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Improvement of in vitro efficacy of a novel schistosomicidal drug by incorporation into nanoemulsions

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Abstract

The aim of this article included the development and evaluation of the capacity of nanoemulsions to improve the activity of the novel schistosomicidal drug—2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid (BphEA). BphEA is a compound with a poor solubility in water, which makes its application as a drug difficult. Nanoemulsion formulations presenting anionic (NANOSTOA, NANOST and NANOLP) and cationic (NANOSTE) interfacial charges were prepared to encapsulate BphEA. These formulations were characterized by the encapsulation rate, diameter, and zeta potential. NANOSTOA, NANOST, and NANOLP presented an entrapment efficiency and zeta potential of $18.7 \pm 1.8\%$ and -33.6 ± 1.2 mV; $20.5 \pm 3.0\%$ and -31.5 ± 5.7 mV; as well as $33.8 \pm 7.2\%$ and -62.6 ± 1.3 mV, respectively. NANOSTE presented an entrapment efficiency of $51.8 \pm 5.0\%$ and a zeta potential of 25.7 ± 3.9 mV. The mean droplet size (between 200 and 252 nm) and polydispersity index (between 0.158 and 0.294) were similar for all formulations. The stability study showed no alteration in these formulations' zeta potential and size. The in vitro schistosomicidal activity of BphEA was higher with the use of NANOSTE than with free BphEA. In addition, release studies revealed a good stability of NANOSTE containing BphEA in a biological medium. These results indicate that cationic nanoemulsions can represent an interesting delivery system for the pharmaceutical formulation of BphEA.

Keywords: Schistosomiasis; 2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid; Nanoemulsions

1. Introduction

Schistosomiasis is a common intravascular trematode infection that represents a serious health problem in tropical countries. This disease still remains as one of the most prevalent parasitic infections and presents significant economic and public health consequences. It is estimated that 200 million people are infected by schistosome worms and more than 600 million people are at risk (Engels et al., 2002; Blanchard, 2004). The only drugs currently available for the treatment of schistosomiasis mansoni are oxamniquine and praziquantel. However, the development of resistance to oxaminiquine has been reported, and its production has decreased due to a lack of demand (Cioli et al., 1993; Fallon and Doenhoff, 1994; Coelho et al., 1997; Sabadini and Dias, 2002; Melo et al., 2003). Resistance to praziquantel, which can lead to failures in treatment, has also been reported in the literature (Fallon and Doenhoff, 1994; Ismail et al., 1999; Liang et al., 2001). Another important limitation of these drugs is their low effectiveness in the treatment of acute toxemic schistosomiasis due to their low activity against immature *Schistosoma mansoni* (Frézard and Melo, 1997). In this context, studies related to the synthesis and improvement of new drugs against schistosomiasis are of great importance. Aminoalkanethiosulfuric acids showed positive results as schistosomicidal agents (Penido et al., 1994; Moreira, 2003). However, the limitation to their use

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as a pharmaceutical product is their poor solubility in water. A strategy for overcoming this inconvenience is the incorporation into drug delivery systems. Nanoemulsions are fine oil-in-water dispersions, with droplets in the 100–600 nm range, designed for transporting oil-soluble materials which can be dissolved in the oil phase of the emulsion or amphiphilic drugs which can be adsorbed at the oil–water interface of the emulsion (Klang et al., 1998; Bouchemal et al., 2004). Moreover, after intravenous administration, the nanoemulsions are taken up by reticular endothelial system organs, such as the liver, where the schistosomes are located. This drug-targeted delivery can contribute to the decrease or elimination of toxicity. In addition, the scaling-up and quality control of these delivery systems is uncomplicated (Sarker, 2005). Thus, one of these derivatives, the 2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid (BphEA), was synthesized as part of the present work and incorporated into anionic and cationic nanoemulsions. Nanoemulsions of different compositions were characterized by the entrapment rate, particle size, zeta potential, and storage stability. The in vitro schistosomicidal activity of the nanoemulsion containing BphEA was also evaluated in comparison with that of free BphEA.

2. Materials and methods

2.1. Materials

Lipoid-S75® (lecithin) and medium chain triglycerides (MCT) were supplied by Lipoid GmbH (Ludwigshafen, Germany). Poloxamer 188® was purchased from BASF (Ludwigshafen, Germany). Tween 80® (polysorbate 80), Span 80® (sorbitan monooleate), ethanol, and oleic acid were obtained from Synth (Diadema, Brazil). Serotonin, stearylamine, penicillin (10000 UI/mL)/streptomycin (10 mg/mL), and Roswell Park Memorial Institute (RPMI)-1640 culture medium were purchased from Sigma Chemical Company (St. Louis, MO, USA). Methanol and acetonitrile were obtained from Fisher Scientific (New Jersey, USA). All solvents used were of analytical grade.

2.2. Synthesis of 2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid (5)

The synthesis of 2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid (**5**, BphEA) was performed as described by Moreira et al. (2000) and is summarized in Fig. 1.

For the synthesis of the first intermediate, 2-(butylamino)-1-phenyl-1-ethanol (**2**), a mixture of 41 mL (0.34 mol) of styrene oxide, 7 mL (0.17 mol) of methanol, and 100 mL (1.02 mol) of butylamine were stirred in a Parr reactor for 4 h at 130 °C. The reactor was cooled to room temperature, and the methanol and excess amine were removed on a rotary evaporator. The residue was recrystallized from petroleum ether. The *N*-butyl-2-hydroxy-2-phenyl-1-ethanaminium bromide intermediate (**3**) was obtained when 40 mL of concentrated hydrobromic acid was added over a 1-h period to a solution of 37.6 g (0.19 mol) of **2** in 100 mL of ethanol with stirring at ice bath temperature and maintained under agitation. The ethanol and excess hydrobromic acid were removed on a rotary evaporator, and the crude product was dried under vacuum. The residue **3** was recrystallized from a 1:1 mixture of ethyl acetate and chloroform. In the synthesis of the *N*-butyl-2-bromo-2-phenyl-1-ethanaminium bromide (**4**), 76 g (0.28 mol) of redistilled PBr_3 was slowly added to a stirred mixture of 52.1 g (0.19 mol) of β-aminoalcohol hydrobromide (**3**) and 50 mL of dry benzene. The system was heated under reflux for 4 h. The mixture was allowed to stand for 24 h, ethanol was added to decompose the excess PBr_3, and the benzene and excess ethanol were removed on a rotary evaporator. The residue was washed with ethyl ether and recrystallized from a 1:1 mixture of ethyl acetate and chloroform. Finally, for the synthesis of **5**, a solution of 31.1 g (0.092 mol) of **4** in 50% ethanol was stirred with an equimolar quantity of a concentrated aqueous solution of sodium thiosulfate for 24 h at room temperature and then heated under reflux for 1 h. The mixture was filtered, and the resulting white solid was recrystallized from methanol. The final product was characterized by melting point determination (Thomas Hoover Uni-Melt melting point apparatus), infrared (Shimadzu IR 408 spectrophotometer using KBr disks) and ^{1}H NMR spectroscopy (Bruker *Avance*

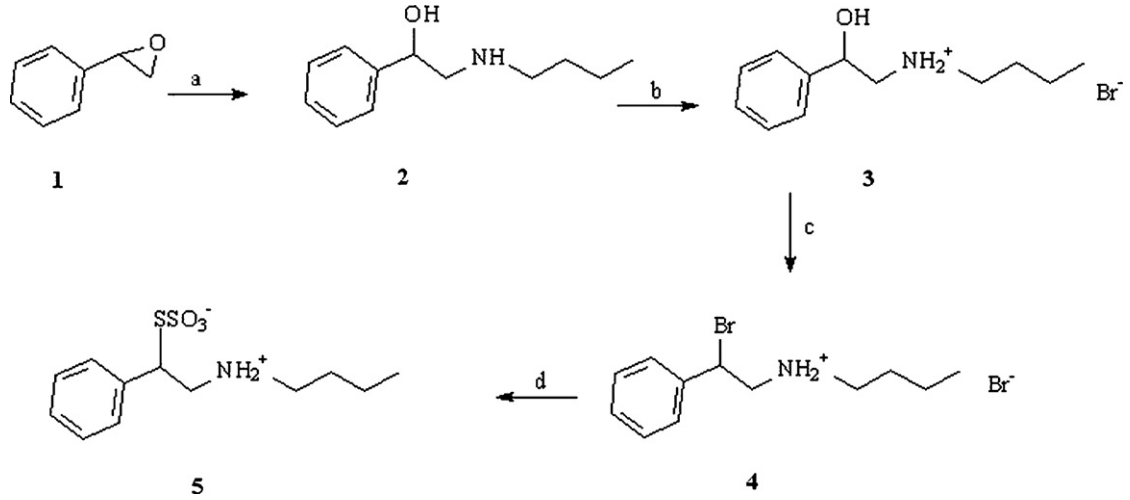

Fig. 1. Synthetic pathway for 2-(butylamino)-1-phenyl-1-ethanethiosulfuric acid (**5**): (a) butylamine/MeOH; (b) HBr; (c) PBr_3; (d) $Na_2S_2O_3$.

DPX 200 spectrometer). Chemical shifts are reported in parts per million (δ units). Coupling constants (J) are reported in Hz. Yield = 13.31 g (49.9%); 174–176 °C; IR (KBr): ν_{max} 3600, 2900, 2600, 1600, 1450, 1222, 1180, 1022, 750, 690 cm^{-1}; ^1H NMR (DMSO-$d6$, 200 MHz): δ 0.87 (t, J = 7.2 Hz, 3 H, CH_3), 1.1–1.4 (m, 2 H, $CH_2CH_2CH_3$), 1.4–1.6 (m, 2 H, CH_2CH_2Et), 2.92 (t, J = 7.4 Hz, 2 H, $^+NH_2CH_2C_3H_7$), 3.55 (dd, J = 5.7 and 12.8 Hz, 1 H, $CH_aH_bNH_2^+$), 3.76 (dd, J = 8.1 and 12.8 Hz, 1 H, $CH_aH_bNH_2^+$), 4.63 (t, J = 7.4 Hz, 1 H, $PhCHSSO_3^-$), 7.40 (s, 3 H, aromatic CH), 8.3 (broad, 2H, NH_2^+).

2.3. Preparation of nanoemulsions

2.3.1. Spontaneous emulsification method

The procedure and the oil and aqueous phase compositions were defined as described by Silva et al. (2006). The concentrations of Poloxamer 188®, Lipoid-S75® and stearylamine were also employed as cited by Silva et al. (2006). The concentration of 0.5% oleic acid was chosen to guarantee its interaction with BphEA. The oleic acid:BphEA stoichiometric ratio was equal to 5. Finally, the concentrations of Span 80® and Tween 80® were determined according to the required HLB of the MCT-based nanoemulsions (HLB = 11.0). These values are in agreement with the studies developed by Bouchemal et al. (2004).

An ethanolic solution (30 mL) composed of 10% (w/w) of MCT, 1.88% (w/w) of Span 80® or 2.0% (w/w) of Lipoid-S75® and 10 mg of BphEA was prepared. The homogeneous aqueous phase was composed of water (60 mL), 2.25% (w/w) of glycerol, 1.68% (w/w) of Poloxamer 188® or 3.3% (w/w) of Tween 80®, as described in Table 1. The organic phase was added to the aqueous phase under magnetic stirring at 150 rpm during 30 min at 25 °C. The oil/water emulsion was formed instantaneously by diffusion of the organic solvent into the external phase. The preparation was evaporated under reduced pressure to a final volume of 10 mL. Size reduction of all formulations was achieved using the tapered microtip ultrasound probe 1/8, with a 3 mm diameter (Ultrasonic Processor Model VC505, Sonics and Materials, Inc., Newtown, USA). The sonication was performed in an ice bath during 2 min. This length of time for sonication was sufficient to obtain small and homogeneous particles. This step was employed for all formulations. However, this procedure was not necessary for the NANOLP. This formulation presented a droplet size of approximately 200 nm and a polydispersity index of 0.158 as soon as it was prepared. A formulation study was performed where the percentage of MCT was fixed in all preparations, and different combinations and concentrations of surfactants were tested as described in Table 1.

Table 1
Composition of formulations of nanoemulsions containing BphEA[a]

	NANOLP	NANOST	NANOSTE	NANOSTOA
Lipoid S-75®	2.00	–	–	–
MCT	10.00	10.00	10.00	10.00
Span 80®	–	1.88	1.88	1.88
Stearylamine	–	–	0.30	–
Oleic acid	–	–	–	0.50
Tween 80®	–	3.3	3.3	3.3
Poloxamer 188®	1.68	–	–	–
Glyceiol	2.25	2.25	2.25	2.25
Water	100.00	100.00	100.00	100.00

[a] The concentrations of the constituents in each formulation are expressed in % (w/w).

2.4. Nanoemulsion characterization

The nanoemulsions were characterized by their percentage of encapsulation, size, and zeta potential. All experiments were performed in triplicate and were expressed as mean values ± S.D.

2.4.1. Encapsulation levels

The evaluation of the entrapment percentage for nanoemulsions was performed as described by Michalowski et al. (2004). The amount of free BphEA (unassociated with the nanostructures) was determined in the ultrafiltrate solution after separation of the nanoparticles using the ultrafiltration/centrifugation technique (Ultrafree-MC, 10,000 MW, Millipore). A 400-μL aliquot of the nanoemulsion sample was added to the Ultrafree-MC device and centrifuged for 20 min at 1800 × g. The percentage of encapsulation of BphEA into nanoemulsions was determined by high performance liquid chromatography (HPLC). The total concentration of BphEA was measured by HPLC after dissolution of the colloidal dispersions in methanol. The concentration of BphEA associated with nanostructures was calculated from the difference between the total concentration and the free drug concentration measured in the ultrafiltrate. The chromatographic apparatus consisted of a Model 515 pump (Waters Instruments, Milford, MA, USA), a Model 717 Plus auto-injector (Waters Instruments, Milford, MA, USA), and a Model 2487 variable wavelength UV detector (Waters Instruments, Milford, MA, USA) controlled by Millenium® software. Separations were performed using a 4 mm × 4 mm, 5 μm Lichrospher® 100 CN guard column (Merck, Darmstadt, Germany) connected to a 25 cm × 4 mm, 5 μm Lichrosorb® RP-18 column (Merck, Darmstadt, Germany). The eluent system was composed of a 1:1 methanol/water mixture, and the flow rate was 1.0 mL min^{-1}. Samples (20 μL) were injected, and the absorbance of the eluate was monitored at 264 nm. A linear response was obtained in the concentration range evaluated (20–60 μg/mL), with a correlation coefficient greater than 0.999 and a linear equation, $y = 1140289.495x + 5182.426$.

2.4.2. Measurements of size and zeta potential

The mean diameter of the nanoemulsions containing BphEA was determined by quasi-elastic light scattering at 25 °C and at an angle of 90°, using the unimodal analysis. The zeta potential was evaluated by determining the electrophoretic mobility at an angle of 90°. The measurements were performed using the 3000HS Zetasizer (Malvern Instruments, England). The samples were diluted with 1.0 mM NaCl solution.

2.5. Stability study

The determination of the storage stability of nanoemulsions containing BphEA was performed at 30 and 60 days after their preparation. These formulations were maintained at 4 °C. The parameters evaluated were mean diameter and zeta potential. The mean values of these parameters were compared with that obtained at time zero. Data were subjected to statistical analysis employing one-way analysis of variance (ANOVA), followed by the Dunnett test, and *P*-values less than 0.05 were regarded as significant (Graphpad Prism 3.0, Graphpad Software Inc., San Diego, CA, USA). This experiment was performed in triplicate and the results were expressed as mean values ± S.D.

2.6. Release study

The cationic nanoemulsion containing BphEA (NANOSTE) was selected for the release study as it presented the highest entrapment level. The release of BphEA associated with a cationic nanoemulsion was evaluated in RPMI-1640 medium, pH 7.4. The nanoemulsion sample was diluted to a final concentration of 6.25 μg/mL of BphEA in RPMI-1640 medium. The experiment was performed at 37 °C with moderate magnetic stirring. The samples ($n = 3$) were collected at intervals of 10 and 30 min as well as at 1, 4, 8, 24, and 48 h. The samples were submitted to ultrafiltration/centrifugation and analyzed by HPLC. A concentration interval lower than that validated in Section 2.4.1 was applied in these analyses. Thus, it became necessary to obtain another calibration curve. A linear response was obtained in the concentration range evaluated (5–15 μg/mL), with a correlation coefficient higher than 0.99 and a linear equation, $y = 1171.97x - 762.96$.

2.7. In vitro schistosomicidal activity

The in vitro studies were performed as described by Oliveira et al. (2006). The adult *S. mansoni* worms (LE strain) were recovered by perfusion of the hepatic portal system of infected female SWISS mice 6 weeks after inoculation. Three pairs of worms were washed three times with an RPMI-1640 medium and then transferred to six-well plates. NANOSTE, a methanolic solution of free BphEA (BphEA-L), and a cationic nanoemulsion without BphEA (NANOSTEb) were diluted 160-fold in 4 mL of an RPMI-1640 medium containing penicillin (100 UI/mL)/streptomycin (100 μg/mL) and supplemented with 5% (v/v) heat-inactivated fetal calf serum. The final concentration of BphEA was equal to 6.25 μg/mL. In the control groups, the worms were maintained in the medium alone or in the medium containing methanol at a concentration equal to that of the BphEA-L sample. The 160-fold dilution was chosen because the cationic nanoemulsion without BphEA presented no schistosomicidal activity at this concentration. The worms were incubated at 37 °C under a humidified 5% CO_2 atmosphere. Some general aspects of the worms, such as loss of motor activity, morphological alterations in the tegument, and death, were monitored. These parameters were evaluated at time intervals of 1, 3, 5, 7, 24, and 48 h by the use of a Nikon Eclipse TS100 inverted optical microscope (Nikon, Tokyo, Japan). The death of parasites was denoted by the absence of any contraction of the worms after having been exposed to a 10 mM serotonin solution, as described by Penido et al. (1994). Microphotographs were taken directly of the culture plates with a standard Nikon Coolpix 4.0 camera (Nikon, Tokyo, Japan) attached to the inverted microscope. These experiments were performed in duplicate.

2.8. Detection of membrane surface damage by fluorescence probe

The determination of the effect of free BphEA or BphEA incorporated into nanoemulsions on *S. mansoni* was performed using the Hoechst 33258 probe, as described by Lima et al. (1994). This probe is hydrophilic and fluoresces when bound to DNA, thus acting as an indicator of membrane integrity. The cultures of worms were incubated with NANOSTE and with NANOSTEb, both diluted 160-fold in a culture medium. The concentration of BphEA was equal to 6.25 μg/mL. The worms were also incubated with free BphEA at concentrations of 6.25 and 100 μg/mL in DMSO. The higher concentration corresponded to the effective dose of free BphEA. The control groups were cultured in pure medium or medium containing 2% (v/v) DMSO. The 2% DMSO solution was used as control because it was employed for the dissolution of BphEA at concentration of 100 μg/mL. After 48 h, the worms were washed with RPMI-1640 medium and incubated for 1 h with 20 μL of Hoechst 33258. The damage to the *S. mansoni* membrane was assessed by fluorescence microscopy after washing the worms with an RPMI-1640 medium to remove the probe. These experiments were performed in duplicate.

3. Results and discussion

3.1. Synthesis of BphEA

BphEA was obtained successfully in agreement with results described by Moreira et al. (2000). The structure of BphEA was confirmed by IR and NMR spectroscopy as described in Section 2.2.

3.2. Characterization of the nanoemulsions

The physico-chemical characteristics of nanoemulsions prepared with different combinations of surfactants are summarized in Table 2. Nanoemulsions containing Lipoid-S75® as a surfactant (NANOLP) presented an encapsulation level of BphEA near 34.0% (0.34 mg/mL) and homogeneous droplets with a mean diameter of 200 nm (polydispersity index equal to 0.158). In spite of the neutral charge of Lipoid-S75®, the zeta potential of −62.6 mV can be attributed to the presence of fatty acids in the Lipoid-S75® composition. Considering that BphEA is a zwitterionic molecule presenting a partition coefficient (log *P*) equal to 0.85 (Moreira, 2003), it may be located either inside the oil core or at the nanoemulsion interface. The adsorption

Table 2
Physico-chemical properties of nanoemulsions containing or not BphEA[a]

Formulation	Encapsulation (%)	Diameter (nm)	Polydispersity (ip)	Zeta potential (mV)
NANOLP	33.8 ± 7.2	200.5 ± 17.6	0.158	−62.6 ± 1.3
NANOST	20.5 ± 3.0	251.8 ± 11.0	0.286	−31.5 ± 5.7
NANOSTb[b]	–	251.8 ± 8.1	0.243	−35.9 ± 5.0
NANOSTOA	18.7 ± 1.8	209.4 ± 4.5	0.244	−33.6 ± 1.2
NANOSTOAb[b]	–	251.9 ± 42.1	0.308	−37.8 ± 5.2
NANOSTE	51.8 ± 5.0	225.8 ± 32.1	0.294	25.7 ± 3.9
NANOSTEb[b]	–	238.4 ± 11.1	0.303	39.0 ± 0.6

[a] Values are expressed as mean ± S.D. ($n = 3$).
[b] Formulations without BphEA.

of BphEA at the NANOLP interface may occur as a result of its interaction with the phosphatidylcholine molecules present in the composition of Lipoid-S75®. The phosphatidylcholine derivatives present anionic phosphate and cationic ammonium groups that can interact with ammonium and thiosulfate groups of BphEA, respectively. Thus, nanoemulsions showing either a negative or positive group at the interface were prepared to evaluate the influence of the interfacial charge. Anionic and cationic nanoemulsions were obtained by the use of oleic acid and stearylamine, respectively. Anionic nanoemulsions (NANOST and NANOSTOA, presenting zeta potentials equal to −31.5 and −33.6 mV, respectively) presented an association level of approximately 20.0% (0.2 mg/mL) with BphEA, whereas cationic nanoemulsions (NANOSTE) were equal to 51.8% (0.52 mg/mL). For both types of nanoemulsions, the mean diameter of the droplets was equivalent (between 209.4 and 251.8 nm; polydispersity index was between 0.244 and 0.294).

It is noteworthy that the zeta potentials were not significantly altered for the anionic nanoemulsions with or without BphEA. However, in the case of cationic nanoemulsions, the presence of BphEA provoked a decrease in the zeta potential (25.7 mV versus 39.0 mV). This finding suggests that the thiosulfate group of BphEA interacts with the ammonium group of stearylamine, reducing the number of positive charges exposed at nanoemulsion interface. The thiosulfate group seems to be less sterically hindered than the ammonium group in BphEA. Thus, the presence of stearylamine favored the interaction with BphEA at the nanoemulsion interface, while the possible interaction between anionic groups and the ammonium group of BphEA was less favorable.

3.3. Nanoemulsion stability

Anionic and cationic nanoemulsions containing BphEA showed a good stability in terms of mean droplet size and zeta potential after storage for 2 months at 4 °C (Table 3). No significant change in the values of these parameters ($p > 0.05$) was observed. This stability can be the result of the presence of negative or positive surface charges in these nanoemulsions, as shown by zeta potential measurements. This interfacial charge contributes to an electrostatic repulsion between the nanodroplets, which prevents coalescence and, consequently, the formation of larger entities (Klang and Benita, 1998). In addition, in the case of NANOLP, the presence of poloxamer molecules can promote steric hindrance and facilitate the control of the nanodroplet size and stability (Teixeira et al., 1999).

3.4. Release study

The release profile of BphEA from NANOSTE in an RPMI-1640 medium is presented in Fig. 2. The release of BphEA was time-dependent, revealing an optimum stability of NANOSTE, which was submitted to a high dilution in this medium. A release of only 5% of the BphEA was observed until 1 h after incubation in the culture medium. The release of approximately 30% of the BphEA associated with the cationic nanoemulsion was observed during the period from 4 to 24 h. At the end of 48 h, the moiety of the BphEA remained associated with the cationic nanoemulsion. A linear regression was applied to the time interval comprised between 10 min and 4 h. The release kinetics was assumed to have an apparent zero-order rate. The adjusted curve for BphEA released (%) versus time (h) was derived from the

Table 3
Physico-chemical stability of nanoemulsions containing BphEA[a]

Formulation	Time 0		30 days		60 days	
	Size (nm)	Zeta potential (mV)	Size (nm)	Zeta potential (mV)	Size (nm)	Zeta potential (mV)
NANOLP	200.5 ± 17.6[b]	−62.6 ± 1.3[b]	196.1 ± 14.7[b]	–	204.6 ± 16.4[b]	−63.4 ± 0.5[b]
NANOST	251.8 ± 11.0[b]	−31.5 ± 5.7[b]	252.4 ± 13.6[b]	−34.5 ± 1.1[b]	258.8 ± 8.5[b]	−33.5 ± 2.8[b]
NANOSTOA	209.4 ± 4.5[b]	−33.6 ± 1.2[b,c]	215.4 + 5.4[b]	−34.6 ± 1.6[b]	208.3 ± 4.9[b]	−40.5 ± 4.4[c]
NANOSTE	225.8 ± 32.1[b]	25.7 ± 3.9[b]	223.4 ± 29.4[b]	24.6 ± 5.4[b]	225.2 ± 33.8[b]	33.3 ± 3.5[b]

[a] Each value represents the mean ± S.D. ($n = 3$).
[b] There is no significant difference among the means indicated at the same line ($p > 0.05$).
[c] There is a significant difference among the means indicated at the same line ($p < 0.05$). All means were compared with time zero.

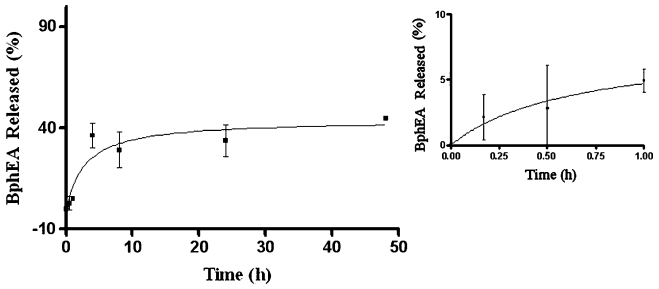

Fig. 2. Amount of BphEA released from the cationic nanoemulsion as a function of time. The insert indicates the percentage of BphEA released from the cationic nanoemulsion up to 1 h. The data were obtained from three experiments.

minimum-squares equation ($-9.3815x + 101.72$, $r^2 = 0.9845$), and the release rate observed was equal to 9.38% BphEA/h. These findings indicate that the cationic nanoemulsion should be an appropriate carrier for BphEA in a biological medium, allowing its delivery associated with this nanostructured system.

3.5. In vitro schistosomicidal activity

The features of the *S. mansoni* worms after their incubation in the presence of BphEA, either free or associated with cationic nanoemulsion, are presented in Fig. 3. After 48 h, the worms of the control groups maintained normal movements with no evident alterations. The aspects of the female and male worms

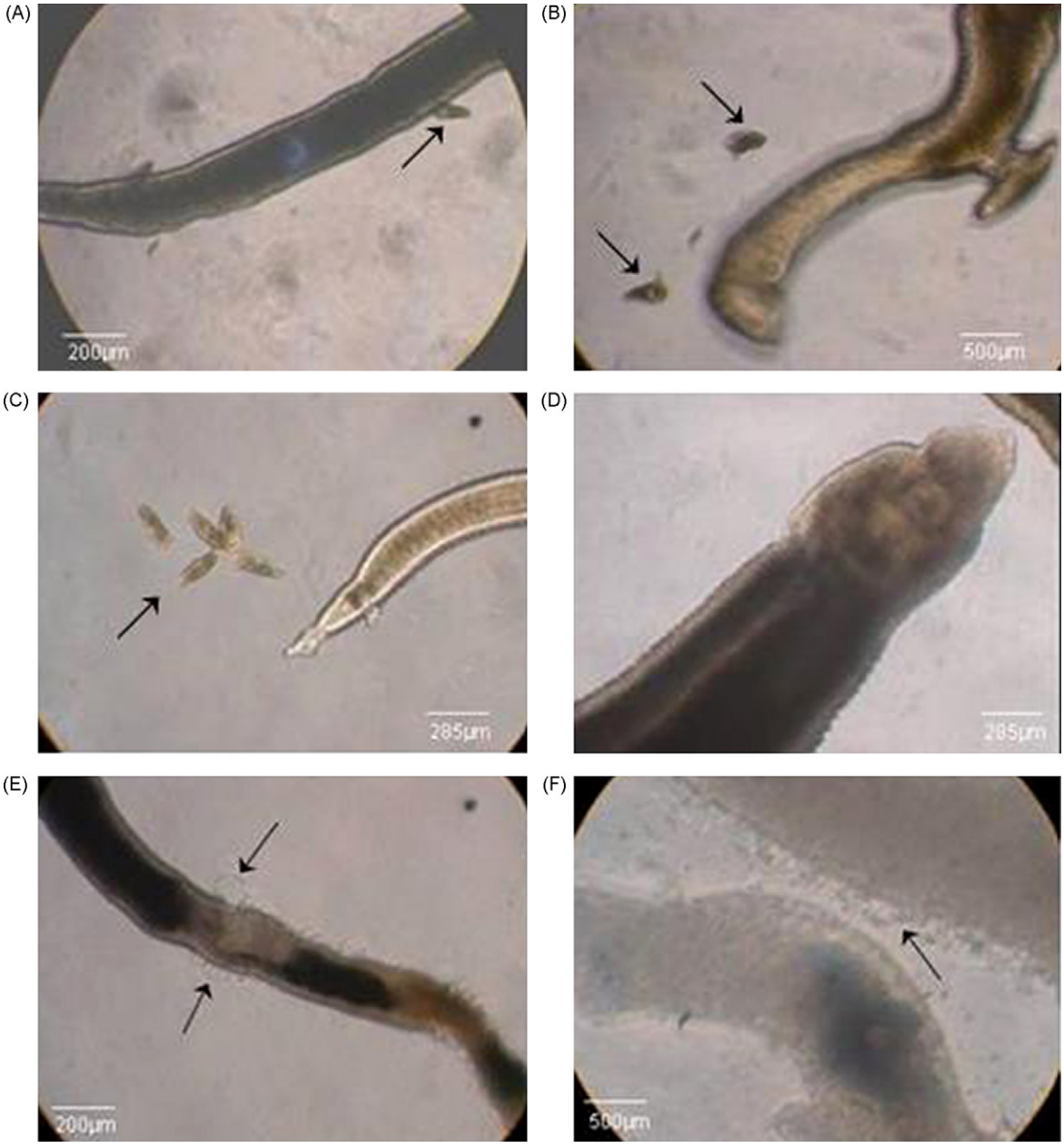

Fig. 3. Adult worms of *Schistosoma mansoni* submitted to the different treatments. Female worm (A) and male worm (B) incubated in a culture medium with no treatment; female worm incubated with free BphEA (6.25 μg/mL) (C) (arrows indicate eggs of *S. mansoni*); male worm incubated with NANOSTEb (D); female (E) and male (F) worms incubated with NANOSTE (arrows indicate vesicles in worm tegument).

incubated in the pure RPMI-1640 medium are shown in Fig. 3A and B. The worms exposed to free BphEA (6.25 μg/mL) and NANOSTEb presented aspects similar to those of the control groups (Fig. 3C and D). The presence of *S. mansoni* eggs could be observed with the worms incubated with free BphEA and with the control group thus confirming their viability (arrows in Fig. 3B and C). However, the *S. mansoni* worms gradually lost their motor activity after 3 h of incubation with NANOSTE. Moreover, at 24 h post-incubation, the tegument of the worms started to suffer from disorganization revealed by the presence of

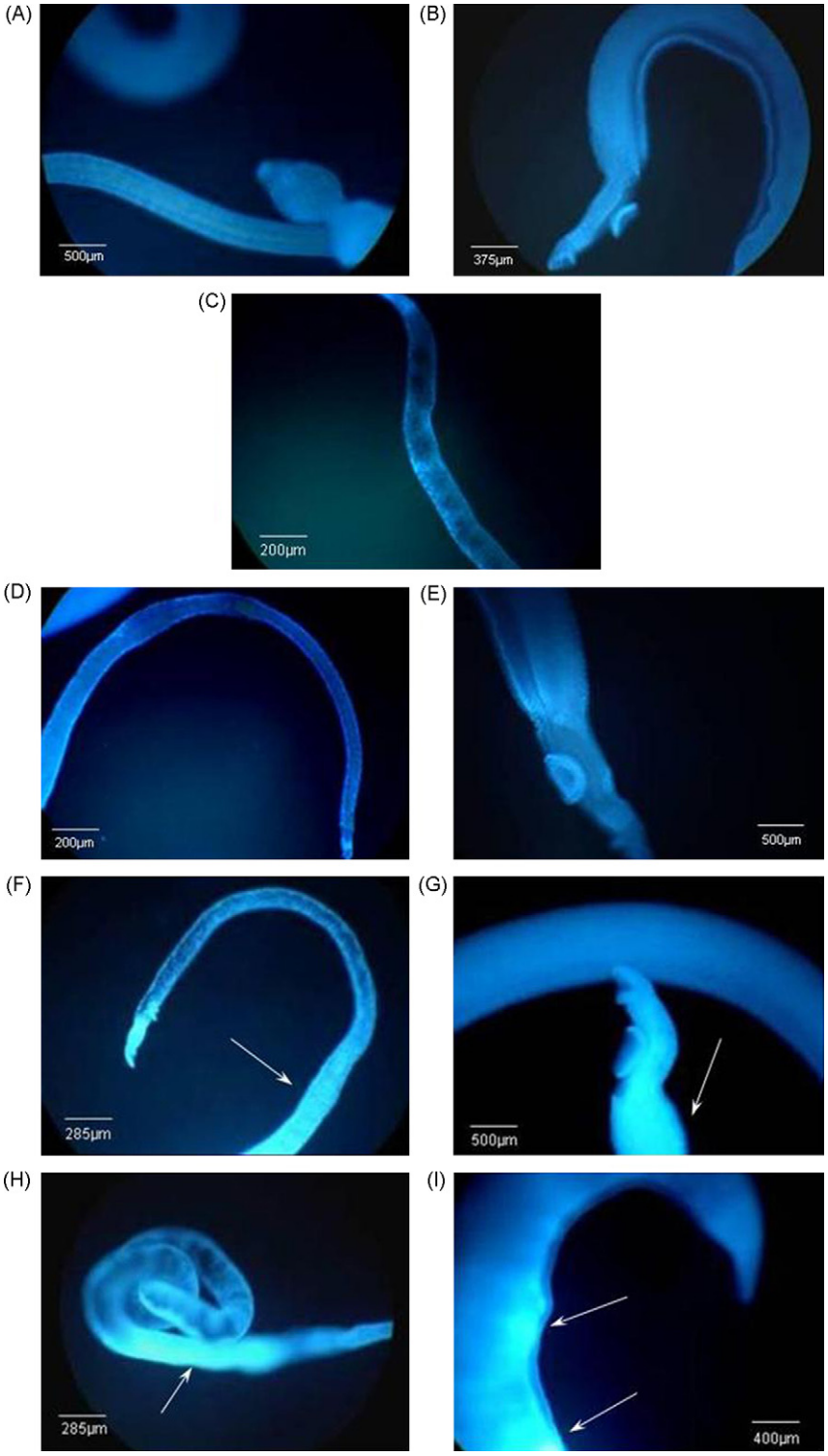

Fig. 4. Worms labeled with Hoechst 33258. A pair of worms incubated in a pure culture medium (A) and male worm in DMSO (B); female worm incubated with NANOSTEb (C); female (D) and male (E) worms incubated with free BphEA (6.25 μg/mL); female (F) and male (G) worms incubated with free BphEA (100 μg/mL); female (H) and male (I) worms incubated with NANOSTE. The arrows show areas of intense fluorescence, indicating surface damage.

vesicles in their structure and this alteration was observed up to 48 h after incubation with NANOSTE (arrows in Fig. 3E and F). Furthermore, all the female worms incubated with NANOSTE died, whereas the male worms moved slowly at the end of 48 h. These findings indicate that NANOSTE is in fact able to induce the death of or damage to the *S. mansoni* worms, thereby improving the effectiveness of BphEA.

3.6. Detection of membrane surface damage by fluorescence probe

The permeability of the worm tegument remained unaltered when submitted to treatment with DMSO and NANOSTEb. No fluorescence inside of the worms was detected, as shown in Fig. 4B and C. Fluorescence inside the worms treated with free BphEA at a concentration of 6.25 μg/mL could not be observed, thereby confirming the findings discussed in section 3.5 (Fig. 4D and E). However, at a higher concentration (100 μg/mL), the appearance of fluorescence inside the female and male worms was observed, indicating the occurrence of damage to the membrane surface (arrows in Fig. 4F and G). Similarly, areas of intense fluorescence were detected in the worms treated with NANOSTE (arrows in Fig. 4H and I), demonstrating that this delivery system was capable of affecting the integrity of the worm membranes. This study shows that the association of BphEA with the cationic nanoemulsions enhanced its efficacy against *S. mansoni* 16-fold. The improvement of BphEA delivery to the parasites by the cationic nanoemulsion may be explained as a function of the structure of the schistosome surface. *S. mansoni*, as well as other intravascular trematodes, is covered by a syncytium, which is in turn covered by a membrane composed of several layers. These layers are organized in two lipid bilayers: an internal one, which is rich in intramembranous particles, and an external one, almost entirely composed by phospholipids and sterols (Lima et al., 1994). This membrane contains conjugated carbohydrates, such as glycoproteins, glycolipids, and mucopolysaccharides, which are responsible for its polyanionic nature. Consequently, the anionic groups on the surface of the parasites can bind electrostatically with the cations in the environment (Georgieva and Mizinska-Boevska, 1999). Thus, it can be suggested that the positively charged nanoemulsion should bind electrostatically with these anionic groups on the worm surface and facilitate the delivery of BphEA.

4. Conclusion

The development of resistance to the conventional schistosomicidal drugs has justified the search for new compounds. However, some compounds present a limitation in their use as pharmaceutical products due to their poor solubility in water. A strategy to overcome this inconvenience is their incorporation into drug delivery systems, such as nanoemulsions. In this study, a novel schistosomicidal drug, BphEA, was synthesized, and formulation studies were performed to incorporate this drug into nanoemulsions. Cationic nanoemulsions showed higher encapsulation levels of BphEA. The size and zeta potential of the cationic nanoemulsion containing BphEA remained constant during a 60 days storage period, indicating a good physical stability. The incorporation of BphEA into the cationic nanoemulsion led to an increase in its schistosomicidal effectiveness. This effect was most likely due to the interaction between the negative membrane surface of the *S. mansoni* and positive particles of the nanoemulsion, thus favoring the entrance of the BphEA into the worms. In future studies, the *in vivo* schistosomicidal efficacy of this cationic nanoemulsion containing BphEA should be evaluated.

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