Journal of Controlled Release 160 (2012) 346–352

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

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

Prolonged analgesia from Bupisome and Bupigel formulations: From design and fabrication to improved stability

Rivka Cohen [a], Hiba Kanaan [a], Gilbert J. Grant [b], Yechezkel Barenholz [a,*]

[a] Laboratory of Membrane and Liposome Research, IMRIC Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel
[b] Department of Anesthesiology, New York University School of Medicine, New York, New York 10016, USA

ARTICLE INFO

Article history:
Received 15 August 2012
Accepted 21 December 2012
Available online 30 December 2011

Keywords:
Alginate hydrogel
Bupivacaine
Controlled slow release
Local anesthetic

ABSTRACT

There is a compelling need for an ultralong-acting local anesthetic. Previously, we demonstrated in mice and humans that encapsulation of bupivacaine into large multivesicular liposomes (Bupisome) prolongs drug residence time and analgesic duration at the injection site while reducing peak plasma concentration. However, we observed considerable leakage of bupivacaine from the liposomes during storage at 4 °C. This deficiency was overcome by modifying the lipid composition of Bupisome and by entrapping them in a Ca-alginate cross-linked hydrogel (Bupigel), forming stable, soft, injectable (3–5 mm) beads. Bupisome are not released from Bupigel, but their encapsulated bupivacaine is released into the bulk solution. Adding 0.5% to 2.0% free bupivacaine to the Bupigel prevented net loss of bupivacaine from the Bupisome after storage at 4 °C for 2 years, and at 37 °C enough bupivacaine was released to prolong analgesia. For injection subcutaneously into mice, the beads are drawn into a syringe, leaving the small amount of free bupivacaine behind. Both Bupisome and Bupigel formulations significantly prolonged analgesia in mice compared to standard bupivacaine, with Bupigel performing better than Bupisome.

1. Introduction

There is a compelling need for an ultralong-acting local anesthetic that would provide prolonged pain relief with a single administration [1,2]. Local anesthetics are relatively small molecules (<400 Da) and are therefore rapidly redistributed from their site of administration, limiting their duration of action. Moreover, the risk of systemic toxicity limits the dose of local anesthetics that may be safely administered. These dual problems of rapid redistribution and systemic toxicity may be solved by incorporation of the local anesthetic within a slow-release depot. Such a depot would enable safe administration of a large dose of drug that would be liberated slowly to minimize the likelihood of systemic toxicity while providing prolonged analgesia. Various delivery systems for local anesthetics have been studied, primarily involving liposomes or polymers [3]. There are numerous reports of liposomes prolonging the duration of local anesthetic action in animals [4–12] and humans [13–16].

Our work differs from most published or patented approaches. While most work of others is focusing on polymer-based DDS, we are using a 2-stage system which includes remotely drug loaded multivesicular liposomes entrapped in cross-linked hydrogels, cross-linked alginate being our preferred hydrogel. All components (lipids and alginate) used in our unique 2-stage DDS are biocompatible and biodegradable. They have drug master files and are prepared under cGMP standards. Based on previous experience in humans from clinical use of multivesicular liposomal drugs for local administration of other formulations (DepoCyt™ and DepoDur™) and our previous experience with liposomal bupivacaine formulations [13,15], our system should not pose the potential problems for local toxicity as seen with the polymer-based delivery systems used by others. This claim is supported by the recent FDA approval of Pacira Pharmacceuticals' EXPAREL™ (bupivacaine liposome extended-release injectable suspension) [17].

We have previously described the use of large multivesicular vesicles (LMVV) in the micron range, which have a structure of smaller liposomes encapsulated in larger liposomes. These LMVV have an exceptionally large aqueous trapped volume (>10 μl/ml), which is much larger than classical multilammelar vesicles, permitting a much higher drug-to-lipid ratio [12,13].

Encapsulation of bupivacaine in LMVV has been shown to be an effective means of prolonging analgesia ($t_{1/2}$ > 12 h compared with 1 h in mice [18] and $t_{1/2}$ > 40 h compared with 2 h in humans [13].

The prolonged retention of the LMVV at the site of administration and the slow release of drug from the liposomal depot reduce the peak plasma levels and, therefore, the toxicity and side effects in humans [15]. This permits administration of a much higher (>4-fold) bupivacaine dose for the management of acute pain after surgery or trauma [15].

Although encapsulation of bupivacaine into LMVV has been shown to result in a high drug-to-phospholipid ratio (>1) and prolonged analgesia in mice and in humans, after prolonged storage at 4 °C there was considerable drug leakage.

* Corresponding author.
E-mail address: yb@cc.huji.ac.il (Y. Barenholz).

0168-3659/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.jconrel.2011.12.030

The overall objective of this study was to overcome the drawback of drug leakage during storage to achieve a "viable" formulation.

As is demonstrated below, this objective was achieved with a better liposome lipid composition and an improved storage medium. In addition, encapsulation of these superior LMVV, termed "Bupisome" in a cross-linked hydrogel of alginate or chitosan, termed a "Bupigel", results in a very low level of free bupivacaine upon injection.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine having iodine value of 1.0 and Tm of 55 °C (HSPC100), 99%, and HSPC3 having iodine value of 3 and Tm of 52.5 °C, 100.7%, are from Lipoid GmbH (Ludwigshafen, Germany); fully synthetic *N*-palmitoyl-D-erythro-sphingosine-1-phosphocholine (C16-SPM) of 98% chemical purity and >99% isomeric purity, from Bio-Lab Ltd. (Jerusalem); cholesterol (CHOL), >99%, from Solvay Pharmaceuticals (Veenendaal, The Netherlands); ammonium sulfate (AS) extra pure ,>99%, from J.T. Baker (Phillipsburg, NJ); and bupivacaine HCl is from Orgamol (Evionnaz, Switzerland). Sodium alginate (PRONOVA™ UP VLVG) is from NovaMatrix FMC Biopolymer(Oslo, Norway). It meets the standards of USP/NF, European Pharma copoeia, and ASTM F2064 for use in tissue engineered products. A drug master file has been submitted to the US FDA. Chitosan from crab shells, minimum 85%, is from Sigma (St. Louis, MO); calcium chloride, 99%–102%, from Merck (Darmstadt, Germany); oxalic acid dehydrate, 99%, from Sigma; and citric acid anhydrous AR, 99.5%–101%, is from Bio-Lab.

2.2. Preparation of bupivacaine-loaded LMVV (Bupisome)

2.2.1. Remote loading of bupivacaine using a transmembrane ammonium sulfate gradient

Bupivacaine-loaded large multivesicular vesicles (LMVV), referred to as Bupisome, were prepared as described previously [6], with a few modifications. In short, lipids were mixed as powders (HSPC100, C16-SPM, and cholesterol at 3/3/4 mole ratio) and dissolved in ethanol at 65 °C. For lipid hydration the ethanolic lipid solution was mixed at 65 °C for 30 min with 127 mM (285 mOsm) aqueous ammonium sulfate to a final ethanol concentration of 10% and phospholipid concentration of 60 mM. Then the lipid dispersion was subjected to 10 freeze–thaw cycles (using liquid nitrogen and 65 °C water) to form LMVV. The high concentration of ammonium sulfate in the hydration medium is responsible for LMVV formation.

The first step for remote loading bupivacaine, which is an amphipathic weak base, is to create a large transmembrane ammonium sulfate gradient, which acts as the driving force for the remote loading [6,19,20].

This requires the removal of ammonium sulfate from the extraliposomal medium, replacing it with 150 mM NaCl (saline, 285 mOsm). For this we used diafiltration at 4 °C with a Labscale TFF System having a Pellicon XL, 500 K polyethersulfone membrane (Millipore Corp., Billerica, MA).

Bupivacaine was remote loaded by mixing and incubating the concentrated dispersion of LMVV in saline with 5.7% bupivacaine HCL solution (1:3 volume ratio) for 1 h at 60 °C, obtaining a final concentration of 25–40 mM phospholipids. Diafiltration at 4 °C against 150 mM NaCl (saline) was applied to replace the non-entrapped bupivacaine. The Bupisome were stored at 4 °C in citrate-buffered saline at pH 5.5 or in free bupivacaine solutions (0.5%–2%) at pH 5.5.

2.2.2. Passive loading of bupivacaine

Bupivacaine loading was done by hydrating the lipid solution in ethanol with an aqueous solution of 5.7% bupivacaine (285 mOsm) at 65 °C for 30 min. The suspension was processed by 10 freeze and thaw cycles and then cooled to 4 °C. The non-entrapped bupivacaine was replaced with saline using diafiltration at 4 °C.

2.3. Preparation of Bupisome entrapped in cross-linked hydrogel (Bupigel)

2.3.1. Preparation of alginate gel beads entrapping Bupisome (alginate-Bupigel)

To obtain cross-linking of the hydrogel, at 4 °C the concentrated Bupisome dispersion (25–40 mM phospholipids) and 2% sodium alginate solution were mixed in 1:1 volume ratio and injected through a 1-ml Becton Dickenson Plastipak syringe into a 1.5% calcium chloride solution (285 mOsm) at 4 °C and stirred slowly for 15 min. The alginate is cross-inked non-covalently with Ca^{++}, forming stable, smooth, and spherical beads of radius 3–5 mm [21]. See Fig. 1.

The beads were washed with cold (4 °C) saline and then stored at 4 °C in citrate-buffered saline or free bupivacaine solutions (0.5%–2%) at pH 5.5.

The determination of water content of beads was done by using a Karl Fischer Coulometer (Metrohm Ltd, Herisau, Switzerland).

Calcium was determined by Zeeman atomic absorption spectrometry (SPECTRA A-300). Quantification of alginate of empty beads and of Bupigel beads was done by using the dye 1,9-dimethyl methylene blue (DMMB) [22].

2.3.2. Preparation of chitosan gel beads entrapping Bupisome

We used the same method as in Section 2.3.1 but replacing alginate as the polymer with chitosan and replacing 1.5% Ca ions as cross-linker agent with 1.5% oxalic acid.

All of the above preparations were done under physiologic (285 mOsm) conditions as measured with a Model 1332 osmometer (Advanced Instrument).

2.4. Liposome characterization

Bupivacaine concentration was determined by HPLC [7]. Phospholipid concentration was determined using a modified Bartlett method [23].

Concentrated liposomes (25–40 mM phospholipids) or Bupigel after passage through a #31 gauge needle, which creates a homogeneous dispersion, were diluted 1:10 (w/v) with cold saline, and then aliquots were used to determine phospholipid concentration and total (liposomal and free) bupivacaine concentrations. The diluted liposomes were centrifuged at 2000 *g* for 10 min at 4 °C using an Eppendorf 5810 R centrifuge to separate liposomes from non-encapsulated drug, and free bupivacaine concentration was assayed in the supernatant. The concentration of liposomal bupivacaine was calculated by subtraction of free from the total bupivacaine concentration. The mole ratio of liposomal drug to phospholipid concentration (D/PL) was calculated for the bupivacaine-loaded LMVV. To determine in vitro release profiles, Bupisome and Bupigel were stored at 4 °C or 37 °C, and free drug in the extraliposomal medium was measured at the desired time intervals.

Liposome size distribution was determined by laser diffraction particle size analyzer (LS13320 Beckman Coulter), which enables measuring particle size in the range of 40 nm to 2 mm [23,24].

For determining trapped volume of the liposomes, usually a tracer (e.g. radioactive, colored, or fluorescent) is passively encapsulated. It should be water-soluble and not interact with, or pass through, the liposome membrane. In the case of Bupisome, which have a transmembrane ammonium sulfate gradient, ammonium ion and sulfate ion are each a candidate for determining trapped aqueous volume prior to bupivacaine loading. We determined ammonium ion concentration using an Orion ammonium electrode with a Cyberscan PCD 6500, Eutech Instruments, (Nijkerk, The Netherlands).

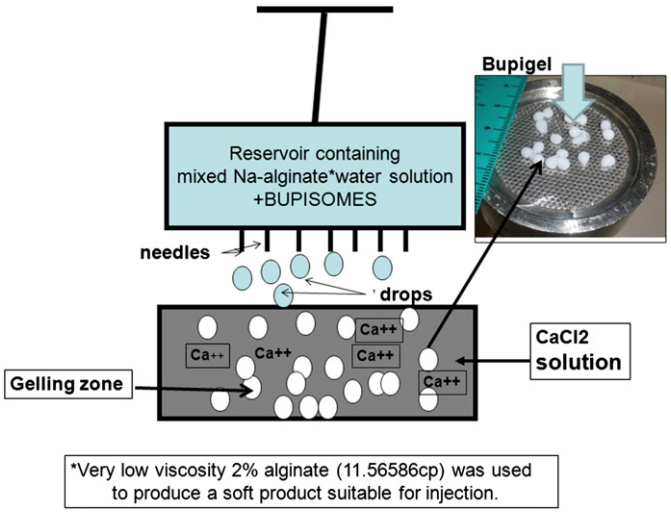


Fig. 1. Fabrication of Bupigel: at 4 °C, a mixture of Bupisome and 2% sodium alginate were mixed in 1/1 vol/vol ratio and dropped into 1.5% calcium chloride solution (4 °C) forming spherical beads.

Trapped volume was calculated by determining ammonium ion concentration in the hydration medium followed by determination ammonium ion concentration of the liposomal dispersion after total removal of un-encapsulated ammonium ion. Trapped volume is described in terms of μl of aqueous medium per μmole of liposome phospholipid (the latter is used to achieve standardization).

2.5. Assessment of analgesia duration in mice

All animal experiments were approved and ratified by the Ethics Committee of the Hebrew-University Hadassah Medical School.

Testing for analgesia was done by a vocal response to electrical stimulation (beginning at 1 mA and increasing to a maximum of 8 mA) at the skin directly overlying the abdomen at the site of injection using a current generator (model S48, Grass Instruments). Electrical stimulation testing has long been used as a means of determining analgesia in animals and humans [1,7,13,25]. Mice (male Swiss-Webster), 26 ± 3 g (n = 6–8) had the hair overlying the abdomen shaved, and were tested prior to injection to determine the vocalization threshold. Mice were injected with a constant dose of 3 mg liposomal bupivacaine/25 g mouse (0.15–0.3 ml according to the liposomal batch concentration) in Bupisome or Bupigel formulations through a 30 G needle (the soft Bupigel reduced to very small gel particles that passed through the needle while the encapsulated liposomes remained intact), followed by determination of analgesia at the desired time points [7]. 100% analgesia indicates that all of the mice group (n = 6–8) did not vocalize to electrical stimulation 2 mA above threshold; 50% analgesia indicates that half of the mice group vocalized to electrical stimulation; and 0% analgesia (return to baseline) indicates that all of the mice group vocalized to electrical stimulation.

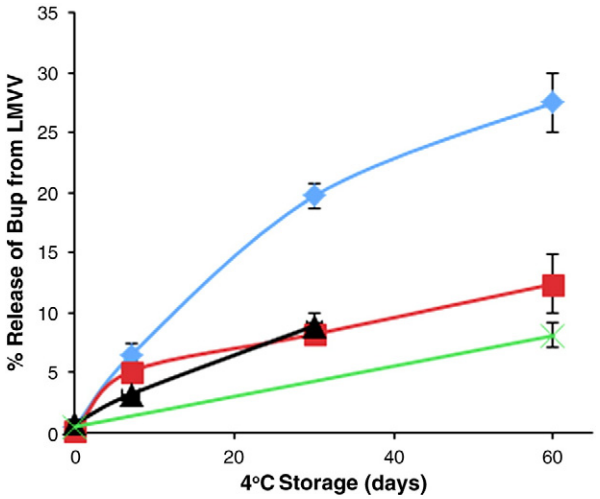
3. Results

3.1. Optimization of bupivacaine release from LMVV as a measure of formulation physical stability

Our studies on the effect of LMVV lipid composition on the release rate and drug-to-lipid ratio demonstrate that among the various LMVV compositions studied, the liposomes with the lipid composition of C16-SPM, HSPC100, and cholesterol in a 3:3:4 mole ratio have the smallest rate of % bupivacaine release at 4c storage, as was shown in Fig. 2.

These LMVV have a high trapped volume (17.2 μl/μmol PL) and thus a bup/PL mole ratio >1 (Fig. 2), very low drug release rate at 4^0 C, and slow drug release at 37 °C but sufficient to produce analgesia in vivo (Fig. 6). These Bupisome are superior to LMVV composed of HSPC3 and cholesterol in a 6:4 mole ratio, described by us previously [13,15] (Fig. 2).

With LMVV of this superior lipid composition, we studied the effect of intraliposome aqueous media on the 4 °C leakage rate by comparing our remotely loaded Bupisome (by transmembrane ammonium sulfate gradient) to Bupisome which were prepared by passive encapsulation of bupivacaine.



LMVV's lipids composition	mole ratio	bup/PL mole/mole
HSPC3/CHOL	6/4	2.26
HSPC3/C16SPM/CHOL	3/3/4	2.23
HSPC100/CHOL	6/4	2.22
HSPC100/C16SPM/CHOL	3/3/4	1.54

Fig. 2. Effect of remote loaded LMVV lipid composition on bupivacaine long-term 4 °C release as a measure of LMVV physical stability.

Table 1
A comparison between LMVV prepared by lipid hydration in 127 mM ammonium sulfate (285 mOsm) and stored in iso-osmotic NaCl (150 mM = 285 mOsm), compared to LMVV prepared at 250 mM AS (560 mOsm) and stored at hypo-osmotic 0.15 M (285 mOsm) NaCl).

Intraliposome AS concentration used in active loading procedure	LMVV D/PL mole ratio	% bupivacaine released from Bupisome after storing 6 months at 4 °C in 0.15 mM NaCl
250 mM; 560 mOsm	1.6 ± 0.14	21 ± 1.0
127 mM; 285 mOsm	2.3 ± 0.25	13 ± 0.6

Remote loaded compared to passive loaded Bupisome show a large advantage of a much slower (5-fold) release rate after 60 days of storage at 4 °C (5% compared with 26%).

The effect of external medium composition also included optimization of the effect of intra/extra liposome osmotic pressure gradient, which may cause swelling or shrinkage (depending on the direction of the gradient). The osmotic behavior of LMVV is explained by the liposomes being almost an ideal osmometer [26,27]. Therefore the presence of an osmotic gradient may accelerate or reduce leakage rate of the liposomes [26,27]. Table 1 clearly shows the advantage of storage in iso-osmotic medium in reducing drug release at 4 °C.

The second aspect of the effect of external media on storage stability was to compare storage in media containing various levels of bupivacaine (all under iso-osmotic conditions of 285 mOsm to the concentration of ammonium sulfate used for lipid hydration).

For this we compared bupivacaine release from LMVV stored in 10 mM citrate buffer pH 5.5, adjusted to 285 mOsm with NaCl, with storage in iso-osmotic media containing different concentrations of bupivacaine (0.2, 0.5, 1.0, and 2.0%, in 10 mM citrate buffer pH 5.5 adjusted to 285 mOsm with NaCl. Above 2%, bupivacaine starts to crystallize). The idea underlying storage in bupivacaine-containing media is to significantly reduce the trans-membrane drug gradient ($[\text{bupivacaine}]_{\text{LMVV}} >> [\text{bupivacaine}]_{\text{medium}}$). Fig. 3 shows that, indeed, storage in iso-osmotic 0.5% and 2.0% solutions of free bupivacaine decreases the rate of bupivacaine leakage from the LMVV. Even after 2 years of 4 °C storage, there is almost no net loss of liposomal bupivacaine from these LMVV, while storage in saline results in a major (>49%) bupivacaine loss (Fig. 3 and Table 2). Table 2 shows 1 year storage results in which 49% bupivacaine loss occurred for LMVV stored in iso-osmotic saline compared with only 5% loss upon storage at iso-osmotic 2% bupivacaine.

This is also expressed in the liposomal bupivacaine/phospholipid (bup/PL) mole ratio (Table 2), which remains much higher during 4 °C storage of Bupisome in bupivacaine solutions than during storage in saline.

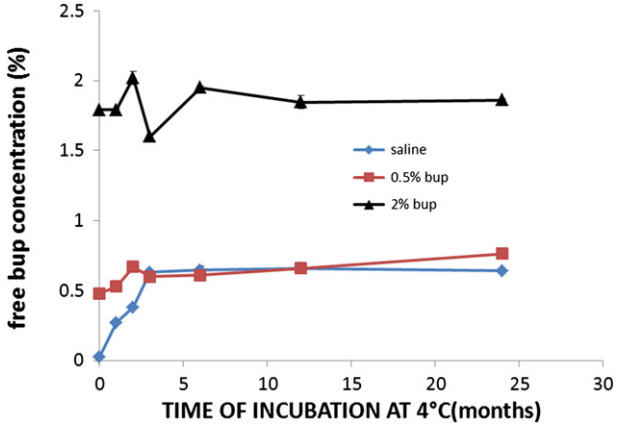


Fig. 3. Effect of storage of Bupisome, with and without added free bupivacaine, on the time-dependent change in level of free bupivacaine in the LMVV storage media. HSPC100/C16SPM/CHOL (3/3/4 mole ratio) LMVV were used, storing in the following iso-osmotic media of saline, 0.5%, and 2.0% bupivacaine were compared.

Table 2
Effect of bupivacaine concentration in storage medium at 4 °C on LMVV bupivacaine/PL mole ratio in LMVV.

LMVV Stored in	Bupivacaine/PL mole ratio Time = 0	Bupivacaine/PL mole ratio 6 months	Bupivacaine/PL mole ratio 12 months	% Bupivacaine accumulated leakage at 12 months
saline	1.63 ± 0.14	0.9 ± 0.15	0.83 ± 0.145	49.1
0.5% bupivacaine	1.8 ± 0.149	1.56 ± 0.189	1.56 ± 0.18	13.4
1% bupivacaine	1.8 ± 0.184	1.65 ± 0.184	1.62 ± 0.183	10.0
2% bupivacaine	1.8 ± 0.242	1.7 ± 0.256	1.71 ± 0.25	5.0

However, this advantageous storage in iso-osmotic bupivacaine solution cannot be applied clinically as there is no easy way to separate the Bupisome from the free drug present in the storage medium before administration to patients. In order to overcome this major obstacle we developed Bupigel.

3.2. Bupigel characterization

Bupigel is a hydrogel composed of a cross-linked biocompatible polysaccharide entrapping Bupisome of 1–10 μm size in beads of 3–5 mm (Fig. 1). The idea behind the development of Bupigel is that the removal of the storage medium from the hydrogel is easy and can be performed clinically at the bedside. Two types of hydrogel beads loaded with Bupisome were prepared. One of them is based on negatively charged alginate cross-linked by Ca ions, and the second is based on the positively charged chitosan cross-linked by oxalic acid.

The composition of alginate Bupigel and the empty alginate is described in Table 3.

Table 3 demonstrates that alginate-based Bupigel is indeed a hydrogel (with water constituting > 70% of the Bupigel beads). Weight ratios of alginate/PL and bupivacaine/PL are both ~0.59.

Phospholipids determination of the storing media of Bupigel demonstrated that all (100%) of the LMVV were entrapped in the beads.

Cryo-TEM of Bupigel (Fig. 4) shows that its LMVV structure is similar to that of Bupisome. There were no crystals of bupivacaine either inside or outside the LMVV.

3.3. Effect of Bupigel storage medium on bupivacaine long-term 4 °C release

Storing Bupigel at 4 °C in bupivacaine solutions prevents the loss of bupivacaine from the encapsulated liposome in an identical way to that in Bupisome (Figs. 3, 5).

Table 3
Characterization of low-viscosity alginate beads encapsulating Bupisome (Bupigel) and of empty alginate beads.

Bupigel components	Alginate Bupigel	Empty beads
Water		
mg/bead	3.085 ± 0.21	2.54 ± 0.2
mg/mg beads	0.722	0.76
Calcium ion		
μg/bead	3.906 ± 0.06	4.35 ± 0.08
μg/ mg bead	0.915	1.64
Phospholipids		
nmoles/bead	51.34 ± 0.93	0
nmoles/mg beads	12.02	
μg/mg beads	9.0	
Alginate		
μg/bead	22.7 ± 0.5	55.7 ± 0.3
μg/mg beads	5.3	16
Bupivacaine		
nmoles/bead	68.06 ± 9.7	0
μg/bead	22.66 ± 3.3	
μg/mg beads	5.3	

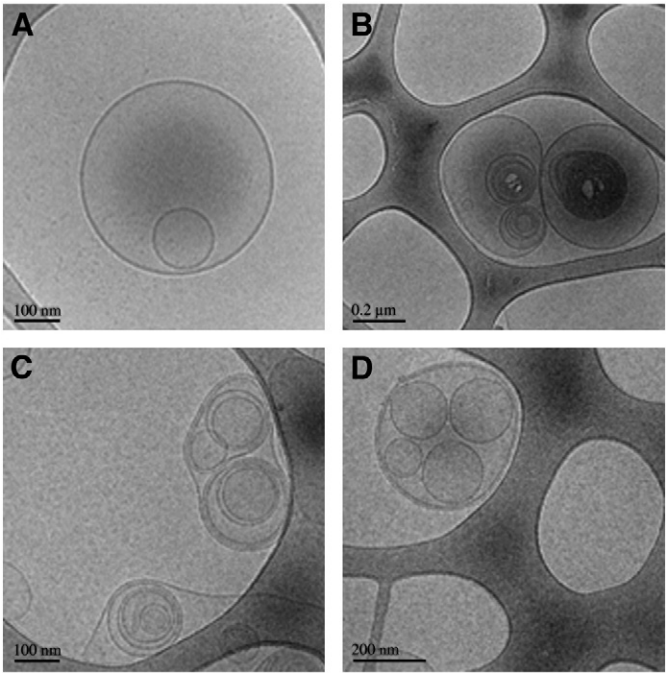


Fig. 4. Cryo-TEM of Bupisome (A, B) and Bupigel (C, D). LMVV having high trapped aqueous volume are shown. The hydrogel itself is not visible due to lack of contrast between the hydrogel and the aqueous phase. The lamellar structure of the LMVV in the Bupigel looks the same as in the Bupisome.Scale bars: 100 nm (A, C); 200 nm (B,D).

Fig. 5 shows that the level of free bupivacaine in the external Bupigel medium increases with storage time when the hydrogel was stored in iso-osmotic saline, but it does not increase when stored in 0.5% or 2% free bupivacaine.

3.4. Therapeutic performance: prolonged local analgesia duration by Bupisome and Bupigel

In vitro bupivacaine release from Bupisome and chitosan or alginate Bupigels at 37 °C (Fig. 6) shows a slow and similar release rate for both Bupigels. As we previously demonstrated, release rate at 37 °C correlates well with duration of analgesia; the slower the release rate, the longer the analgesic duration [28]. The two Bupigel release rates resemble that of Bupisome.

In vivo results (Fig. 7) indicate that a single administration of Bupisome or Bupigel to mice provides significantly prolonged analgesia compared with free (non-liposomal) bupivacaine. The median duration of analgesia after 0.75 mg/25 g mouse of free bupivacaine was 1 hour, while the median durations of analgesia after liposomal bupivacaine in Bupisome or Bupigel of 3 mg bupivacaine/25 g mouse were 24 and 35 hours, respectively.

The free bupivacaine present in the Bupigels was removed before administration to mice.

The Bupisome were stored at 4 °C for 2 months in saline before administration to the mice. The free bupivacaine present in Bupisome was not removed.

It is worth noting that no systemic toxic effects were observed after administration of free bupivacaine, Bupisome, or Bupigel. Administration of more than 0.75 mg /mouse of free bupivacaine caused severe systemic toxicity.

4. Discussion

The overall objective of this study was to achieve LMVV that have high "energy of activation" in the Arrhenius plot describing bupivacaine release [19]; namely, to develop LMVV encapsulating

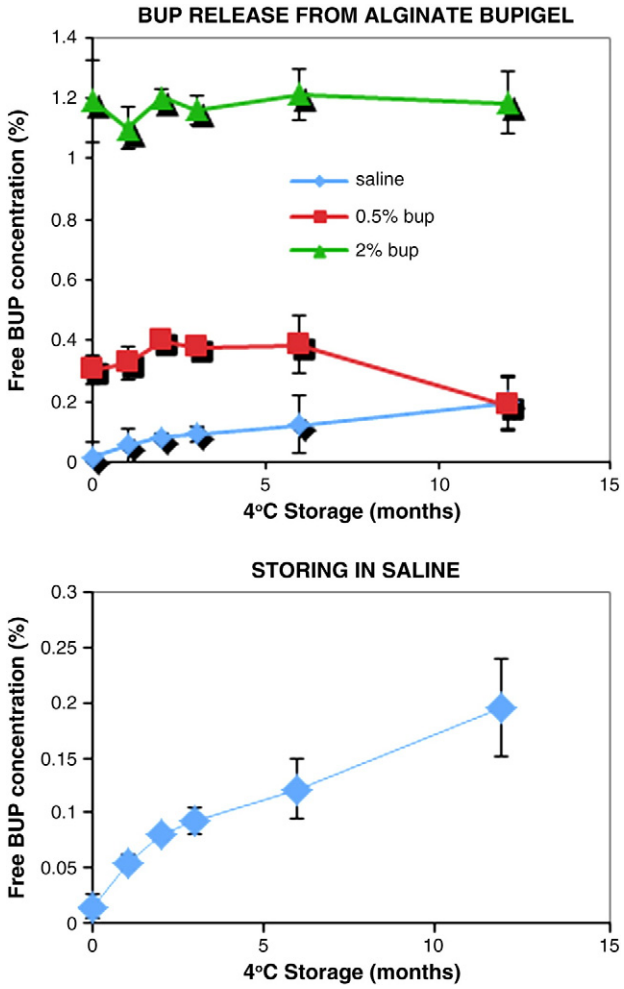


Fig. 5. Effect of storage of Bupigel in iso-osmotic extralipsome media, with and without added free bupivacaine, on the time-dependent change in level of free bupivacaine in the hydrogel storage media.

bupivacaine that have a very low level of free drug and exhibit very low drug leakage when stored at 4 °C and, concomitantly, provide long-lasting analgesia at 37 °C. This requires a release rate at 37 °C which has to be slow, though sufficient to be efficacious without compromising safety. In addition, the LMVV should have a very high bupivacaine/PL ratio, which is essential to have a sufficient amount of depot drug required for prolonged analgesia.

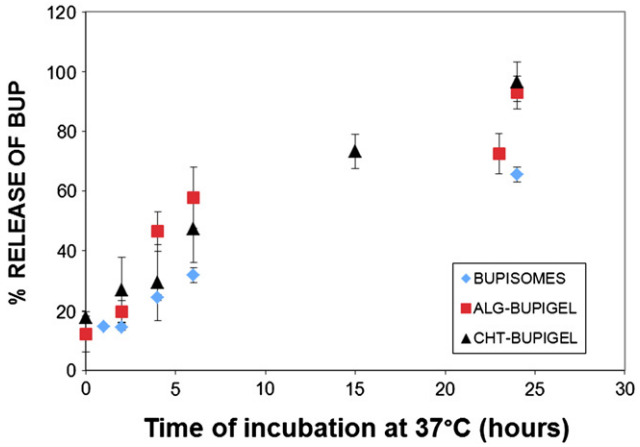


Fig. 6. *In vitro* study: bupivacaine release at 37 °C from Bupigels based on alginate (ALG) or chitosan (CHT) compared with Bupisome stored in saline.

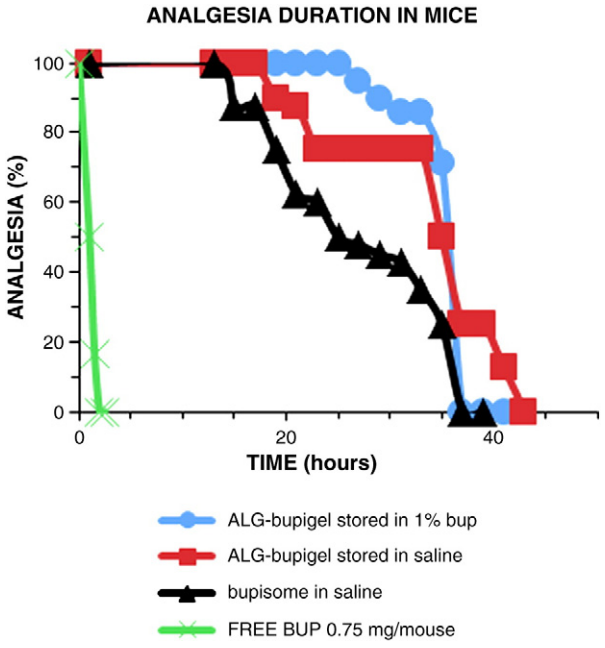


Fig. 7. *In vivo* study: duration of sensory block of the skin over the abdomen after subcutaneous injection (duration of analgesia) of free bupivacaine 0.75 mg/mouse (maximal nontoxic dose); 3 mg/mouse bupivacaine in Bupisome stored at 4 °C for 2 months in saline, or in alginate Bupigel in saline, or in 1% bupivacaine (n = 6–8 mice per group). The free bupivacaine present in the Bupigels was removed before administration to mice (as described in Methods) while the free bupivacaine present in Bupisome was not removed. Data analysis of *t*-test for paired two sample for means:

1- $P(T \leq t) = 0.092$ one-tail for ALG-bupigel stored in 1%bup and ALG-bupigel stored in saline. ($P > 0.05$ non-statistical significance).
2- $P(T \leq t) = 1.4E-04$ one-tail for *ALG*-bupigel stored in 1%bup and bupisome in saline ($P < 0.05$ statistical significance)
3- $P(T \leq t) = 2.9E-06$ one-tail for *ALG*-bupigel stored in saline and bupisome in saline ($P < 0.05$ statistical significance)

The data analysis results show that analgesic duration was: BupiGel stored in 1% bupivacaine = Bupigel in saline > Bupisome in saline >>> free bupivacaine.

However, this is not an easy task, as bupivacaine is an amphipathic weak base having a relatively high octanol/aqueous phase logD value, as well as a high ratio of nonpolar/polar surface area (295.3/32.3 = 9.14), Table 4. These properties result in an inherent high permeability and therefore large and unwanted bupivacaine leakage [29]. This explains why all our past efforts to "tame" the release rate failed (Grant and Barenholz, unpublished).

Basically, the release rate of a given molecule encapsulated in liposomes at a given temperature is controlled by: (i) liposome lipid composition [26,29,31–34], (ii) the intraliposome aqueous medium, (iii) the extra-liposome aqueous medium [33,35]. The difference in composition, together with the type of ions in the intra- and extra-liposome aqueous media determines if there is a transmembrane ion gradient that can act as the driving force responsible for remote (active) drug loading. Such a gradient requires that one of the ions used for lipid hydration is able to diffuse through the lipid bilayer [20,30].

The results of our current studies demonstrate that Bupisome composed of C16-SPM, fully hydrogenated soy PC-100 (HSPC100), and cholesterol in a mole ratio of 3:3:4, when having high drug/lipid mole ratio (>1), show dramatic prolongation of local analgesia combined with much superior physical stability over the Bupisome which lack the C16-SPM and in which the HSPC is HSPC-3 (see Materials and methods). The selection of C16-SPM as a major component stems from previous data that demonstrate that sphingomyelin reduces membrane permeability better than PC [27,32]. This also holds for systems in which the PC T_m is higher than the T_m of the SPM [36]. The lower permeability of a bilayer enriched with SPM is related to its ability to act as a donor and acceptor in hydrogen bonding, yielding tighter packing, which results in a low level of bilayer free volume [32]. These are also responsible in part for "raft" formation [32]. Our previous data also suggest that SPM becomes dominant when the SPM composes 50 mol% or more of the liposome membrane lipids (Barenholz, unpublished).

This explains why LMVV composed of C16-SPM and cholesterol (6/4 mole ratio) were worse than SPM/HSPC100/cholesterol (3/3/4 mole ratio) with respect to release rate during 4 °C storage. Also, the use of SPM as the single liposome-forming lipid will be much more expensive (due to the very high cost of C16-SPM). Reduction in the level of C16-SPM much below 50% of the liposome-forming lipids led to increase in 4 °C release rate (data not shown).

The use of fully synthetic *N*-palmitoyl-D-erythro-sphingosine-1-phosphocholine instead of naturally occurring SPM is important for clinical use to avoid contaminants from SPM obtained from natural sources such as egg SPM or brain SPM.

Our study shows that, in addition to the major reduction in bupivacaine leakage during long-term 4 °C storage, loss of bupivacaine from the LMVV can be further reduced by storing the Bupisome in media with a solution of free bupivacaine.

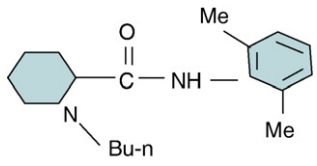
This approach, which works well in the lab, is not applicable for clinical use as it is very cumbersome to remove the extra-liposome media as a bedside procedure. This led us to the development of Bupigel beads, from which free drug in the storage media can be easily removed prior to administration. Cryo-TEM photos, release rate studies at 4 °C and 37 °C, and analgesic efficacy data, in which Bupisome and Bupigel are compared, clearly demonstrate that the entrapment process has no effect on structure and function of the bupivacaine-loaded LMVV.

The Bupisome and Bupigel developed in this study showed a much prolonged analgesia when compared with free bupivacaine, with Bupigel being superior to Bupisome (Fig. 7). This, together with the ease of removal of the free bupivacaine in the medium, make the use of Bupigel a feasible and attractive option.

Although our human studies with our HSPC/cholesterol (6/4 mole ratio) show lack of systemic toxicity [15] and our mice studies also lack toxic effects, there is a need to extend the toxicity studies of Bupigel, with particular emphasis on potential toxicity to nerves at the site of injection before initiating clinical trials with Bupigel.

Table 4
Relevant physicochemical properties for remote loading and release of bupivacaine [20,30].

Physicochemical properties of bupivacaine (25 °C)	
• pKa 1	14.85
• pKa 2	8.17
• logD pH4	0.57
• logD pH7	2.45
• logP	3.63
• Solubility pH4	49g/L
• Solubility pH7	0.66g/L
• Polar surface area	32.3A^2
• Nonpolar area	295.3A^2



References

[1] G.J. Grant, M. Bansinath, Liposomal delivery systems for local anesthetics, Reg. Anesth. Pain Med. 26 (1) (2001) 61–63.
[2] K. Karashima, M. Taniguchi, T. Nakamura, M. Takasaki, K. Matsuo, M. Irikura, T. Irie, Prolongation of intrathecal and sciatic nerve blocks using a complex of levobupivacaine with maltosyl-beta-cyclodextrin in rats, Anesth. Analg. 104 (5) (2007) 1121–1128.
[3] C.F. Weiniger, M. Golovanevski, M. Sokolsky-Papkov, A.J. Domb, Review of prolonged local anesthetic action, Expert Opin. Drug Deliv. 7 (6) (2010) 737–752.
[4] G.J. Grant, K. Vermeulen, L. Langerman, M. Zakowski, H. Turndorf, Prolonged analgesia with liposomal bupivacaine in a mouse model, Reg. Anesth. 19 (4) (1994) 264–269.
[5] H.Y. Yu, S.D. Li, P. Sun, Kinetic and dynamic studies of liposomal bupivacaine and bupivacaine solution after subcutaneous injection in rats, J. Pharm. Pharmacol. 54 (9) (2002) 1221–1227.
[6] G.J. Grant, K. Vermeulen, M.I. Zakowski, M. Stenner, H. Turndorf, L. Langerman, Prolonged analgesia and decreased toxicity with liposomal morphine in a mouse model, Anesth. Analg. 79 (4) (1994) 706–709.
[7] G.J. Grant, Y. Barenholz, B. Piskoun, M. Bansinath, H. Turndorf, E.M. Bolotin, DRV liposomal bupivacaine: preparation, characterization, and in vivo evaluation in mice, Pharm. Res. 18 (3) (2001) 336–343.
[8] G.J. Grant, J. Lax, L. Susser, M. Zakowski, T.E. Weissman, H. Turndorf, Wound infiltration with liposomal bupivacaine prolongs analgesia in rats, Acta Anaesthesiol. Scand. 41 (2) (1997) 204–207.
[9] J.J. Mowat, M.J. Mok, B.A. MacLeod, T.D. Madden, Liposomal bupivacaine. Extended duration nerve blockade using large unilamellar vesicles that exhibit a proton gradient, Anesthesiology 85 (3) (1996) 635–643.
[10] T. Mashimo, I. Uchida, M. Pak, A. Shibata, S. Nishimura, Y. Inagaki, I. Yoshiya, Prolongation of canine epidural anesthesia by liposome encapsulation of lidocaine, Anesth. Analg. 74 (6) (1992) 827–834.
[11] J. Boogaerts, N. Lafont, M. Donnay, H. Luo, F.J. Legros, Motor blockade and absence of local nerve toxicity induced by liposomal bupivacaine injected into the brachial plexus of rabbits, Acta Anaesthesiol. Belg. 46 (1) (1995) 19–24.
[12] S. Mantripragada, A lipid based depot (DepoFoam technology) for sustained release drug delivery, Prog. Lipid Res. 41 (5) (2002) 392–406.
[13] G.J. Grant, Y. Barenholz, E.M. Bolotin, M. Bansinath, H. Turndorf, B. Piskoun, E.M. Davidson, A novel liposomal bupivacaine formulation to produce ultralong-acting analgesia, Anesthesiology 101 (1) (2004) 133–137.
[14] N.D. Lafont, F.J. Legros, J.G. Boogaerts, Use of liposome-associated bupivacaine in a cancer pain syndrome, Anaesthesia 51 (6) (1996) 578–579.
[15] E.M. Davidson, Y. Barenholz, R. Cohen, S. Haroutiunian, L. Kagan, Y. Ginosar, High-dose bupivacaine remotely loaded into multivesicular liposomes demonstrates slow drug release without systemic toxic plasma concentrations after subcutaneous administration in humans, Anesth. Analg. 110 (4) (2010) 1018–1023.
[16] J.G. Boogaerts, N.D. Lafont, A.G. Declercq, H.C. Luo, E.T. Gravet, J.A. Bianchi, F.J. Legros, Epidural administration of liposome-associated bupivacaine for the management of postsurgical pain: a first study, J. Clin. Anesth. 6 (4) (1994) 315–320.
[17] http://www.pacira.com.
[18] G.J. Grant, B. Piskoun, M. Bansinath, Analgesic duration and kinetics of liposomal bupivacaine after subcutaneous injection in mice, Clin. Exp. Pharmacol. Physiol. 30 (12) (2003) 966–968.
[19] G. Haran, R. Cohen, L.K. Bar, Y. Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, Biochim. Biophys. Acta 1151 (2) (1993) 201–215.
[20] D. Zucker, D. Marcus, Y. Barenholz, A. Goldblum, Liposome drugs' loading efficiency: a working model based on loading conditions and drug's physicochemical properties, J. Control. Release 139 (1) (2009) 73–80.
[21] L. Xing, C. Dawei, X. Liping, Z. Rongqing, Oral colon-specific drug delivery for bee venom peptide: development of a coated calcium alginate gel beads-entrapped liposome, J. Control. Release 93 (3) (2003) 293–300.
[22] J.C. Richardson, P.W. Dettmar, F.C. Hampson, C.D. Melia, A simple, high throughput method for the quantification of sodium alginates on oesophageal mucosa, Eur. J. Pharm. Biopharm. 57 (2) (2004) 299–305.
[23] S. Amselem, Y. Barenholz, Quality control assays in the development and clinical use of liposome-based formulations, in: G. Gregoriadis (Ed.), second ed., Liposome Technology, Vol. 1, CRC Press, Boca Raton, FL, 1993, pp. 527–616.
[24] O. Even-Or, S. Samira, E. Rochlin, S. Balasingam, A.J. Mann, R. Lambkin-Williams, J. Spira, I. Goldwaser, R. Ellis, Y. Barenholz, Immunogenicity, protective efficacy and mechanism of novel CCS adjuvanted influenza vaccine, Vaccine 28 (39) (2010) 6527–6541.
[25] G.J. Grant, B. Piskoun, A. Lin, M. Bansinath, An in vivo method for the quantitative evaluation of local anesthetics, J. Pharmacol. Toxicol. Methods 43 (1) (2000) 69–72.
[26] J. de Gier, Osmotic behaviour and permeability properties of liposomes, Chem. Phys. Lipids 64 (1–3) (1993) 187–196.
[27] R. Hertz, Y. Barenholz, Permeability and integrity properties of lecithin–sphingomyelin liposomes, Chem. Phys. Lipids 15 (2) (1975) 138–156.
[28] Y. Barenholz, G. Grant, H. Turndorf, B. Piskoun, E. Bolotin, Liposomal Analgesic Drug Compositions Prepared in GMV Using an Ammonium Sulfate Gradient, U.S. Patent 7357944, April 15 2008.
[29] W.D. Stein, Transport and Diffusion across Cell Membranes, Academic Press, Orlando, FL, 1986.
[30] Scifinder, Calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris, , 1994–2009.
[31] O.G. Mouritsen, K. Jorgensen, A new look at lipid–membrane structure in relation to drug research, Pharm. Res. 15 (10) (1998) 1507–1519.
[32] Y. Barenholz, Sphingomyelin and cholesterol: from membrane biophysics and rafts to potential medical applications, in: P.J. Quinn (Ed.), Membrane Dynamics and Domains, Subcellular Biochemistry, Vol. 37, Kluwer Academic/Plenum, New York and London, 2004, pp. 167–215.
[33] Y. Barenholz, G. Cevc, Structure and properties of membranes, in: A. Baszkin, W.N. (Eds.), Physical Chemistry of Biological Surfaces, Marcel Dekker, New York, 2000, pp. 171–241.
[34] O.G. Mouritsen, in: D. Dragoman, M. Dragoman, A.C. Elitzur, M.P. Silverman, J. Tuszynski, H.D. Zeh (Eds.), Life as a Matter of Fat: the Emerging Science of Lipidomics, Springer, Berlin, 2005, pp. 129–136.
[35] Y. Barenholz, Liposome application: problems and prospects, Curr. Opin. Colloid Interface Sci. 6 (2001) 66–77.
[36] I.V. Zhigaltsev, N. Maurer, Q.F. Akhong, R. Leone, E. Leng, J. Wang, S.C. Semple, P.R. Cullis, Liposome-encapsulated vincristine, vinblastine and vinorelbine: a comparative study of drug loading and retention, J. Control. Release 104 (1) (2005) 103–111.