in the diet of mice causes a decrease in the replication of \textit{Leishmania} spp. in different tissues of infected animals due to the interaction with reactive oxygen and nitrogen species [31,32].

Several studies have shown the potential of using nanoparticles as a new method for treating leishmaniasis. However, only a few studies report the effects of using iron oxide nanoparticles [11,12,15,33-35]. Recently, the effects of magnetic iron oxide nanoparticles were demonstrated in \textit{L. mexicana} axenic amastigotes. First, the amastigotes were treated with 200 µg/mL of magnetic nanoparticles. Subsequently, magnetic hyperthermia was applied using an alternating field of 30 mT with a frequency of 452 kHz for 40 min. The results showed that magnetic hyperthermia was efficient in killing \textit{L. mexicana} axenic amastigotes [12]. Another study demonstrated the anti-\textit{Leishmania} effect of magnetic nanoparticles synthesized by green chemistry in \textit{L. major} promastigotes [35]. Finally, a study showed the effect in vitro and in vivo of amphotericin B encapsulated in magnetic iron oxide nanoparticles coated with glycine-rich peptides for treating visceral leishmaniasis caused by \textit{L. donovani} [12]. All these studies demonstrated the potential gain of drug conjugation with magnetic nanoparticles for treating leishmaniasis.

<table>
<thead>
<tr>
<th>Time</th>
<th>Estimated cytotoxic concentration of 50% ($CC_{50}$) for macrophages</th>
<th>Estimated selective index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>1271.5 µg/mL</td>
<td>1054</td>
</tr>
<tr>
<td>48 h</td>
<td>2250.6 µg/mL</td>
<td>2654</td>
</tr>
<tr>
<td>72 h</td>
<td>3420.0 µg/mL</td>
<td>5119</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Compound</th>
<th>SI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>amphotericin B</td>
<td>16</td>
<td>[26]</td>
</tr>
<tr>
<td>48 h</td>
<td>TC95</td>
<td>24</td>
<td>[23]</td>
</tr>
<tr>
<td>48 h</td>
<td>KH-TFMDI</td>
<td>81</td>
<td>[22]</td>
</tr>
<tr>
<td>72 h</td>
<td>itraconazole</td>
<td>103.17</td>
<td>[25]</td>
</tr>
<tr>
<td>72 h</td>
<td>ravuconazole</td>
<td>28.9</td>
<td>[24]</td>
</tr>
<tr>
<td>72 h</td>
<td>miltefosine</td>
<td>34.2</td>
<td>[27]</td>
</tr>
</tbody>
</table>
Conclusion

The use of SPIONs synthesized with coconut water to treat macrophages infected with *Leishmania amazonensis* intracellular amastigotes revealed a significant anti-*Leishmania* effect with a selectivity index more than 240 times higher than those of other currently used drugs. Furthermore, it was also observed that the SPIONs could be directed into the parasitophorous vacuoles of infected cells and parasites. Thus, this new nanomaterial is a promising new therapeutic alternative as (1) an active treatment agent because of its intrinsic properties, (2) a treatment agent associated with heating through alternating current magnetic fields, and (3) a drug carrier.

Finally, SPIONs can be considered a strong candidate for a new therapeutic approach to treating cutaneous leishmaniasis, that is, an accessible and low-cost topical treatment.

Experimental SPIONs

The SPIONs used in the present study were synthesized as described in [9] (patent application registration BR 10 2020 015814 [36]). For assays, after synthesis and purification, the SPIONs were dispersed in a 70% ethanol solution (Merck, Germany). The maximum ethanol concentration in cultures did not exceed 0.5%, which did not interfere with cell growth. The nanoparticles used in the biological tests were stored at −20 °C.

Ethics committee for the use of laboratory animals

The assays that used mammalian macrophages and parasites from animal models were approved by the Ethics Committee for the Use of Laboratory Animals (CEUA) of the Centro de Ciências da Saúde from the Universidade Federal do Rio de Janeiro according to the Brazilian Federal Law (11794/2008, Decreto No. 6,899/2009). For the use of peritoneal macrophages resident in mice and the maintenance of *Leishmania amazonensis* species in Balb/C mice, the protocol number was UFRJ/CCS-142/21. Furthermore, all animals received human care according to the guide published by the Brazilian Society of Zootechnics of Laboratory and Council National Control of Animal Experimentation.

Cell culture

The immortalized murine macrophages RAW 264.7 were grown in 25 cm² bottles in RPMI 1640 medium (Cultilab, Brasil) supplemented with 2% sodium bicarbonate, 10% fetal bovine serum, and 100 U/mL penicillin. Cells were cultured at 37 °C in 5% CO₂ atmosphere, and the medium was changed three times a week; cells were passed when they reached confluence in the bottles. In addition, primary cultures of murine macrophages were obtained from the peritoneal cavity of CF1 mice by washing with Hanks’ balanced solution. Then, they were plated on coverslips in a 24-well culture plate and placed to adhere for 24 h at 37 °C in an atmosphere of 5% CO₂. For the microscopic analyses, macrophages were grown in 25 cm² bottles or on glass coverslips in 24-well plates; after 24 h of culture, they were treated for 24 h with different SPION concentrations. This study used the WHOM/BR/75/JOSEFA *Leishmania amazonensis* strain as a standard model for cutaneous leishmaniasis. The parasites were maintained according to previously published protocols [22].

Prussian blue staining

For staining with Prussian blue (Sigma-Aldrich, Germany), promastigote and intracellular amastigotes were treated with 100 µg/mL of SPIONs for 24 h. The promastigotes (control and treated cells) were washed in phosphate-buffered saline (PBS) pH 7.2 and adhered for 10 min on glass coverslips previously coated with poly-L-lysine (Sigma-Aldrich, Germany). The intracellular amastigotes were obtained after infection of RAW 264.7 macrophages at a ratio of ten parasites to one macrophage. After treatment, cells were washed in PBS pH 7.2, fixed, and dehydrated, as described in [9]. Finally, cells were observed using a DM2500 optical microscope (Leica Microsystems, Germany) in bright-field mode.

Electron microscopy analysis

Control and treated cells were washed in PBS pH 7.2, fixed, and post-fixed according to previously published protocols [23]. Then, cells were processed for scanning electron microscopy and chemical element mapping analysis as described in [9]. The micrographs were obtained using a TESCAN VEGA 3 LMU scanning electron microscope operating at 20 kV equipped with an OXFORD X-MaxN 20 mm² detector (Oxford Instruments, United Kingdom) for energy-dispersive X-ray spectroscopy. For transmission electron microscopy, after fixation, samples were dehydrated in increasing acetone concentrations and embedded in Epon. Ultrathin sections were obtained using a PT-PC PowerTome ultramicrotome (RMC Boeckeler, USA) stained with uranyl acetate and lead citrate and observed using a FEI TECNAI SPIRIT transmission electron microscope operating at 120 kV.

Antiproliferative effects of SPIONs in *Leishmania amazonensis* promastigotes and intracellular amastigotes

To evaluate the effect of the SPIONs on the growth of *L. amazonensis* promastigotes, cell density experiments were initiated with an inoculum of 1.0 × 10⁶ parasites/mL in M199 culture medium supplemented with 10% fetal bovine serum and cultivated at 25 °C. After 24 h of growth, different concentra-
tions of SPIONS (1, 5, 10, 50, and 100 µg/mL) were added, and cells were cultured for 96 h. The cell density was calculated every 24 h by counting the number of cells in a Neubauer chamber using contrast-phase light microscopy. Besides, SPIONS were also evaluated against intracellular amastigotes, the clinically relevant stage of leishmaniasis. For this analysis, murine macrophages and parasites were obtained as previously published [23]. After 24 h of the initial infection, different concentrations of SPIONS (1, 5, 10, 25, and 50 µg/mL) were added, and the medium with the nanoparticles was changed every day for three days. The IC₅₀ was calculated using the linear regression method defined in [37].

Statistical analysis
Statistical analysis was conducted using GraphPad Prism with one-way analysis of variance (ANOVA). The results were considered statistically significant for cases of p ≤ 0.05 (*).

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ORCID® iDs
Brunno R. F. Verçoza - https://orcid.org/0000-0001-6117-7879
Robson R. Bernardo - https://orcid.org/0000-0002-0035-5149
Luiz Augusto S. de Oliveira - https://orcid.org/0000-0002-4034-8365
Julkany C. F. Rodrigues - https://orcid.org/0000-0001-6887-8101

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