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Research Paper

Anaesthetic benefits of a ternary drug delivery system (Ropivacaine-in-Cyclodextrin-in-Liposomes): *in-vitro* and *in-vivo* evaluation

Ana Laís Nascimento Vieira[a], Michelle Franz-Montan[b], Luís Fernando Cabeça[c] and Eneida de Paula[a]

[a]Department of Biochemistry and Tissue Biology, Institute of Biology, Campinas [b]Department of Physiological Sciences, Piracicaba Dental School, University of Campinas – Unicamp, Piracicaba and [c]Department of Chemistry, Federal Technological University of Paraná, Londrina, Brazil

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Correspondence
Eneida de Paula, Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, UNICAMP, Rua Monteiro Lobato 255, 13083-862 Campinas, São Paulo, Brazil.
E-mail: depaula@unicamp.br

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Abstract

Objectives To evaluate whether a ternary system composed of hydroxypropyl-β-cyclodextrin (HP-βCD) further encapsulated into egg phosphatidylcholine liposomes (LUV) could prolong the action and reduce the toxicity of ropivacaine (RVC).

Methods Dynamic light scattering and NMR were used to characterize the inclusion complex (RVC : HP-βCD), liposomal (RVC : LUV) and ternary (LUV : RVC : HP-βCD) systems containing 0.25% RVC. Their encapsulation efficiency, release kinetics, *in-vitro* cytotoxicity and *in-vivo* anaesthetic effect (paw-withdraw tests in mice) were also evaluated.

Key findings 1 : 1 RVC : HP-βCD inclusion complex was encapsulated in liposomes (220.2 ± 20.3 nm size, polydispersity <0.25, zeta potentials = −31.7 ± 1.4 mV). NMR (diffusion-ordered spectroscopy (DOSY)) revealed stronger anaesthetic binding to LUV : RVC : HP-βCD (K_a = 342 M^{-1}) than to RVC : HP-βCD (K_a = 128 M^{-1}) or liposomal formulation (K_a = 22 M^{-1}). The formulations promoted *in-vitro* sustained drug release and partially reverted the cytotoxicity of RVC against 3T3 fibroblasts in the profile: LUV : RVC : HP-βCD ≥ RVC : HP-βCD > RVC : LUV. Accordingly, *in-vivo* sensory block of free RVC (180 min) was prolonged *ca*. 1.7 times with the ternary system and RVC : HP-βCD (300 min) and 1.3 times with RVC : LUV (240 min).

Conclusions These results confirm the suitability of this double-carrier system in clinical practice, to decrease the toxicity and prolong the anaesthesia time evoked by RVC.

Introduction

The pain relief provided by local anaesthetics (LA) came from their ability to bind to the sodium channel of excitable membranes, thus blocking sodium influx and the propagation of nervous impulse.[1–3] The desirable characteristics for an anaesthetic molecule include long duration of action, reduction of local and systemic toxicity and increased selectivity for sensory nerve block.[4]

Ropivacaine (RVC) belongs to the cyclic aminoamide local anaesthetics family. Within this series, RVC has longer action than mepivacaine and is less toxic to the CNS/CVS[5] than bupivacaine. Studies in pigs showed that RVC caused less hemodynamic repercussions than bupivacaine when equivalent doses were intravenously injected.[6] Furthermore, studies in animals and humans suggest that RVC produces less motor block compared to bupivacaine, given its greater selectivity on the sensory nerve fibres,[7] since it is synthesized and marketed in the *S* isomer form.[2]

The use of sustained-release formulations has been proposed to improve solubility and bioavailability of low solubility anaesthetics, including systems based on cyclodextrins[8] and liposomal formulations.[9] In fact, among the commercially available dosage forms and patents for carrying of LA, there are many formulations based on these two carriers.[10] Considering the possibility to enhance the potency of RVC,

liposomal and cyclodextrin inclusion complexes have been developed as described in the literature.[11–16]

An innovative approach proposed by Loukas *et al.*[17] was the combination of these two carriers (liposomes and cyclodextrins) in an optimized ternary system. Drug-in-CD-in liposomes have been proven potential carriers to increase the encapsulation efficiency, prolonging the release and modulating the pharmacokinetics and biological activity of hydrophobic drugs.[18] As for local anaesthetics, previously reports in the literature showed that prilocaine, benzocaine and butamben when incorporated in such ternary system exhibited faster onset of anaesthesia, sustained release and significant improvement of both intensity and duration of the *in-vivo* anaesthetic effect in rabbit and pig models.[19,20]

In this way, we tested the hypothesis of whether a double-carrier system for the local anaesthetic RVC, containing 1 : 1 RVC : hydroxypropyl-β-cyclodextrin inclusion complex encapsulated into extruded egg phosphatidylcholine liposomes (Figure 1) would be able to prolong the kinetics of RVC release, lower its systemic toxicity and increase its antinociceptive activity. Besides the novelty of the ternary system *per se*, we were curious to see if RVC, that has previously shown to take advantages from both carriers, separately,[13,14] would equally benefit from liposomes and cyclodextrins, in the same formulation.

Materials & Methods

Ropivacaine hydrochloride (98.5% pure) was donated by Cristália Prod. Quím.Farm. Ltda (Itapira-SP, Brazil); HP-βCD (Kleptose HP®) was obtained from Roquette Serv.-Tech. Lab (Lestrem-Cedex, France). Egg phosphatidylcholine, EPC (purity >99%), α-tocopherol and HEPES buffer were purchased from Sigma Chem. Co. (St. Louis-MO, USA); acetonitrile (HPLC grade) was from Merck® (Darmstadt, Germany).

Ropivacaine quantification

Quantification of RVC was done by HPLC in a Merck-Hitachi equipment with autosampler and diode array detector. For data collection and calculation, the EzChrom Software was used. The chromatographic conditions were optimized in a C18 column (Purospher Star RP 18 end-capped – 150 × 4.6 mm – particle size 5 μm; Merck). The mobile phase (acetonitrile : phosphate buffer pH 8.0, 60 : 40 *v* : *v*) was filtered through 0.45 μm Millipore® (Bedford, MA, USA) membrane filters. The measurements at 30°C were conducted at 240 nm, at a flow rate of 1.2 ml/min, injection volume = 10 μl; the samples containing RVC (0.08 or 1.16 mM) were diluted in 20 mM HEPES buffer pH 7.0.[21]

RVC : HP-β cyclodextrin inclusion complexation

Solid inclusion complexes in 1 : 1 stoichiometry were obtained by mixing appropriate amounts of HP-βCD and RVC in water, as previously reported.[14] The samples were shacked for 24 h at room temperature. The suspension was then lyophilized and stored for later use.[8,22] The RVC : HP-βCD complex was obtained by suspension of the freeze-dried powder in HEPES buffer (20 mM HEPES pH 7.0 with 150 mM NaCl) to a final concentration of 8 mM (0.25%) RVC.

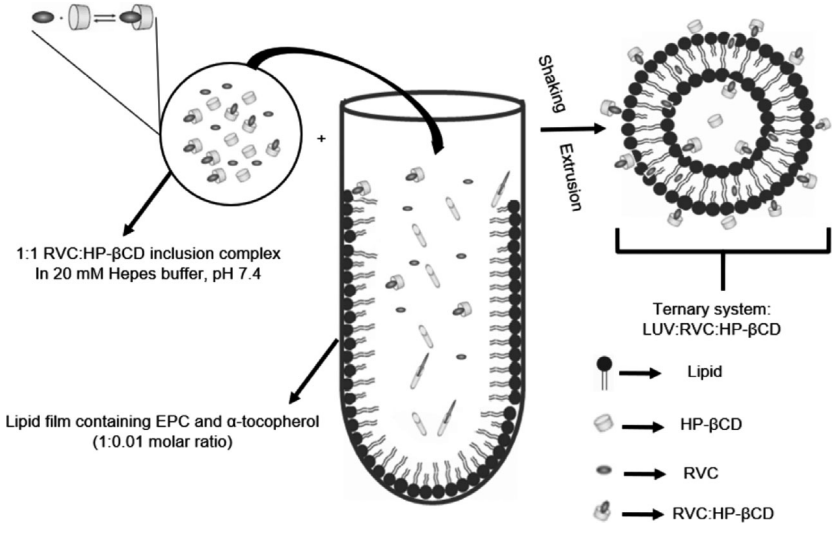

Figure 1 Schematic representation of the LUV : RVC : HP-βCD formulation.

Liposomes preparation

Liposomes composed of EPC plus α-tocopherol (1 mol%, used to prevent lipid peroxidation) were prepared by the dry film method.[13] Briefly, the lipid film was suspended in HEPES buffer, containing or not (LUV) ropivacaine, free (RVC : LUV) or complexed with cyclodextrin (LUV : RVC : HP-βCD). Large multilamellar vesicles were obtained by vortexing (3 min). Further extrusion trough polycarbonate membranes (0.4 nm; Poretics, Livermore, CA, USA) under N_2 flux (40 psi or 3.0 kgf/cm^2) at room temperature gave rise to large unilamellar vesicles (LUV). The final phospholipid concentration was set to 8 mM, measured according to Brito *et al.*[23] The final RVC concentration was also 8 mM (0.25%).

Size, polydispersity and zeta potential of liposomes

Dynamic light scattering (DLS) measurements were performed in liposomes, in the presence and absence of RVC (free or complexed with HP-βCD), to verify the mean size, polydispersity (PDI) and zeta potential (ZP) of the vesicles, before and after RVC encapsulation (n = 3). The size (in scattered light intensity), PDI and ZP were obtained at 25°C with a detection angle of 173° in a Zeta Sizer NanoZS (MALVERN Instr. Co, Worchestershire, UK) equipment. The mean size, PDI and ZP of the liposomes were followed at time zero and after 15, 30 and 60 days of storage at 4°C. The formulations were diluted in Milli-Q (5 : 955, v : v), in triplicates.

Encapsulation efficiency of ropivacaine in liposomes

The encapsulation efficiency (%EE) of RVC, free and complexed with HP-βCD, was determined by phase separation of liposomes (submitted to ultracentrifugation at 120 000 *g*, for 2 h at 10°C). Aliquots of the supernatant were analysed by UV absorption at 260 nm[13] and subtracted from the initial (total RVC) in the sample, according to:

$$\%EE = [\text{encapsulated RVC}]/([\text{total RVC}]) \times 100 \quad (1)$$

Transmission electron microscopy

Transmission electron microscopy (TEM) images were obtained in a Zeiss Leo 906 equipment, to access the morphology of RVC : LUV and LUV : RVC : HP-βCD samples. One drop of each formulation was added to a gold grid. After 10 s, excess volume was withdrawn with filter paper, and a drop of (2% *w*/*w*) aqueous uranyl solution was added to improve the contrast. After 8 s, the excess was withdrawn and a drop of Milli-Q water was added to the grid, with removal of the excess volume after 5 s.

Nuclear magnetic resonance: diffusion-ordered spectroscopy measurements

Ropivacaine association with cyclodextrin and liposomes was studied by hydrogen nuclear magnetic resonance (^{1}H-NMR). In the diffusion-ordered spectroscopy (DOSY)-NMR experiments, the diffusion coefficient ($D_{observed}$) of ligands associated to macromolecular systems (cyclodextrin or liposomes) can be measured. $D_{observed}$ reflects the weighted average of the coefficients of the populations of the species in exchange (macromolecular system and drug), according to Equation 2 [24]:

$$D_{observed} = (f_{bound}.D_{macromolecule}) + (f_{free}.D_{free}) \quad (2)$$

Assuming that the exchange occurs between a free and bound state (drug complexed or encapsulated in the macromolecule), the fraction of drug either free or bounded is represented by f_{free} and f_{bound}, such that D_{free} and D_{bound} are the diffusion coefficient of the free ligand and of the macromolecular complex, respectively. The bound fraction of the drug (f_{bound}) and the association constant (K_a) can be calculated from Equations 3 and 4, respectively:

$$f_{bound} = \frac{(D_{free} - D_{observed})}{(D_{free} - D_{macromolecule})} \quad (3)$$

$$Ka = \frac{f_{bound}}{\{(1 - f_{bound}).([\text{macromolecule}] - (f_{bound}.[\text{drug}]))\}} \quad (4)$$

The spectra were run in a Varian 600 MHz spectrometer at the Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil), at 25°C. The following samples were used in the study: RVC : HP-βCD; RVC : LUV, LUV : RVC : HP-βCD, and their controls: RVC, HP-βCD and LUV. Freeze-dried samples were suspended in 20 mM phosphate buffer pH 7.0 prepared in deuterated water (D_2O) at the time of use. DOSY spectra were obtained using a DgcteSL (gradient compensated stimulated echo spin lock) pulse sequence[25] with a diffusion time of 0.06 s. The processing program (macro DOSY) used fn = 32 K.

Estimation of phospholipid peroxidation

Lipid peroxidation of the liposomes (RVC : LUV, LUV : RVC : HP-βCD, and LUV) was measured by the

thiobarbituric acid test[26] in freshly prepared samples and after 15, 30 and 60 days of storage at 4, 25 and 37°C. A calibration curve was determined with tetraethoxypropane (0.01–0.12 mM).

In-vitro release tests

Release Kinetic tests were performed at 37°C with RVC either free, complexed with cyclodextrins or encapsulated in liposomes as described before.[27] 1000 Da molecular exclusion cellulose membranes (Spectrapore®) were used. Samples (RVC, RVC : HP-βCD, LUV : RVC, and LUV : RVC : HP-βCD) were withdrawn from the receptor compartment (with replacement of the volume – 300 μl) at the following times: 15 and 30 min, 1, 2, 3, 4, 6 and 8 h, and analysed by HPLC as described (item 3.1).

In-vitro toxicity evaluation

In-vitro tests were conducted on Balb/c 3T3 mouse fibroblasts. The cells were incubated for 2 h with either LUV or HP-βCD, RVC, RVC : HP-βCD, LUV : RVC or LUV : RVC : HP-βCD at concentrations from 0.4 to 7.2 mM RVC. The formed formazan was detected at 570 nm.[28]

In-vivo assays: pharmacological evaluation

Swiss albino mice (25 g) were submitted to 12-h light–dark cycles, with water and feed *ad libitum*. All experiments were in accordance with the Ethical Principles in Animal Experimentation adopted by the Brazilian Society of Laboratory Animal Science (SBCAL), which followed the Helsinki principles, and were approved by the Committee on Ethics in the Use of Animals of the University of Campinas (CEUA/Unicamp), protocol #2596-1. The following groups (seven animals/group) were analysed: LUV, HP-βCD and LUV : HP-βCD (controls), 0.125% RVC; 0.25% RVC; 0.125% LUV : RVC; 0.25% LUV : RVC; 0.125% RVC : HP-βCD; 0.25% RVC : HP-βCD; 0.125% LUV : RVC : HP-βCD and 0.25% LUV : RVC : HP-βCD.

The intensity of the motor block was evaluated according to the score: 0 (normal use of the paw), 1 (inability to fully flex the limb) and 2 (inability to use the paw).[29,30] The evaluation was done every minute during the first 5 min after administration and later, in intervals of 5–10 min until total recovery of movements (minimum 1 h). The measured parameters were the onset of blockage (in s), the duration ($T_{recovery}$) and the potency of motor block, from the area under the curve, AUC.[13]

To measure the sensory block, the paw-removal threshold of the animals against a mechanical stimulus was used. The test was performed with a gradual increase of force (g) on the dorsal surface of the animal's paw.[31] A maximum cut-off of 150 g was established to avoid foot injury. The measurements were carried out fixed (20, 30, 40, 60, 90, 120, 150, 180, 240 and 300 min) intervals.

Statistics

Size, polydispersity, zeta potentials, endoperoxides concentration of fresh and throughout storage (15, 30 and 60 days) liposomes, *in-vitro* release kinetics, cell viability assay and *in-vivo* sensory blockade were analysed by ANOVA followed by Tukey's *post hoc* test. *In-vivo* motor blockade was evaluated by Kruskal–Wallis. The significance level was set at 5%.

Results

A successful liposomal formulation for the upload of ropivacaine has been previously reported, with vesicles composed of EPC and cholesterol.[13,32] But since cholesterol is able to complex with cyclodextrins to form inclusion complexes that destabilize lipid membranes,[18,33] in this work we prepared liposomes without cholesterol.

Size, polydispersity, zeta potential and encapsulation efficiency of the liposomes

Table 1 shows the mean vesicle size, polydispersity and ZP of the prepared extruded liposomes, as determined by DLS.

The low polydispersity index (PDI < 0.25) indicated good homogeneity of the particles' population,[34,35] and no significant changes in the size of the liposomes was

Table 1 Average size, polydispersity (PDI), zeta potential (ZP) and encapsulation efficiency of the extruded liposomal formulations composed of EPC : α-tocopherol 1 : 0.01 mol%. $n = 3$, $T = 25$°C

Sample	Size (nm)	PDI	ZP (mV)	%EE
LUV	221.4 ± 6.2	0.252 ± 0.004	−20.8 ± 1.0	–
LUV : RVC	224.2 ± 19.7	0.256 ± 0.016	−18.6 ± 1.5	25.0 ± 0.8
LUV : HP-βCD	244.3 ± 9.2	0.242 ± 0.011	−12.1 ± 0.7	–
LUV : RVC : HP-βCD	220.2 ± 20.3	0.250 ± 0.010	−31.7 ± 1.1*	33.0 ± 2.4

Statistical analysis – ANOVA/Tukey–Kramer. *$P < 0.05$: LUV : HP-βCD vs LUV : RVC : HP-βCD.

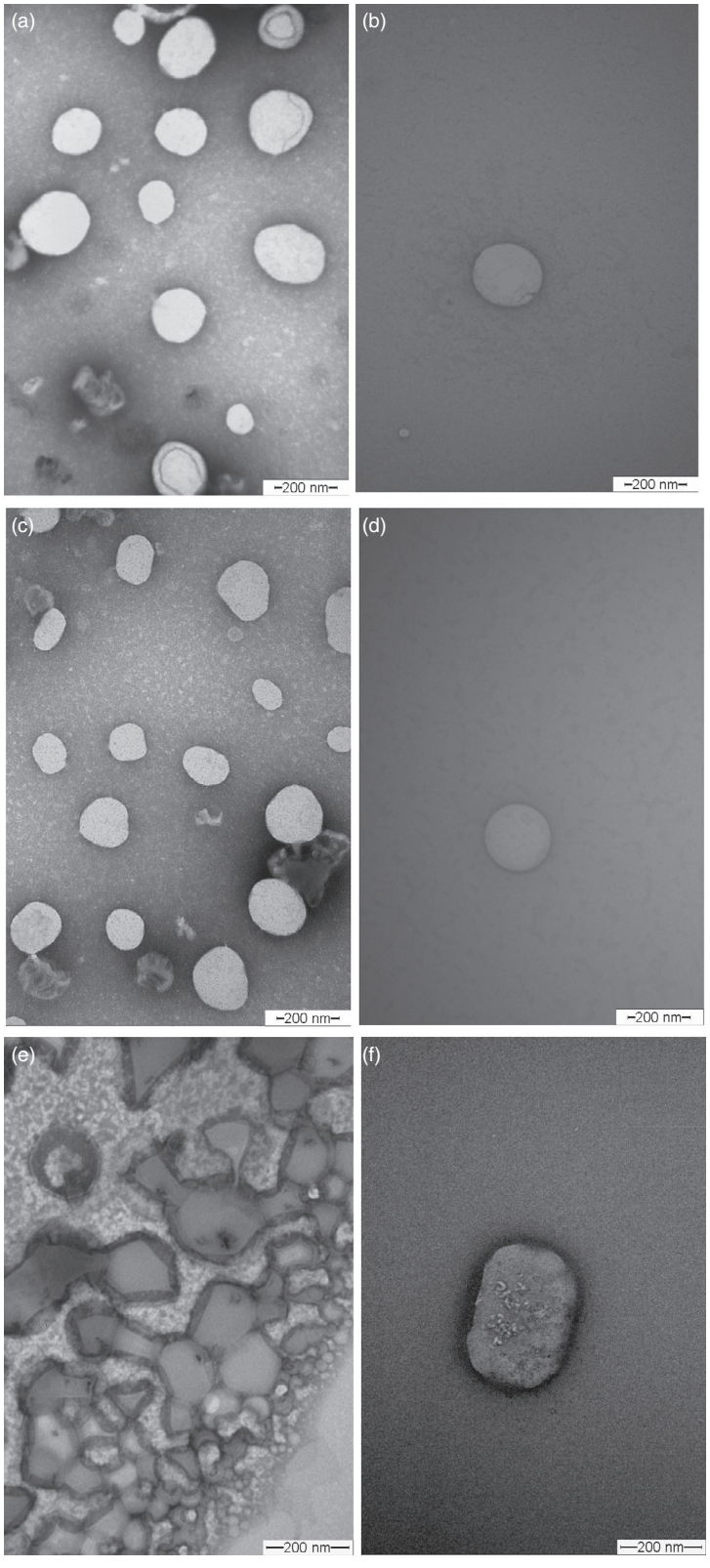

Figure 2 TEM images: (A) LUV (60 000×); (B) LUV (100 000×); (C) LUV : RVC (60 000×); (D) LUV : RVC (100 000×); (E) LUV : RVC : HP-βCD (60 000×); (F) LUV : RVC : HP-βCD (100 000×).

observed after encapsulation of RVC or the inclusion complex (RVC : HP-βCD).

Regarding zeta potentials, addition of RVC did not change the negative surface charge of the liposomes (LUV vs LUV : RVC). When HP-βCD alose was added to the liposomes (LUV : HP-βCD) the zeta value decreased in modulus |12.1 mV|, showing that the cyclodextrin somehow interact with the vesicles. Fortunately, in the ternary system (LUV : RVC : HP-βCD) the surface charge of the vesicles was larger in modulus |31.7 mV|, confirming the stability of the formulation.

The encapsulation efficiency of the anaesthetic by LUV : RVC determined by phase separation at pH 7.0 (25.0 ± 0.8%, Table 1) significantly increased (33.0 ± 2.4%) in the presence of cyclodextrin.

Liposomes morphology

Figure 2 shows TEM images – in two magnifications – of liposomes prepared without (Figure 2A and 2B), with RVC (Figure 2C and 2D) and in the presence of RVC and HP-βCD (Figure 2E and 2F). Vesicles of spherical shapes and *ca*. 200 nm, compatible to the sizes determined by DLS (Table 1), were detected. Neither RVC nor the presence of HP-βCD in the formulations significantly changed the vesicles size, although the morphology of the vesicles became spherical in the ternary system (Figure 2E and 2F).

Stability analysis of liposomes during storage

To check for the chemical stability of the EPC molecules in the liposomes, lipid peroxidation tests were performed at 4,

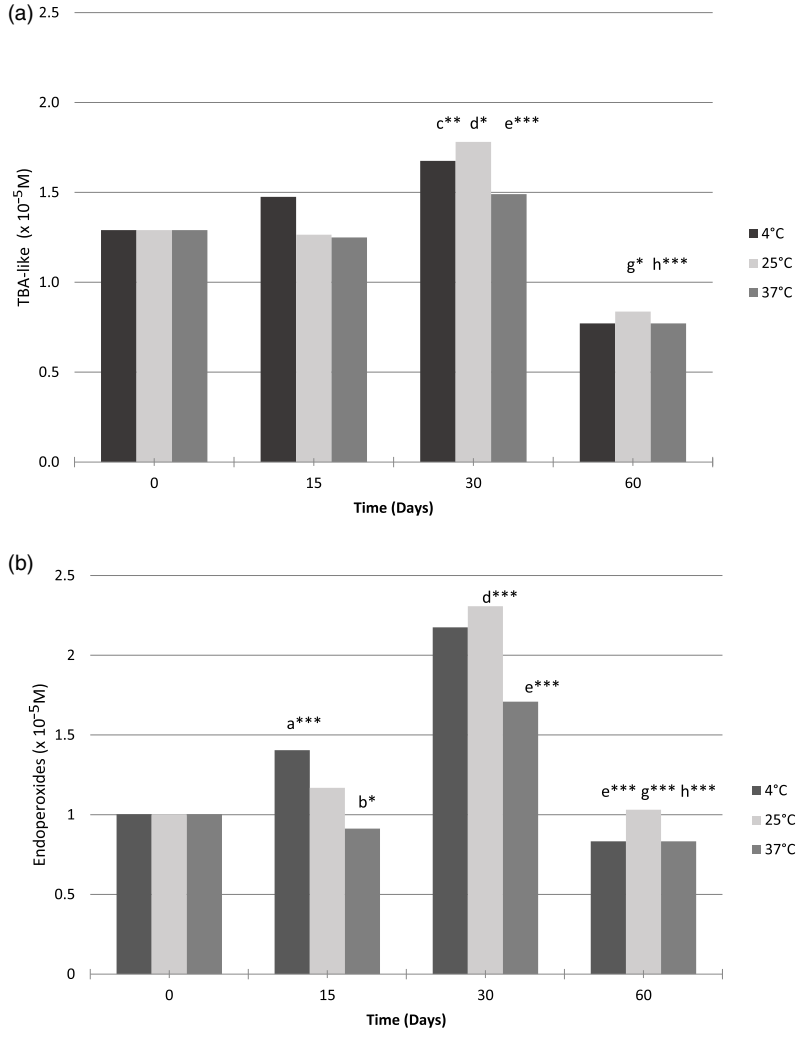

Figure 3 Endoperoxides concentration in LUV : RVC (A) and LUV : RVC : HP-βCD samples (B) stored at 4, 25 or 37°C. Statistical analysis by ANOVA/Tukey–Kramer: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (a) initial vs 15 days, 4°C; (b) initial vs 15 days, 37°C; (c) initial vs 30 days, 4°C; (d) initial vs 30 days, 25°C; (e) initial vs 30 days, 37°C; (f) initial vs 60 days; 4°C (g) initial vs 60 days, 25°C; (h) initial vs 60 days, 37°C.

25 and 37°C. The results showed significant increases in lipid peroxidation levels after 60 days, both for LUV : RVC (Figure 3A) and LUV : RVC : HP-βCD (Figure 3B), at the different temperatures (4, 25 and 37°C). Despite of that, the concentration of endoperoxides in LUV never exceeded 2.10^{-5} mol/l in LUV : RVC (3.10^{-5} mol/l in LUV : RVC : HP-βCD), that is 0.5% of the total lipids in the formulation (8 mM), indicating good chemical stability of the liposomes.[13] Moreover, temperature variations (4, 25 and 37°C) did not influence the formation of endoperoxides in any of the formulations.

The size and ZP of the main population of liposomes in the formulations were followed at 4°C, at time zero and after 15, 30 and 60 days of storage (Table 2).

While LUV : RVC and LUV : HP-βCD samples showed vesicles with significantly higher diameters after 60 days, the average size of the liposomes in the ternary system was slightly smaller ($P < 0.001$), a change possible explained by their less spherical (squared) shapes – see Figure 2, not appropriate for DLS measurement.[36] Nevertheless, the ternary system presented the higher proportion (99.2%) of vesicles with the average size, in relation to other formulations. Finally, and as sign of the physical stability of the liposomes, for all the samples ZP values were kept high, in modulus, even after 60 days of storage (Table 2).

Interaction of RVC with cyclodextrin and liposomes, determined by ^{1}H-NMR

In a previous work, using ^{1}H(DOSY)NMR experiments we have shown that the interaction of RVC with EPC liposomes (K_a = 22 l/mol) was weaker than that with HP-βCD (K_a = 128 l/mol).[21] As for the ternary system, Table 3 shows that the association constant of RVC increased to 342 l/mol, confirming the synergistic effect between both carriers (liposomes and cyclodextrin) towards the anaesthetic upload.

In-vitro release tests

The cumulative *in-vitro* release of RVC, either free, complexed with HP-βCD, encapsulated into liposomes or in the ternary system is given in Figure 4.

All the formulations promoted sustained release of RVC in comparison to free RVC (in solution), for which 50% release was achieved after 30 min, in good agreement with the literature.[14,17] The time for 50% release was prolonged to 2 and 4 h, when the anaesthetic was encapsulated in LUV : RVC and RVC : HP-βCD systems, respectively. Once local anaesthetics are regionally applied, these *in-vitro* results indicate that the formulations will probably provide increased drug concentration at the site of action, prolonging anaesthesia. Among the formulations, the profile of the

Table 2 Mean diameters and zeta potentials (ZP) of the main population of liposomes in LUV, LUV : HP-βCD, RVC : LUV and LUV : RVC : HP-βCD samples, measured by DLS during 60 days of storage at 4°C; mean and standard deviation (n = 3)

Time of storage:	0 day		15 days		30 days		60 days	
Sample	Diameter (nm) Proportion (%)	ZP	Diameter (nm) Proportion (%)	ZP	Diameter (nm) Proportion (%)	ZP	Diameter (nm) Proportion (%)	ZP
LUV	221.4 ± 6.2 (100)	−20.8 ± 0.9	220.9 ± 18.5 (98.3 ± 1.7)	−25.5 ± 2.5	202.3 ± 15.4 (98.3 ± 1.6)	−28.3 ± 1.4	213.4 ± 66.5 (97.3 ± 4.0)	−40.7 ± 2.9
LUV : HP-βCD	244.3 ± 9.2 (100)	−12.1 ± 0.7	232.8 ± 22.0 (97.4 ± 2.4)	−18.7 ± 0.3	186.9 ± 9.1 (98.7 ± 1.4)	−35.6 ± 2.5	291.3 ± 19.8 (91.8 ± 4.3)	−46.1 ± 0.6
LUV : RVC	224.2 ± 19.7 (100)	−18.6 ± 1.5	212.4 ± 12.9 (98.5 ± 2.2)	−13.0 ± 0.8	186.7 ± 12.4 (98.8 ± 1.2)	−27.8 ± 1.5	357.1 ± 16.2$^{b\ ***}$ (92.9 ± 8.6)	−46.9 ± 1.9$^{b\ **}$
LUV : RVC : HP-βCD	220.2 ± 20.3 (99.6 ± 0.6)	−30.7 ± 1.1	188.9 ± 19.3 (98.5 ± 1.3)	−22.6 ± 10	166.7 ± 4.3$^{a\ *}$ (99.2 ± 1.2)	−31.7 ± 2.3	162.1 ± 9.6$^{b\ ***}$ (98.9 ± 1.1)	−32.1 ± 3.7

^{a}Initial vs 30 days. ^{b}Initial vs 60 days. ^{g}*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$: Statistical analysis by ANOVA/Tukey–Kramer.

Table 3 Diffusion coefficients (D) measured by ^{1}H-DOSY-NMR for RVC, carriers (HP-βCD, LUV), and formulations (RVC : HP-βCD, LUV : RVC and LUV : RVC : HP-βCD. The fraction of RVC bound to the carrier (molar fraction) and the association constants (K_a) were measured using Equations 4 and 5

Sample	D (10^{-10} m^2/s)	Molar fraction (%)	K_a (l/mol)
RVC	5.21 ± 0.04	–	–
HP-βCD	2.47 ± 0.01	–	–
LUV	0.80 ± 0.02	–	–
RVC : HP-βCD	4.47 ± 0.01	27	128[a]
LUV : RVC	4.61 ± 0.02	14	22[a]
LUV : RVC : HP-βCD	4.20 ± 0.01	36	342

The fraction of RVC bound to the carrier (molar fraction) and the association constants (K_a) were measured using Equations 4 and 5. [a]As previously determined.[21]

equilibrium was RVC < LUV : RVC < RVC : HP-βCD ≤ LUV : RVC : HP-βCD. No significant change in the release rate of the anaesthetic ($P > 0.05$) was observed when the ternary system was compared with RVC : HP-βCD (Figure 4).

In-vitro cytotoxicity of the formulations

Figure 5 shows the results obtained with *in-vitro* cytotoxic tests over 3T3 fibroblasts in culture.

The concentration-dependent toxicity of RVC was significantly reduced when the anaesthetic was encapsulated into liposomes, complexed with HP-βCD or inside the ternary system (Figure 5). Once more there was no significant changes ($P > 0.05$) between the effects of RVC : HP-βCD and LUV : RVC : HP-βCD.

Evaluation of sciatic nerve blockade

As shown in Table 4, after sciatic nerve infiltration, all formulations changed the motor function of treated animals, in a dose-dependent manner, with reversible loss of motor reflexes. But despite the apparent faster onset of anaesthesia observed with RVC : HP-βCD and LUV : RVC : HP-βCD, none of the formulations were able to significantly modify the onset, duration (T_{max}, $T_{recovery}$) or potency (area under the curve, AUC) of motor block, neither at 0.125 nor at 0.25% RVC. This is a very interesting result, since local anaesthetics are expected to selectively block sensory rather than motor pathways, and the formulations did not affect the motor block induced by the anaesthetic.[2]

As for the sensory block, both RVC : HP-βCD and LUV : RVC : HP-βCD group of animals showed significantly greater anaesthesia than the free drug and LUV : RVC. The infiltration of LUV : RVC : HP-βCD at 0.125 and 0.25% (Figure 6A and 6B, respectively) promoted equivalent effects, with statistical differences to free RVC, from 40 min ($P < 0.05$) up to 180 min ($P < 0.01$) after injection. The endpoint of induced analgesia by both RVC : HP-βCD complex and LUV : RVC : HP βCD at 0.25% occurred after 300 min (5 h).

Discussion

It has been previously shown that RVC was able to benefit both from liposomes[13] and cyclodextrin formulations.[14] To see if this local anaesthetic would also profit from the combination of both carrier systems, a novel formulation was prepared.

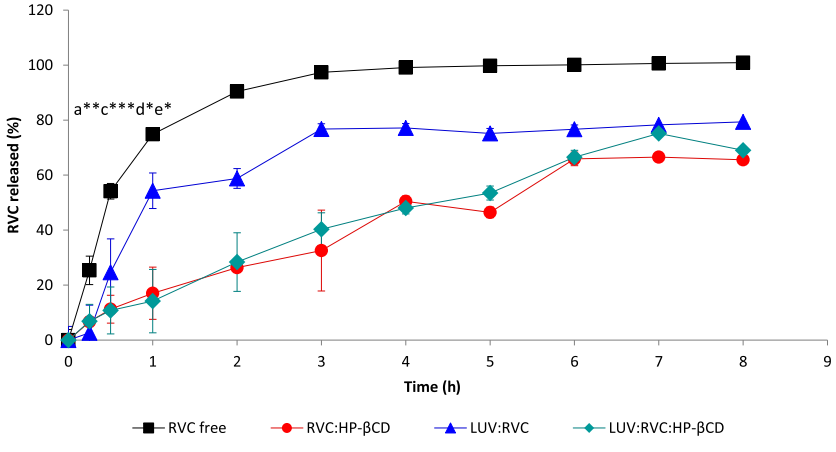

Figure 4 *In-vitro* release kinetics of RVC in solution (RVC_{free}), complexed with cyclodextrin (RVC : HP-βCD), encapsulated in liposomes (LUV_{RVC}) or in the ternary system (LUV : RVC : HP-βCD) at pH 7.0 and 37°C; (RVC) = (EPC) = (HP-βCD) = 8 mM. Data represented as mean ± SD ($n = 3$). Statistical analysis by ANOVA/Tukey–Kramer: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (a) RVC_{free} vs RVC : HP-βCD; (b) RVC_{free} vs LUV : RVC; (c) RVC_{free} vs LUV : RVC : HP-βCD; (d) RVC : HP-βCD vs LUV : RVC; (e) LUV : RVC vs LUV : RVC : HP-βCD.

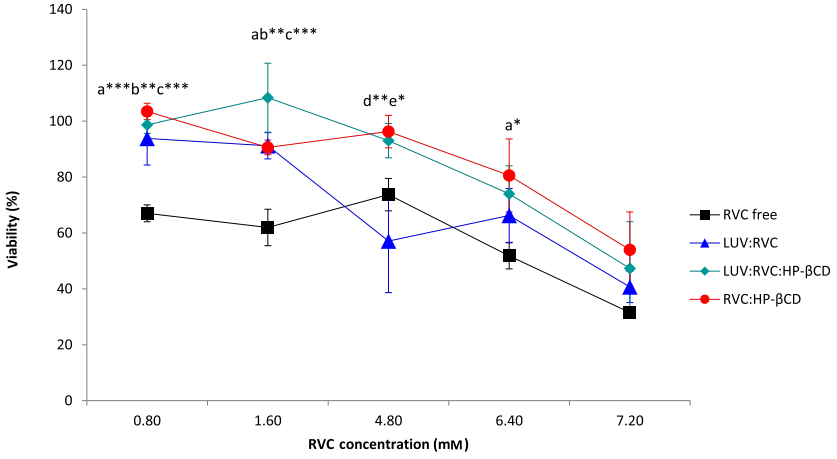

Figure 5 Cell viability assay. Statistical analysis by ANOVA/Tukey–Kramer: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (a) RVC_{free} vs RVC : HP-βCD; (b) RVC_{free} vs LUV : RVC; (c) RVC_{free} vs LUV : RVC : HP-βCD; (d) RVC : HP-βCD vs LUV : RVC; (e) LUV : RVC vs LUV : RVC : HP-βCD.

Table 4 Motor blockade of the sciatic nerve in mice, induced by RVC formulations

RVC (%)	Groups	Onset (s)	Tmax (min)	Trecovery (min)	AUC (score/min)
0.125	RVC	50.0 (20.0–60.0)	1 (1–3)	34.3 (20.0–50.0)	26.9 (14.0–44.0)
	LUV : RVC	40.0 (20.0–60.0)	1 (1–4)	27.1 (10.0–40.0)	26.9 (14.0–44.0)
	RVC : HP-βCD	25.0 (15.0–40.0)	1 (–)	22.9 (10.0–30.0)	15.8 (6.5–24.0)
	LUV : RVC : HP-βCD	25.0 (15.0–45.0)	1 (–)	21.4 (10.0–30.0)	12.2 (6.5–24.0)
	RVC	35.0 (30.0–50.0)	1 (1–4)	48.6 (40.0–60.0)	32.9 (14.5–46.5)
0.250	LUV : RVC	40.0 (25.0–60.0)	1 (1–5)	50.0 (40.0–60.0)	48.8 (24.0–108.0)
	RVC : HP-βCD	20.0 (15.0–25.0)	1 (1–5)	41.4 (30.0–50.0)	32.4 (14.0–45.5)
	LUV : RVC : HP-βCD	20.0 (10.0–25.0)	1 (1–5)	41.4 (30.0–50.0)	29.6 (14.0–48.0)

Data expressed in median (minimum and upper limits) (n = 7/group). Statistical analysis by Kruskal–Wallis: not significant.

Liposomes of spherical morphology, with average sizes (221.4 ± 6.2 nm) and low polydispersity (< 0.25), were successfully prepared. As expected, the average size of these cholesterol-free vesicles prepared was significantly smaller than those reported in the literature for EPC : cholesterol 3 : 2 mol% (356 ± 83 nm) liposomes,[13] reflecting the increased membrane fluidity of the EPC liposomes without the sterol.[37,38]

When HP-βCD was added to the liposomes (LUV : HP-βCD) the zeta values came close to zero showing rearrangement of the vesicles surface charges. That is explained by cyclodextrin interaction with molecules at the bilayer surface.[33] Luckily, greater ZP values (in modulus) were observed in the ternary system, confirming the stability of the LUV : RVC : HP-βCD formulation.

The encapsulation efficiency of RVC in the liposomes prepared without cholesterol (25.0 ± 0.8%, Table 1) was slightly higher than that observed in EPC : cholesterol liposomes (23%), confirming that RVC partition into the bilayer is favoured in the absence of cholesterol and very similar to that determined for bupivacaine in EPC : cholesterol vesicles (24.8 ± 4.2%), as expected by the related chemical structures of these local anaesthetics.[13] The level of encapsulation with the ternary system was higher (% EE = 33.0 ± 2.4%) than that achieved with the liposomal and cyclodextrin systems separately, a typical effect observed in drug-in cyclodextrin-in liposome systems[18–20] that is due to the increased drug solubility promoted by complexation with cyclodextrin. Although discrete, such enhancement in drug upload is enough to change the release kinetics of local anaesthetics, and to significantly improve their antinociceptive action, as observed in previous works.[39,40] Additionally, it is important to note that in such formulations the free anaesthetic fraction (not bound to any carrier) is responsible for the fast onset of anaesthesia.[20,40]

The higher upload capacity of the ternary system was confirmed by NMR experiments that proved that RVC interacts with both carriers (cyclodextrin and liposomes). The association constant determined for the ternary system LUV : RVC : HP-βCD increased relatively to the binary systems (RVC : HP-βCD and LUV : RVC), separately. NMR data clearly showed that RVC binds strongly to HP-βCD than to EPC liposomes (Table 3). Such higher affinity

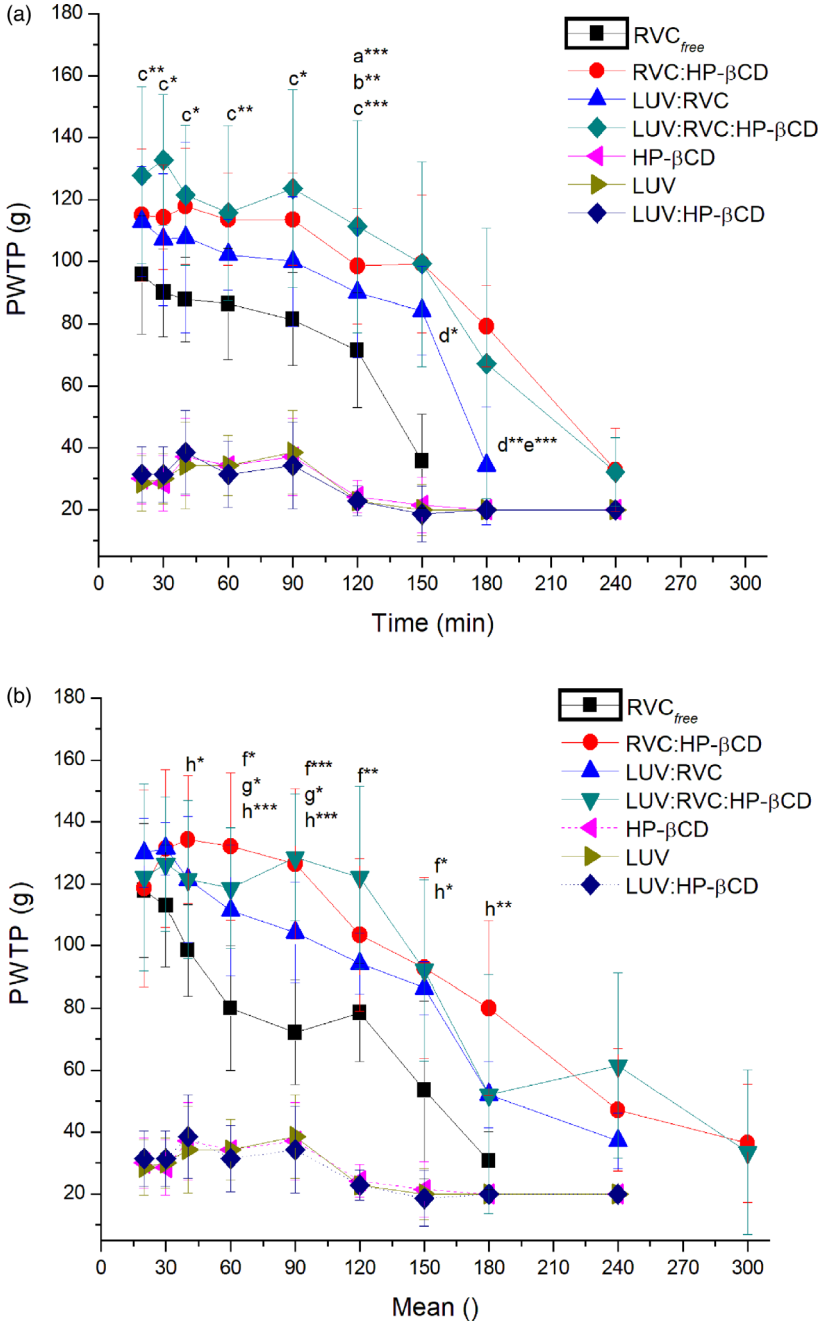

Figure 6 PWPT – Anaesthetic effect vs time: 0.125% (A) and 0.25% (B) RVC. Statistical analysis by ANOVA/Tukey–Kramer: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (a) RVC_{free} vs RVC : HP-βCD; (b) RVC_{free} vs LUV : RVC; (c) RVC_{free} vs LUV : RVC : HP-βCD; (d) RVC : HP-βCD vs LUV : RVC; (e) LUV : RVC vs LUV : RVC : HP-βCD.

for HP-βCD has also been observed for other local anaesthetics such as proparacaine[41] and prilocaine.[42] It explains why changes in release kinetics (Figure 4), cytotoxicity (Figure 5) and analgesia (Figure 6) were more influenced by cyclodextrin than by liposomes – see discussion below. At this point, the higher %EE and association constant pointed out advantages for the drug-in-cyclodextrin-in-liposomes formulation in relation to each individual carrier system.

Stability tests indicated no significant increase in the levels of endoperoxides in the formulation (<1% of the total lipid concentration) after 60 days (Figure 3), and DLS confirmed the stability of the vesicle for up to 60 days in the ternary system (Table 2).

RVC molecules partitioned in the lipid bilayer are expected to slowly dissociate in the aqueous media, extending its release time from liposomes.[4,13] In the release kinetic experiments (Figure 4), both charged and uncharged forms of RVC were present at pH (7.0), contributing to the fast equilibrium (in/out the bilayers) observed with RVC : LUV. Additionally, unilamellar liposomes without cholesterol were employed, facilitating RVC permeation trough the bilayer. As a result, the release of RVC from liposomes was faster than expected, and shorter than that observed with HP-βCD (Figure 4). Such prolonged release of the anaesthetic from HP-βCD over LUV formulation corroborates the stronger association of RVC to cyclodextrins, revealed by NMR (Table 3). Even though the interaction forces between guest-host molecules in cyclodextrin inclusion complexes are weak – mainly van der Waals interactions,[43] the results in Figure 4 show that HP-βCD more than liposomes was responsible for the lower sustained release of RVC from the ternary system.

Ropivacaine, as well as other local anaesthetics, had an intrinsic cytotoxic effect,[44,45] but complexation with cyclodextrin or encapsulation into the liposomes significantly decreased such cytotoxicity, as previously observed.[13,14,46–48] The protective effect of RVC : HP-βCD and LUV : RVC : HP-βCD formulations were similar and most evident (Figure 5), followed by RVC : LUV.

Interestingly, none of the formulations significantly change the duration and potency of motor block, neither at 0.125 nor at 0.25% RVC. This is a highly desirable result, since local anaesthetics are expected to selectively block sensory rather than motor pathways. Indeed, reports on the differential sensory-to-motor block by mepivacaine[49] and RVC[2] have been reported and attributed to their lower partition into lipids that could prevent them to penetrate the larger AB motor fibres.

Improvement in sensory block (shortest onset and prolonged duration) has been previously reported after encapsulation of local anaesthetics (prilocaine, benzocaine and butamben) in ternary system prepared with HP-βCD and deformable, multilamellar liposomes[19,20] Here, the analgesia tests in mice revealed significantly greater sensory block with the ternary system and RVC : HP-βCD than with LUV : RVC of free RVC treatment.

Surprisingly, the anaesthetic effect evoked by the ternary system did not surpass that of RVC : HP-βCD (no statistical differences between them, at 0.125 and 0.25% ropivacaine), although each carrier (HP-βCD and liposomes), independently, were found able to prolong RVC effect. The lack of a more pronounced anaesthesia with the ternary system (regarding RVC : HP-βCD) was also observed in the release kinetics and cytotoxicity tests, and it reflects the stronger interaction of RVC and HP-βCD revealed by NMR (K_a values). In a previous report,[42] similar constants were observed for the association of prilocaine with these two carrier systems: K_a = 36 for βCD and 21 l/mol for liposomes. Correspondingly, when prilocaine was incorporated in a ternary system, an improvement in anaesthesia was observed.[20]

In any case, the ternary system provided increased upload and sustained release of RVC, and prolonged the sensory block by 1.7 times in comparison to free RVC and 1.25 times in respect to LUV : RVC. This ternary drug delivery system of RVC opens perspective for future clinical use since it has the potential to promote long-lasting anaesthesia with reduced cytotoxicity.

Conclusion

A novel drug-in-cyclodextrin-in-liposomes formulation for the local anaesthetics RVC is proposed, that was found to be less cytotoxic, and to promote prolonged release and more effective anaesthesia than each drug delivery system, separately. The improvements achieved points the developed formulation as suitable for future therapeutic application.

Declaration of interest

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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