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RESEARCH PAPER

# Sustained Release from Ionic-Gradient Liposomes Significantly Decreases ETIDOCAINE Cytotoxicity

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## ABSTRACT

***Purpose*** Etidocaine (EDC) is a long lasting local anesthetic, which alleged toxicity has restricted its clinical use. Liposomes can prolong the analgesia time and reduce the toxicity of local anesthetics. Ionic gradient liposomes (IGL) have been proposed to increase the upload and prolong the drug release, from liposomes.

***Methods*** First, a HPLC method for EDC quantification was validated. Then, large unilamellar vesicles composed of hydrogenated soy phosphatidylcholine:cholesterol with 250 mM $(NH_4)_2SO_4$ - inside gradient - were prepared for the encapsulation of 0.5% EDC. Dynamic light scattering, nanotracking analysis, transmission electron microscopy and electron paramagnetic resonance were used to characterize: nanoparticles size, polydispersity, zeta potential, concentration, morphology and membrane fluidity. Release kinetics and *in vitro* cytotoxicity tests were also performed.

***Results*** $IGL_{EDC}$ showed average diameters of 172.3 ± 2.6 nm, low PDI (0.12 ± 0.01), mean particle concentration of $6.3 \pm 0.5 \times 10^{12}$/mL and negative zeta values (−10.2 ± 0.4 mV); parameters that remain stable during storage at 4°C. The formulation, with 40% encapsulation efficiency, induced the sustained release of EDC (*ca.* 24 h), while reducing its toxicity to human fibroblasts.

***Conclusion*** A novel formulation is proposed for etidocaine that promotes sustained release and reduces its cytotoxicity. $IGL_{EDC}$ can come to be a tool to reintroduce etidocaine in clinical use.

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**KEY WORDS** drug-delivery · etidocaine · ionic gradient liposomes · local anesthesia

## ABBREVIATIONS

| | |
|---|---|
| Cho | Cholesterol |
| DDS | Drug delivery system |
| DLS | Dynamic light scattering |
| EDC | Etidocaine |
| EPR | Electron paramagnetic resonance |
| HSPC | Hydrogenated soy phosphatidylcholine |
| $IC_{50}$ | Half maximal inhibitory concentration of cell viability |
| IGL | Ionic gradient liposomes |
| $IGL_{EDC}$ | Etidocaine-containing sulphate gradient liposomes |
| LA | Local anesthetic |
| LUV | Large unilamellar vesicle |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NTA | Nanotracking analysis |
| TEM | Transmission electron microscopy |

## INTRODUCTION

Local anesthetics (LA) prevent neural conduction in a reversible way, by impeding the influx of sodium ions trough the voltage-gated channels of excitable membranes (1,2).

N-(2,6-dimethylphenyl)-2-[ethyl(propyl)amino]butanamide, etidocaine (EDC) is an aminoamide LA, with a pKa = 7.8 (3). Its structure is similar to that of lidocaine, only differing by the propyl substituent linked to the amine group and the ethyl group on the α-carbon of the intermediate chain (4). Such hydrocarbon groups led to an increase in lipophilicity (regarding lidocaine), higher plasma protein bound fraction and longer time of action (4,5).

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EDC has an onset of action of 3–5 min and anesthesia time around 4 h. Being introduced to the medical practice in 1972 (5,6) etidocaine (Duranest®) was lately discontinued by the FDA in 2008, for alleged lack of "efficacy and security" (7) (US FDA 2008). In 2012 the FDA revised this issue, but neither more data nor any other information of problems related to EDC use has been added. Etidocaine remains in the FDA Orange Book of products, but such prohibition is a matter of controversy, since the Federal Register document of 2012 clearly states that "safety or effectiveness" were not the reasons for its discontinuity (8).

Among the strategies to improve the selectivity, effectiveness, to prolong the effect and to reduce the toxicity of drugs, incorporation into liposomes has been widely used (9–12). Liposomes are versatile drug delivery systems (DDS) composed of one or more phospholipid bilayers. Such spherical vesicles can be a carrier for a wide range of hydrophobic and hydrophilic bioactive substances. Besides, liposomes are biocompatible, biodegradable and have low toxicity (13,14).

There are several liposomal-based DDS described for the sustained release of LA (see de Paula *et al.* (12), for a review), prolonging the anesthesia time and decreasing side effects. Since LA prefer to be inserted in the lipid bilayer than in the inner aqueous core of the vesicles, drug upload is limited by the low lipid/water phase ratio of liposomes (15). So, in order to improve drug upload, ionic-gradient liposomes (IGL) have been proposed (16).

In IGL (Fig. 1) the drug is added to pre-formed vesicles that exhibit a transmembrane (pH or ionic) gradient, among which the ammonium sulphate one is the most studied (16). The anesthetic, in its basic form (EDC, Fig. 1), crosses the membranes of pre-formed liposomes, reaching the inner aqueous core where $(NH_4)_2SO_4$ has been entrapped. Such inner compartment has an acidic pH (due to the dissociation of ammonium ion: $NH_4^+ \rightarrow NH_3 + H^+$) that causes protonation of the anesthetic ($EDC\text{-}H^+$), which remains in the vesicle due to the low diffusion coefficient of the charged species across the membrane (17,18). Thus, IGL show a high drug-upload capacity for amphipathic weak bases such as LA, leading to sustained release, and increasing its therapeutic potential (18–20).

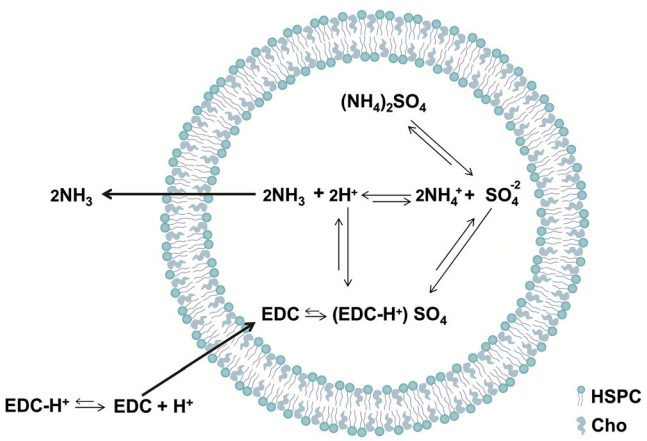


**Fig. 1** Schematic representation of an ionic-gradient liposome prepared with ammonium sulphate for the loading of the local anesthetic Etidocaine.

The clinical demand for long-acting anesthetics to be used in surgical procedures, post-operative and chronic pain management, justifies reconsidering EDC. Being aware of that, this work describes an IGL-based formulation for etidocaine that, exhibiting a sustained release profile and minimizing its cytotoxic effects, can come to be an interesting alternative to reintroduce the anesthetic in the clinical use.

## MATERIALS AND METHOD

### Materials

Acetonitrile (HPLC grade) and chloroform were purchased from Merck (São Paulo, SP, Brazil); phosphoric acid from Cetus (Santo Amaro, SP, Brazil); Bis-Tris and Hepes buffer, cholesterol (Cho), 5-doxyl stearate (5-SASL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, uranyl nitrate and ammonium sulphate were bought from Sigma Chem. Co. (St. Louis, MO, USA); etidocaine hydrochloride was synthesized by Finetech Industry Limited (Wuhan, China); hydrogenated soy phosphatidylcholine (HSPC) was obtained from Avanti Polar Lipids (Alabaster, AL,USA).

### Validation of a HPLC Methodology for the Quantification of Etidocaine

The validation of the analytical methodology for EDC quantification was performed by high performance liquid chromatography, HPLC in a Waters Breeze 2 System (Waters Technol., São Paulo, SP, Brazil). For this purpose, a reversed-phase C18 column was used (Waters 18e - Symmetry® 75 × 4.6 mm, particle size = 3.5 μm). The mobile phase was composed (80:20, v/v) of 0.1% phosphoric acid and acetonitrile. EDC detection was carried out at 260 nm (21). The flow rate was set to 1.0 mL/min, the injection volume was 30 μL, and all samples were previously filtered through 0.22 mm polyethersulfone membranes (Millipore, Bedford, MA). The determined parameters were: linearity, precision, accuracy, limit of detection (LD), limit of quantification (LQ) and specificity (22).

Linearity was measured from the curves of peak area *vs.* EDC concentration (from 0.4 to 1580 μM), determined in 3 consecutive days; the linear regression was calculated by the least square method.

Precision, determining the method capacity to give repetitively accurate results, were calculated from the intra-day and

inter-day variability, and expressed as RSD (relative standard deviation), according to eq. 1:

$$RSD = \frac{SD}{MCD}\ x\ 100 \quad (1)$$

where SD is the standard deviation and MCD is the mean concentration determined.

Accuracy (A) was obtained by the difference between the MCD and the theoretical drug concentration (TC), according to:

$$A = \frac{MCD}{TC} \quad (2)$$

The limit of detection (LD), i.e. the lower concentration of the analyte being detected - but not necessarily quantified, and the limit of the quantification (LQ), defined by the smallest quantified analyte concentration, were also determined. The results were treated by linear regression, where the slope of the average analytical curve and the standard deviation (SD) of the Y-intercept of three analytical curves were used to calculate LD and LQ, according to eqs. 3, 4:

$$LD = \frac{SD.3}{slope}\ x\ 100 \quad (3)$$

$$LQ = \frac{SD.10}{slope}\ x\ 100 \quad (4)$$

## Preparation of Ionic Gradient Liposomes

The first step for the vesicles preparation was the lipid film formation. For that, lipid aliquots (HSPC:Cho, 6:4 mol%) dissolved in chloroform were dried under $N_2$ flux and kept in vacuum for 2 h, at room temperature. The lipid film was then hydrated with 250 mM ammonium sulphate solution, and vortex (3 min.) to form large multilamellar vesicles (final lipid concentration, HSPC+Cho = 10 mM). Multilamellar liposomes were extruded 12 times through polycarbonate membranes with pore size of 100 nm, under 3.0 kgf / $cm^2$ or 40 psi $N_2$ pressure in an (Lipex Biomembranes Inc.® - Canada) extruder, at 60°C. To remove the non encapsulated $(NH_4)_2SO_4$ the extruded liposomes were then centrifuged at 120,000 g (Optima L 90 K ultracentrifuge, Beckman Coulter Inc., Pasadena, USA) for 2 h at 4°C (23). The supernatant was discarded and the pellets were suspended in 50 mM Bis-TRIS buffer, pH 6.2 (control - without etidocaine – liposomes, referred to as IGL) or buffer plus 0.5% EDC ($IGL_{EDC}$). The active incorporation of etidocaine was achieved by incubating $IGL_{EDC}$ samples for 2 h, at room temperature.

## Phosphate and Cholesterol Determination

The phospholipid concentration was quantified by detection of the released inorganic phosphate, after acid digestion (24).

The cholesterol content in the IGL was determined by the cholesterol oxidase enzymatic method (25), using a (Laborlab®, São Paulo, Brazil) kit.

## Liposomes Characterization / Physicochemical Stability Study

Diluted samples (100 times) of the $IGL_{EDC}$ formulation and its control (IGL) were characterized in triplicate during 60 days of storage at 4°C, in terms of average diameter (size, in nm) polydispersity index (PDI) and zeta potential (ZP, in mV). Such parameters, obtained by DLS (Nano ZS90 equipment, Malvern, Worcestershire, England) were measured in function of the intensity of light scattered by the nanoparticles (size and PDI), and by nanoparticle electrophoretic mobility values, expressed in modulus (ZP).

Liposomes concentration (number or vesicles/mL) was determined by nanotracking analysis (NTA). Samples of $IGL_{EDC}$ and IGL, diluted $10^4$ times, were measured with a NanoSight NS300 equipment (Malvern, Worcestershire, England).

## Liposomes Morphological Analysis

The morphological analysis of liposomes was carried out by transmission electron microscopy (TEM 906 equipment, Zeiss-LEO® - Germany). For sample preparation 50 μL of the liposomal sample (10 mM final lipid concentration) was added to the cooper grids (200 mesh) for 10 s, and the liquid excess was removed with filter paper. One drop of uranyl acetate (2%) was added to the grid, and the liquid excess was removed with filter paper. Subsequently, the samples were dried at room temperature before the TEM measurements, run at 60 kV.

## Encapsulation Efficiency Determination

The percent encapsulation efficiency (EE%) of EDC into conventional and ionic-gradient liposomes was determined by ultrafiltration-centrifugation, at 4.000 g for 30 min at 4°C, using 10 kDa regenerated cellulose membranes (Milex, Milipore). The anesthetic was quantified by HPLC, using the described validated method. The filtrates, containing the unencapsulated etidocaine (EDC free) were diluted and analyzed, according to eq. 5:

$$EE\% = \frac{\text{EDC total} - \text{EDC free}}{\text{EDC total}}\ x\ 100 \quad (5)$$

where EDC total refers to the initial amount of anesthetic added to the formulation and detected by HPLC.

From the EE% data we have also calculated the partition coefficient of EDC, according to Eq. 6 (21):

$$P = \frac{n_{m/V_m}}{n_{w/V_w}} \quad (6)$$

where, n = number de moles of etidocaine, V = volume, m and w refer the membrane and aqueous phase, respectively.

## Evaluation of the Membrane Fluidity of IGL

The compactness of the membrane is a crucial parameter for IGL to keep their integrity and ionic gradient (26). To estimate that, an aliquot of the liposomal formulation was added into tubes containing the 5-SASL probe, at 0.8 mol% of the total lipids concentration, and incubated for 30 min at 37°C. After that, the samples were analyzed by electron paramagnetic resonance (EPR) in a Bruker EMX spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany) operated (X band) at 9.4 GHz, at 21°C. Through the spectrum, the segmental order parameter (S) measuring the orientation of the spin probe inserted in-between the lipids regarding the bilayer normal, was calculated according to (27):

$$S = \frac{2A// - 2A\perp}{2\left[A_{zz} - \left(A_{xx} + A_{yy}\right)/2\right]} \quad (7)$$

where A// and A⊥ refer to the maximum and minimum hyperfine splitting in the spectra, corresponding to the spin label long axis orientation to the external magnetic field. $A_{zz}$, $A_{yy}$, and $A_{xx}$ correspond to the maximum values of the hyperfine tensor, measured in single crystals, at low temperature. S values vary from 0 (non oriented) to 1, in highly oriented systems (28).

## Release Assays

*In vitro* release tests were performed in Franz vertical diffusion cells (29). For this purpose, a cellulose (Spectrapore) membrane with 12,000–14,000 Da pores were used to separate the sample (200 μL, in the donor compartment) from the acceptor compartment, containing 50 mM Hepes buffer pH 7.4, at 37°C. Aliquots of 200 μL were withdrawn from the acceptor compartment, at regular intervals, and analyzed by HPLC at 260 nm.

The release profile curves were analyzed with the KinetDS 3.0 software (30). Several kinetic models were tested (zero order, Kosmeyer-Peppas, Weibull), and based on the coefficient of determination ($R^2$) the best fitting model was achieved with the Weibull treatment (31):

$$Q = 1 - \exp\left[\frac{-(t)^b}{a}\right] \quad (8)$$

where Q is the amount of EDC released at the time "t"; "a" is the time constant and "b" is the release exponent (curve shape parameter).

## Cell Viability Assay

Human fibroblasts cells, previously isolated by (32) were grown on 96-well plates (Corning, NY, USA) at an initial density of 110 cells/mm$^2$ per well. After 24 h, the medium was changed, and supplemented with different EDC concentrations, free or encapsulated into IGL. After 4, 6 and 24 h, 10 μL of MTT solution (5 mg/mL in PBS) and 90 μL of base medium were added to each well. Cells were incubated for 3 h at 37°C in 5% $CO_2$, 95% air, and 100% humidity. After 3 h, the MTT solution was removed and replaced with 100 μL of dimethyl sulfoxide (DMSO). The plate was incubated for a further 15 min at room temperature (RT), and the optical density (OD) of the wells determined using a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA), at a test wavelength of 590 nm. For accuracy, the

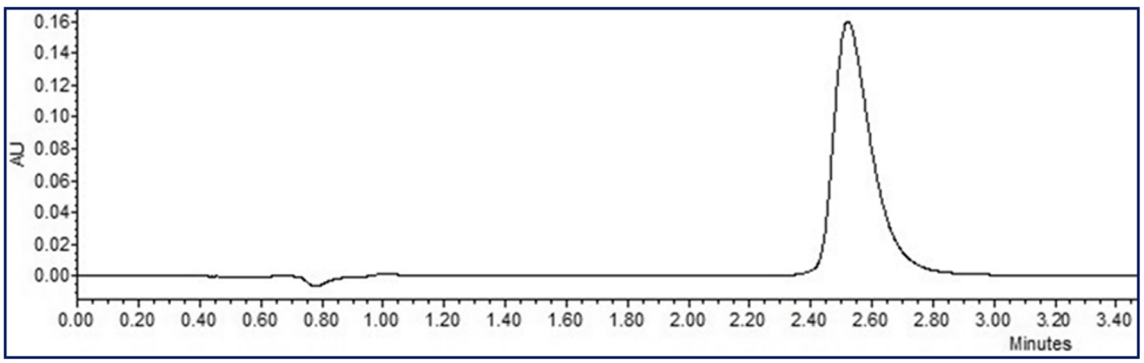


**Fig. 2** HPLC chromatogram showing the EDC peak at 2.5 min retention time, at 260 nm, 30°C.

**Table I** HPLC method for EDC quantification: recovery accuracy test

| [EDC] added (μg/mL) | | [EDC] intraday (μg/mL) | | | [EDC] interday (μg/mL) | Recovery (%) |
|---|---|---|---|---|---|---|
| 49 | | | | | | |
| | Day 1 | 48.43 | 48.22 | 48.22 | 47.69 ± 0.23 | |
| | Day 2 | 49.73 | 49.69 | 49.49 | 49.64 ± 0.25 | 100.83 |
| | Day 3 | 50.32 | 50.18 | 50.25 | 50.25 ± 0.61 | |
| 247 | | | | | | |
| | Day 1 | 242.48 | 242.72 | 241.71 | 242.31 ± 0.55 | |
| | Day 2 | 241.71 | 241.10 | 241.90 | 241.57 ± 0.47 | 98.34 |
| | Day 3 | 242.17 | 245.26 | 245.51 | 244.31 ± 1.53 | |
| 494 | | | | | | |
| | Day 1 | 494.83 | 493.44 | 495.18 | 494.49 ± 1.05 | |
| | Day 2 | 485.11 | 489.28 | 486.50 | 486.97 ± 2.16 | 99.46 |
| | Day 3 | 489.57 | 492.04 | 490.67 | 490.76 ± 1.23 | |

experiments were performed in triplicate in the same conditions.

### Statistical Analyses

Statistical analyses of size, PDI, ZP and EPR data were calculated with the help of Origin pro 8 software (Microcal Software Inc., Northampton/USA), through the unpaired t-test and one-way analysis of variance (ANOVA). Statistical significance was indicated by $p$ value: $(p < 0.05)$*, $(p < 0.01)$**, $(p < 0.005)$***, $(p < 0.001)$****.

## RESULTS AND DISCUSSION

### Validation of a HPLC Method for the Quantification of EDC

Validation of the HPLC methodology proposed to quantify EDC considered the parameters (linearity, precision, accuracy, LD, LQ and specificity) preconized by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines (22).

**Table II** RSD values: results of intra-day and inter-day precision tests, obtained in triplicate, from 3 different (49, 247 and 494 μg/mL) EDC concentrations

| EDC (μg/mL) | RSD Day 1 (%) | RSD Day 2 (%) | RSD Day 3 (%) | Inter-days RSD (%) |
|---|---|---|---|---|
| 49 | 0.328 | 0.138 | 0.264 | 2.318 |
| 247 | 0.126 | 0.114 | 0.167 | 1.010 |
| 494 | 0.185 | 0.248 | 0.148 | 0.828 |

In the column and condition described in methods, a single peak with retention time of 2.5 min was recorded for EDC (Fig. 2). The analytical curve was obtained in the concentration range of 0.4 to 1580 μg/mL (regression equation: Y = 9,000,000x + 47,759, correlation coefficient = 0.9996). The average recoveries were quite satisfactory (98.3–100.8) demonstrating the method's accuracy from 98 to 100% (Table I).

Precision, expressed by RSD, was calculated from three levels of measurements, as described in methods. The

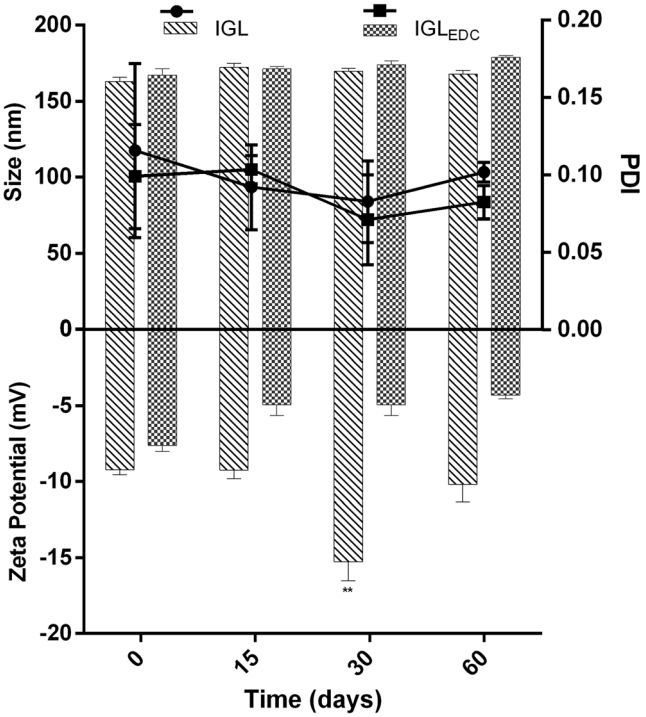


**Fig. 3** Stability tests for IGL and IGL$_{EDC}$ samples, in terms of size (upward bars), PDI (line) and ZP (downward bars), assessed by DLS, during 60 days of storage at 4°C. ** IGL *vs.* IGL$_{EDC}$: statistically significant difference ($p < 0.01$), unpaired t-test.

obtained values were lower than 3% (Table II), in accordance with the pre-established parameters for validation (22).

## Liposomes Characterization and Physicochemical Stability Study

A formulation of $IGL_{EDC}$ containing 0.5% (0.5 g/dL or 16 mM) EDC was prepared, and different techniques (DLS, NTA, TEM and EPR) were used for its characterization. First, quantification of phospholipids (5.9 ± 0.2 mM) and cholesterol (3.9 ± 0.1 mM) revealed a slight loss (ca. 0.1%) of total lipids during the preparation process, probably in the ultracentrifugation step, as also observed before (22). Then, the physicochemical stability of the liposomes (60 days) was followed in terms of size, PDI, and ZP (Fig. 3).

The vesicles were found stable for 60 days under refrigeration, with no significant differences (IGL *vs.* $IGL_{EDC}$) in size and PDI, but slightly smaller absolute ZP values for $IGL_{EDC}$ with the time ($p < 0.01$ in comparison to IGL, at day 30). The average liposome diameter was in the range of 170 nm, for the two formulations. IGL and $IGL_{EDC}$ presented low polydispersity values (0.12 and 0.10, respectively), denoting monodisperse size distribution systems (33). ZP values around −10 mV for IGL and − 5 mV for $IGL_{EDC}$ were determined, indicating good colloidal stability in both cases (15,34). The less negative ZP of $IGL_{EDC}$ (than IGL) is suggestive that a fraction of the (positively charged) EDC molecules were uploaded on the surface of the vesicles, causing a decrease in modulus of the ZP values (35). This result is in accordance with the literature, in which EPR, fluorescence and Nuclear Magnetic Resonance data on liposomes were used to show that EDC and other local anesthetics could insert in between the lipids (21,36).

Additionally, the number of liposomes in suspension was determined by NTA. The particles concentration was in the range of $8.5 \pm 1.24 \times 10^{12}$ for IGL, and it did not change significantly ($IGL_{EDC} = 6.3 \pm 0.5 \times 10^{12}$) after incorporation of etidocaine. The number of particles in the formulation is considered an important parameter to describe the biological activity of drug-delivery systems (37).

TEM analyses revealed particle sizes compatible to those determined by DLS, i.e., around 200 nm (Fig. 4). The vesicles

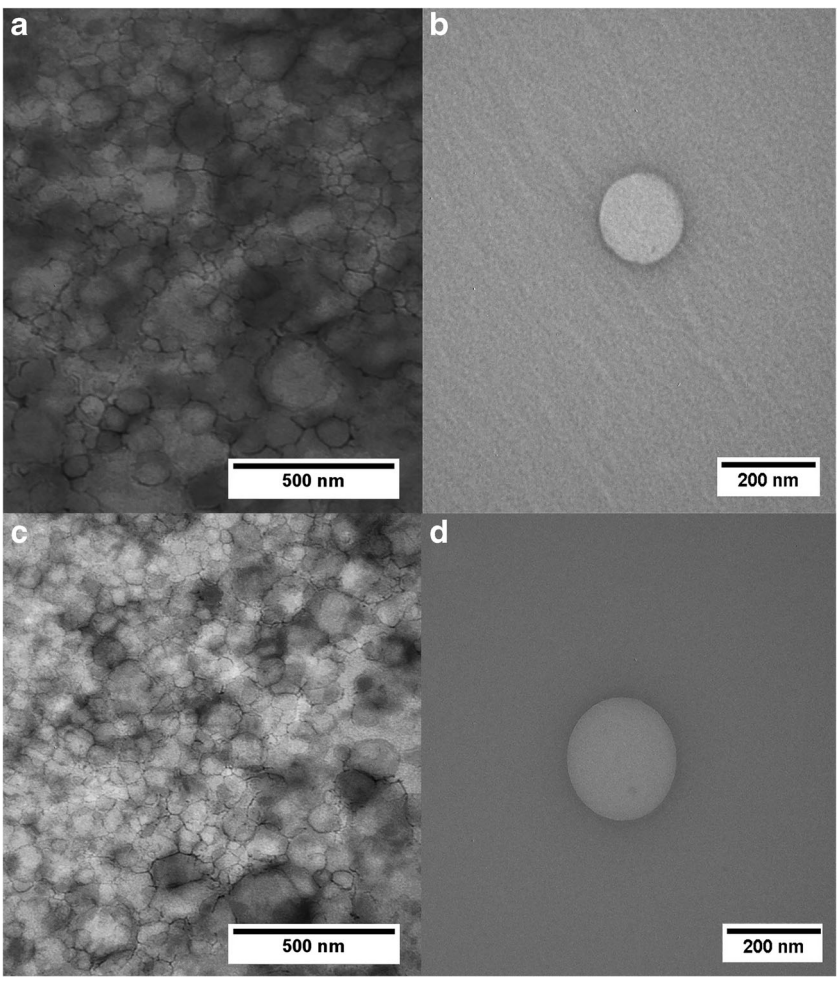


**Fig. 4 TEM**: Representative images of IGL **(a,b)** and $IGL_{EDC}$ **(c,d)** samples, with two different magnification: 60,000 x**(left – a,c)** and 100,000 x**(right – b,d).**

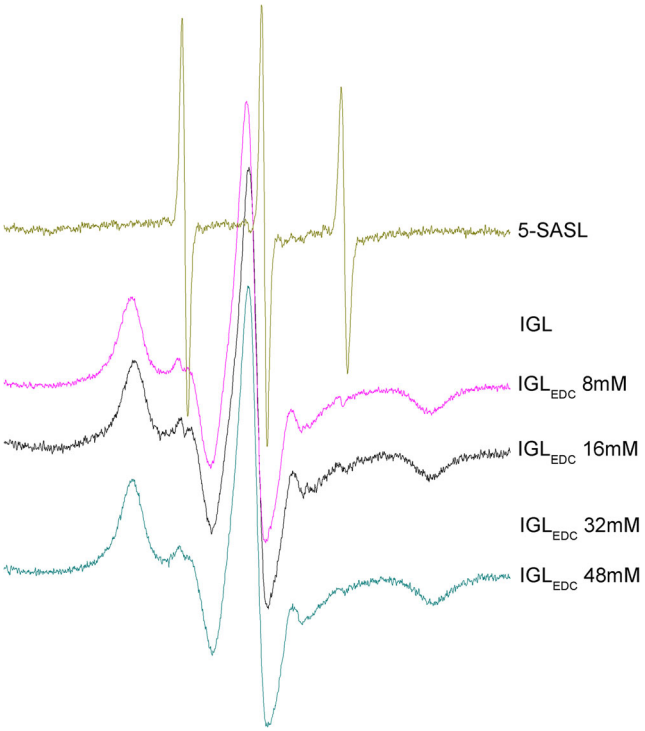


**Fig. 5** EPR spectra of ionic-gradient liposomes (IGL) doped with 0.8 mol% 5-SASL probe, in the absence and presence of increasing EDC concentrations, at 21°C.

have a spherical morphology and EDC entrapment did not change the liposome diameter and shape.

The encapsulation efficiency of EDC into the ionic-gradient liposomes was determined as described in methods, in freshly prepared samples (40.0 ± 1.3%) and after 60 days of storage, with no significant differences in $IGL_{EDC}$ upload. For the sake of comparison, the EE% of etidocaine in conventional liposomes – i.e. vesicles of equivalent composition (HSPC:Cho, 6:4 mol%) but prepared with no ionic gradient – was also measured: 11.0 ± 2.7%. Such difference in EE% is due to the large amount of protonated EDC entrapped into the acidic inner core of IGL (38), while in conventional liposomes encapsulation depends exclusively on the drug partition in the lipid bilayer (39).

Zucker and coworkers (Zucker *et al.* 2009) have reported similar increased encapsulation of betamethasone succinate, vincristine and tempamine into IGL composed of HSPC:Cho 59:41 mol% plus 250 mM ammonium sulphate. This and other studies in the literature revealed that at least two structural properties determine the success of an IGL formulation: vesicle size and compactness of the lipid bilayer. Yet, the larger the liposomes the greater the drug upload (18,40). The other important parameter for drug entrapment and prolonged release is the compactness of the lipid bilayer, for which the lipid composition is essential. Membranes composed of saturated acyl chain (high gel/fluid transition temperature, Tm) lipids, as HSPC (Tm = 52°C), tend to be less permeable than those prepared with unsaturated lipids, such as egg phosphatidylcholine (Tm = −5 to −15°C). Cholesterol also plays a modulatory effect in membrane fluidity (41) and IGL prepared with 40 mol% Cho have been reported to have high compactness (26). Therefore, liposomes prepared with lipids with high Tm and optimal cholesterol levels, will have more organized bilayers to keep the ionic gradient, reduce membrane permeability to the drug, and provide sustained drug release (42).

## Membrane Fluidity of Ionic-Gradient Liposomes

EPR analysis (Fig. 5) was used to access the membrane compactness of ionic-gradient liposomes, as well as the possible fluidizing effect of increasing (0, 8, 16, 32, 48 mM) EDC concentrations.

Liposomes composed of HSPC:Cho showed immobilized 5-SASL spectra, revealing the high orientation level of the saturated (HSPC) molecules arranged in a lamellar phase. The order parameter (S values, Table III) of the ionic gradient liposomes was high (> 0.8) and no changes in the bilayer packing were noticed after EDC addition. The compactness of the HSPC-based IGL (S values close to unit) is evinced when they are compared to liposomes prepared with unsaturated phospholipids, such as egg phosphatidylcholine, which segmental order parameter (S) is lower than 0.7 (20).

In Table III, the etidocaine concentrations inside the lipid bilayer (corresponding to the each of the total EDC concentrations in Fig. 5: 8, 16, 32, 48 mM) were calculated from the partition coefficient of EDC ($P = 19.8$) at pH 6.2, determined in conventional liposomes, as described in methods.

Considering the lipid concentration (10 mM) of the formulation, it seems surprising that such amounts of the anesthetic (1:3–2:1 EDC:lipid or 1:2–3:1 EDC:HSPC, molar ratios) would not disturb the membrane, since previous work have shown significant reduction in the S values after insertion of uncharged local anesthetics (lidocaine, prilocaine) in the bilayers (21). Nonetheless, EDC seems to have a lower disorganizing effect than lidocaine and prilocaine, as evaluated by the $h_{+1}/h_0$ ratio in the EPR spectra of the 5-doxyl methyl stearate probe in EPC liposomes (21). Moreover, the prevalent species of EDC at pH 6.2 is the protonated (charged) one,

**Table III** EPR: Segmental order parameter (S) values measured in the membrane of ionic-gradient-liposomes doped with 0.8 mol% 5-SASL, and exposed to increasing etidocaine concentrations. [total lipid] = 10 mM

| [EDC] total (mM) | 0 | 8 | 16 | 32 | 48 |
|---|---|---|---|---|---|
| [EDC] inside the membrane (mM) | 0 | 3.2 | 6.4 | 12.8 | 19.2 |
| Order parameter (S) | 0.82 | 0.83 | 0.83 | 0.84 | 0.84 |

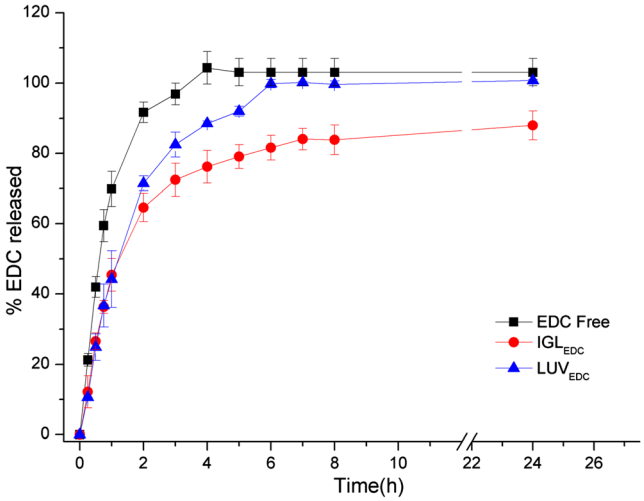


**Fig. 6** *In vitro* release of EDC (16 mM), free or encapsulated into conventional ($LUV_{EDC}$) or ionic-gradient ($IGL_{EDC}$) liposomes, as determined at 37°C.

which interacts less strongly with the membrane. So, the EDC molecules inside the liposomes should comprise the anesthetic molecules embedded in the membrane plus those solubilized in the inner aqueous IGL compartment.

In fact, the lack of changes in membrane fluidity observed here was acknowledged, because it indicates that etidocaine will not disrupt the vesicles nor impair the ionic-gradient. EPR data proved the high compactness of the IGL formulation and that EDC does not destabilize it, helping in the stabilization of the ionic-gradient, responsible for the prolonged release of the anesthetic.

## Release Assays

The liberation of etidocaine from the $IGL_{EDC}$ formulation was compared to that of a hydrochloride solution (EDC free), in release kinetic essays (Fig. 6). The amount of released EDC was quantified using the validated HPLC method.

The curve to the left in Fig. 6 reveals that 100% of the anesthetic in solution (EDC free) was released after 3 h, in agreement with results obtained with other LA agents such as ropivacaine (15) and dibucaine (45). On the other hand, it took ca. 6 h for the total release of etidocaine from conventional liposomes ($LUV_{EDC}$), and more than 24 h with the $IGL_{EDC}$ formulation, indicating the ability of the liposomal systems to promote sustained drug liberation (12), mainly ionic-gradient liposomes (20,43–45). As expected, an initial burst release, probably related to the unencapsulated EDC fraction and that is essential for the onset of anesthesia was observed in the first hour of the experiment, both for $IGL_{EDC}$, and $LUV_{EDC}$.

The EDC free, $LUV_{EDC}$ and $IGL_{EDC}$ curves were analyzed with several kinetic models (31) and the best fittings ($R^2 = 0.99$ for the three curves) were found with the Weibull treatment. Accordingly to eq. 8 the release exponent b values for the liposomal systems were: 0.34 for $LUV_{EDC}$, and 0.30 for $IGL_{EDC}$. For both liposomal systems it was observed an exponential release decay after the initial burst effect, what is compatible with Fickian diffusion and sustained release of etidocaine from the liposomes (46). The prolonged (more than a day) release achieved with $IGL_{EDC}$ is explained by its increased EDC encapsulation efficiency (40%) over $LUV_{EDC}$ (11%).

## Cytotoxicity Tests

The viability of cultured human fibroblast cells exposed to different concentrations of EDC, either free or encapsulated into IGL has been evaluated (Fig. 7).

The different times tested took into consideration that literature reports diverse possible mechanisms for the intrinsic cytotoxicity of local anesthetics: membrane solubilization (47), dose-dependent inhibition of electron-transfer chain mitochondrial enzymes (48), or apoptosis (DNA fragmentation) induction (49). Figure 7 shows that cell survival decreased in

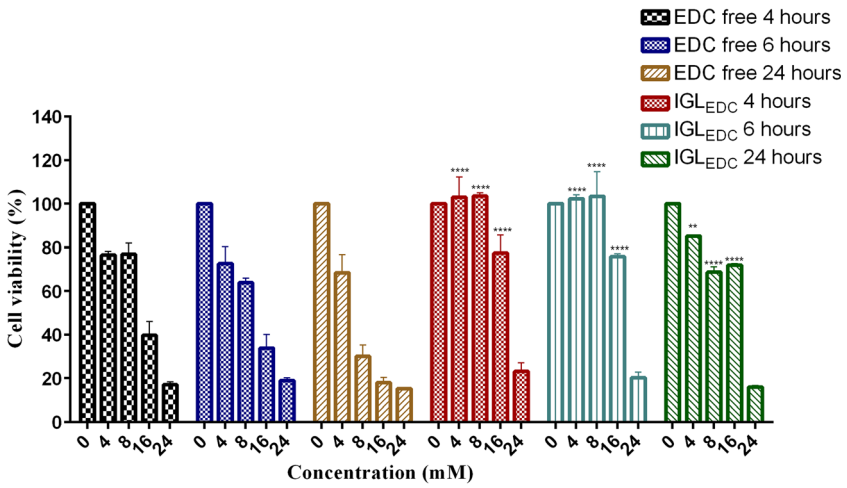


**Fig. 7** Cell viability (%) of human fibroblasts exposed to increasing EDC concentrations, either free or encapsulated into ionic-gradient liposomes, for 4, 6 or 24 h. Statistical analysis: EDC free *vs.* $IGL_{EDC}$ at equivalent condition, unpaired t-test (**$p < 0.01$, ****$p < 0.001$).

a (EDC) concentration, and time-dependent manner. As expected, IGL was not cytotoxic to the cells, confirming the literature data about liposomes safety (12,50).

After 4 h treatment there was a significant difference ($p < 0.05$) between the groups treated with 4 and 8 mM EDC: the cell viability of EDC free treated group was ca. 80% and that of $IGL_{EDC}$ sample was 100%. At 16 mM etidocaine the cell survival of $IGL_{EDC}$ (80%) was twice that of EDC free (40%), even after 6 h of treatment. Within 24 h, the cells treated with $IGL_{EDC}$ in the concentration 4, 8 and 16 mM remained 80–65% viable, while with EDC-free at equivalent concentrations cell viability fallen to 75–20%.

In terms of the half maximal inhibitory concentration ($IC_{50}$), in the groups treated for 4 h, 6 h and 24 h ($IC_{50}$ = 14, 12 and 6 mM in EDC free samples, respectively) there was an increase (to 20, 20 and 19 mM, respectively) in $IGL_{EDC}$ samples. Therefore in any of the observed times, $IGL_{EDC}$ formulations induced a significant increase in human fibroblasts survival, as previously observed in 3T3 cells, for ropivacaine encapsulated in ionic- and pH- gradient liposomes (15).

## CONCLUSIONS

An IGL formulation containing 0.5% EDC was successful prepared. The liposomes had suitable structural properties for drug-delivery and were found stable for up to 60 days of storage under refrigeration. The ammonium sulfate gradient increased the upload capacity of the liposomes (EE% = 40%) and EPR results confirmed the higher degree of bilayer order (S > 0.8), necessary to sustain the ionic-gradient. The bilayer packing was maintained even in the presence of high salt concentration, or after addition of EDC.

$IGL_{EDC}$ significantly prolonged *in vitro* the release time of etidocaine (24 h) and reduced the intrinsic cytotoxic effect of etidocaine over human fibroblasts in culture.

Altogether the promising results achieved with $IGL_{EDC}$ (upload of high amounts of etidocaine associated with sustained release and reduced cytotoxicity) support the idea of reintroducing EDC in surgical procedures, post-operative and chronic pain treatment. Further tests are still in progress to evaluate the *in vivo* (local and systemic toxicity, plus antinociceptive) effects of EDC encapsulated into IGL.

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