Liposphere Local Anesthetic Timed-Release for Perineural Site Application

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Purpose. This investigation determines the drug delivery capacity of Lipospheres, which are drug-containing solid-filled vesicles made of triglyceride with a phospholipid outer covering, to release local anesthetic *in vitro* and to produce sustained peripheral nerve block *in vivo*.
Methods. The local anesthetic, bupivacaine, was loaded into Lipospheres in several dosage forms, characterized, and measured for *in vitro* release. In rats, Lipospheres were administered into a large space between muscle layers surrounding the sciatic nerve to assess sensory and motor block *in vivo*.
Results. The particle size of Lipospheres was determined to be between 5 and 15 μm, with over 90% surface phospholopid. Lipospheres released bupivacaine over two days under ideal sink conditions. Liposphere nerve application produced dose-dependent and reversible block. Indeed, sustained local anesthetic block (SLAB) was observed for 1–3 days in various *in vivo* tests: a) Hind paw withdrawal latency to noxious heat was increased over 50% for 96 hr period after application of 3.6% or 5.6% bupivacaine-Lipospheres. The 3.6% and 5.6% doses were estimated to release bupivacaine at 200 and 311 μg drug/hr, respectively, based on release spanning 72 hr. Application of 1.6% bupivacaine-Lipospheres increased withdraw latency 25–250% but for only a 24 hr duration; b) Similarly, vocalization threshold to hind paw stimulation was increased 25–50% for 72 hr following application of 3.6% bupivacaine-Lipospheres; c) Finally, sensory blockade outlasted or equaled corresponding motor block duration for all Liposphere drug dosages.
Conclusions. Liposphere delivery of local anesthetic drugs may be well suited for site-specific pharmacotherapy of neural tissue to produce SLAB. Dose-dependent effects in duration of action may include lipophilic tissue storage.

KEY WORDS: bupivacaine; marcaine; liposomal; nerve; controlled release; analgesia.

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ABBREVIATIONS: EPC, egg phosphatidyl choline; HPLC, high performance liquid chromatography; MWCO, molecular weight cut-off; NSAIDS, non-steroidal anti-inflammatory drugs; p-CPP:SA, poly-carboxyphenoxy-propane:sebacic acid; PBS, phosphate buffered solution; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PLAM, polymer-local anesthetic matrix; SLAB, sustained local anesthetic block; THF, tetrahydrofuran; TNBS, trinitrobenzene-sulfonic acid; TS, tristearin.

INTRODUCTION

Application of a local anesthetic via a catheter can produce neural blockade for days to weeks (1), however, catheters may cause infection, become blocked or migrate, thus requiring constant monitoring by a health care professional. Therapeutic development of a timed-release local anesthetic system would be a great improvement over catheter applications or other currently available therapies (e.g., neurolytic blocks from repeated injections). Previously, a long-acting local anesthetic system was made from bioerodible, biodegradable polyanhydride copolymers (poly-carboxyphenoxy-propane:sebacic acid, p-CPP:SA) impregnated with the local anesthetics, dibucaine or bupivacaine (2–5). These polymer-local anesthetic matrix (PLAM) implants made from p-CPP:SA-bupivacaine formulations produced sciatic nerve blockade for 3–5 days in rats (2–5). Although this PLAM system can potentially become a useful therapeutic tool, its use thus far is limited to surgical implantation (6).

Liquid drug-delivery systems, which do not require surgical implantation, may use vesicles made up of microparticulates or colloidal carriers composed of lipids, carbohydrates or synthetic polymer matrices. Liposomes, the most widely studied of these vesicles, can be formulated to include a variety of compositions and structures that are potentially nontoxic, degradable and nonimmunogenic. Furthermore, studies are in progress to create liposomes that release more drug in response to changes in their environment, including enzymes, polycations or pH (for review see references (7,8)). To produce a long-acting local anesthetic effect, vesicles have been used to entrap dibucaine (9), methoxyflurane (10), tetracaine (11), and lidocaine (12), using formulations with polylactic acid, lecithin, iophendylate and phosphatidyl choline & cholesterol, respectively. With varying degrees of success, these treatments have provided neural blockade for periods far outlasting that which is produced by any drug given alone.

The present study reports on an injectable drug delivery system that uses Lipospheres (13) to release the local anesthetic, bupivacaine, from a liposomal matrix that is both biodegradable and biocompatible to produce sustained local anesthetic blockade (SLAB). Bupivacaine was used because it has minimal vasodilating properties, in contrast to other local anesthetics (e.g., lidocaine), allowing the released drug to remain at the site of injection longer (14). Lipospheres are an aqueous microdispersion of water insoluble, spherical microparticles (0.2 to 100 μm in diameter), each consisting of a solid core of hydrophobic triglycerides and drug particles that are embedded with phospholipids on the surface. Previous *in vivo* studies with Lipospheres have shown that a single bolus injection can deliver antibiotics and anti-inflammatory agents for 3 to 5 days (15) and also, control the delivery of vaccines (16,17). Here we report that bupivacaine-Liposphere formulations produce 1–3 days of reversible sensory and motor SLAB when applied directly to the rat sciatic nerve. The present study extends the findings of a previous short communication which reported that an infiltrate injection of an approximate 2% bupivacaine-Liposphere formulation into the rat tail can maximally produce 3–6 hr of local anesthetic effect, measuring 6–12 times longer than a standard Marcaine solution (bupivacaine 0.5% with 1:200,000 epinephrine) (18).

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MATERIALS AND METHODS

Materials

Tristearin, 99% pure, was purchased from Hulls (Hulls, NJ). Bupivacaine free base was prepared by chloroform extraction of a basic solution of bupivacaine-HCl (Sigma Chemical Co., St. Louis). Egg phosphatidyl choline (EPC, lecithin), 99% pure, was purchased from Princeton Lipids (Princeton, NJ). Phosphate buffer solution (PBS) was prepared by dissolving monosodium phosphate (3.40 g) and dipotassium phosphate (14.44 g) into 1 liter of distilled water. Methylparaben (0.1% by weight) was added to the PBS release buffer as a preservative. Phosphatidyl ethanolamine and phosphatidyl ethanol amine dimyristoyl-^{14}C (99% pure), was purchased from Sigma Chemical Co. (St. Louis).

Preparation of Lipospheres

Liposphere formulations using 1, 2, 5, and 10% bupivacaine free base in the process, were prepared by the following protocol: Melt a mixture of tristearin and bupivacaine at approximately 75°C and add hot 0.1M PBS (pH 7.4) at once along with phospholipid powder. Homogenize the hot mixture for 2 to 5 min using a Silverson L4 portable homogenizer (Silverson, MA) to form a uniform emulsion that appears white and creamy. Without any delay, rapidly cool the milky formulation down to approximately 20°C by immersing the formulation vial in an acetone-dry ice bath. Store the uniform dispersion at 4°C. The compositions of the formulations used in this study for *in vitro* and *in vivo* analyses are given in Table I.

In addition to the melt method described above, a solvent method of Liposphere preparation was used to compare formulation techniques (Table II). This process consisted of adding bupivacaine (200 mg), tristearin (400 mg), and egg phospholipid (200 mg) to a 50 ml round bottom glass flask. Chloroform (10 ml) was added and mixed to form a clear solution. The solvent was evaporated to dryness and hot 0.1M phosphate buffer solution, pH 7.4 (75°C, 9.3 ml), was added and homogenized for 2 minutes to form a uniform, milky formulation. The hot formulation was rapidly cooled to room temperature by immersing the flask in an ice bath with continued mixing to yield a white thin dispersion.

Characterization of Lipospheres

Particle size of the Liposphere formulations, which were made by the melt method only, was determined by using a Coulter LS100 particle size analyzer (Hialeah, FL). Bupivacaine was determined by high performance liquid chromatography (HPLC, Shimatzu C-R4AX system) according to USP XXII, 1990, using a C18 reverse phase column (Supelcosil LC-18, Supelco Bellefonte, PA). The mobile phase consisted of a mixture of acetonitrile and 0.01M phosphate buffer, pH adjusted to 7.7. Viscosity measurements of formulations were conducted using a RTV Brookfield viscometer (Brookfield Labs. Stoughton, MA) with RV spindle-#4 for the 0.84% and 1.6% formulations, and Halipath spindle-#B for the 3.6% and 5.6% formulations. The sperical shape was confirmed by transmission electron microscopy (Figure 1A) using a Philips electron microscope (Philips CM 12 at 100KV) and a previously described procedure (19). Recently published from our laboratory (19), blank Lipospheres in light microscopy pictures, which are similar to bupivacaine loaded Lipospheres, show a uniform distribution approximately 10 μm in size. The content of phospholipid on the surface was determined by the trinitrobenzene-sulfonic acid (TNBS, Sigma Chem. Co., St. Louis) method using Lipospheres containing phosphatidyl ethanolamine (PE) (20). Liposphere water content was determined by the Calcein method (21).

In short, Calcein was dissolved in buffer solution (2.5 mg/ml) used for the preparation of lipospheres. The fluorescence of liposphere dispersion was determined and quenched with 10 mM cobalt chloride solution (Jasco-FP-770 spectrofluorometer, 490 nm excitation and 520 nm emission). Then, Triton X-100 (10%) detergent was added to break apart liposphere particles and fluorescence was determined again. Liposphere water content was determined from total fluorescence and fluorescence after quenching, as previously described (21).

Determination of Drug Loading

Drug distribution in the formulation was determined by the following protocol: (a) isolate Lipospheres from the aqueous vehicle by centrifugation (14,000 RPM for 5 min), (b) measure the drug concentration in the supernatant by HPLC, (c) isolate the solid phase by extraction with a 0.01N HCl solution to

Table I. Bupivacaine-Liposphere Formulations[a]

Formulation Composition				Formulation Measurements			
Nominal Drug (%)	Added Bupivacaine (mg)	Added TS (mg)	Added EPC (mg)	Viscosity cps	Avg. Particle size (μm)	Loading Drug (%)	Actual Drug (%)
0	0	200	100	<100	12 ± 5	—	0
1	100	200	100	<100	14 ± 5	84 ± 5	0.84 ± 0.05
2	200	400	200	200	13 ± 6	80 ± 6	1.6 ± 0.12
5	500	1000	500	25,000	18 ± 15	72 ± 5	3.6 ± 0.25
10	1000	1000	500	80,000	16 ± 12	56 ± 7	5.6 ± 0.70

Note: TS = tristearin; EPC = egg phosphotidylcholine; CPS = centipoise.

[a] Buffer volume for each formulation was brought to exactly 10 ml during processing to produce the reported drug percentages (e.g., 1 g drug/10 ml buffer = 10% drug). Actual drug percent within the Lipospheres is the drug loading percent multiplied by the added bupivacaine percentage (e.g., 84 × .01 = 0.84).

Table II. Percentage of Drug Disposition in Liposphere Formulations: A Comparison Between Melt and Solvent Preparations Using Varying Ratios of Formulation Components

Ratio of Drug: Tristearin:Egg PC	Technique	Region 1[a] (Aqueous Phase)	Region 2[b] (Precipitated)	Region 3[c] (Bilayers)	Region 4[d] (Solid Core)
1:8:1	Melt	5.0%	21.2%	4.0%	72.7%
	Solvent	[e]	[e]	[e]	[e]
1:10:1	Melt	6.4%	21.0%	9.9%	70.4%
	Solvent	[e]	[e]	[e]	[e]
2:8:1	Melt	10.0%	18.9%	5.7%	61.1%
	Solvent	[e]	[e]	[e]	[e]
2:8:8	Melt	14.2%	20.7%	5.4%	61.3%
	Solvent	[e]	[e]	[e]	[e]
2:2:2	Melt	2.0%	12.5%	19.1%	47.0%
	Solvent	1.9%	14.3%	32.3%	26.9%
2:4:1	Melt	1.6%	24.9%	18.4%	48.2%
	solvent	1.7%	25.4%	25.2%	6.0%
2:2:1	Melt	2.2%	12.5%	31.6%	55.0%
	Solvent	2.3%	16.2%	41.7%	55.0%

[a] Region 1 represents the drug in the continuous aqueous phase at the conclusion of the formulation process. The amount of drug in solution was determined by HPLC after separating out the solids.
[b] Region 2 represents the drug which was precipitated or recrystallized out of solution during formulation or storage, respectively. This was determined by freeze-drying the solids, resuspending in fresh phosphate buffer (pH 2.0), separating out buffer by centrifugation, and HPLC analysis of buffer in which the unencapsulated drug is highly soluble and the drug in the bilayers or core is not soluble.
[c] Region 3 represents the drug in the bilayers which includes entrapped aqueous phase or drug between the hydrophobic tails. This amount was determined by HPLC after the addition of acetonitrile buffer (pH 6.8) to the freeze-dried solids and then subtracting region 1 and 2.
[d] Region 4 represents the drug incorporated into the solid core. This amount was determined by adding acetonitrile to the freeze-dried solids to dissolve the unincorporated drug. Since tristearin (the core material) is not soluble in acetonitrile, the insoluble freeze-dried solids represent the Liposphere core. (Preliminary experiments show that it is possible for 0-2% of the solid core bupivacaine to be extracted by acetonitrile during this procedure.) This insoluble material was separated, dissolved in tetrahydrofuran and HPLC analyzed for bupivacaine content.
[e] Liposphere formulation was unsuccessful.

dissolve unincorporated drug and (d) measure the drug content in isolated Lipospheres dissolved in tetrahydrofuran (THF). In a typical experiment, about 500 mg of the formulation is centrifuged for 2–3 min in an microfuge tube and the clear supernatant is isolated from the solid pellet precipitant. The solid phase is washed once with 2 ml of 0.01N HCl solution to dissolve unincorporated drug and the solid fraction that contains the incorporated drug is isolated by centrifugation and dissolved in THF. The solutions were analyzed for bupivacaine content by HPLC. Drug loading was determined to be 56% to 84% by weight of total drug used in the preparation with approximately 20–40% of drug found unincorporated (Table I).

To determine the form of the unincorporated drug, a bupivacaine Liposphere formulation was prepared without the tristearin component. A uniform and stable submicron dispersion was obtained. Microscopic examination of this fat free Liposphere formulation showed that the dispersed drug microparticles are non-spherical, in the form of long needles. It should be noted that bupivacaine free base is not dispersible in buffer solution without a surfactant such as phospholipids. The unincorporated bupivacaine, which results from tristearin Liposphere formulations, is in a form of dispersible microparticles made of solid drug and phospholipids.

At the completion of the Liposphere formulation process bupivacaine was found to reside not only in the solid core but also in three other compartments making up the liquid-solid mixture. Bupivacaine, tristearin and egg phosphatidyl choline (epc) ratios were varied to assess their effects on drug distribution (Table II). The preparation of these Lipospheres used 200 mg bupivacaine in 10 ml buffer solutions with respective amounts of tristearin and epc.

In Vitro Release Experiments

Release studies were conducted in dialysis tubing with a molecular weight cut-off (MWCO) of 300,000 (Spectrum, CA). One ml of Liposphere formulation (1.6% and 3.6% formulations depicted in Table I) was placed into the dialysis tubing previously presoaked in PBS (pH 7.4). The clamped dialysis tubing was placed in a jar containing 800 ml of PBS. The jars were agitated on an orbital shaker at 100 rpm, in a 37°C oven. Samples were taken at discrete times and analyzed by HPLC to determine the drug release rates. In controlled experiments, bupivacaine-HCl release was not appreciably altered by the dialysis membrane or by gamma-irradation using two formulations (Bup:TS:PC: 1:4:2, 2:4:2).

In Vivo Studies

Neural Block Paradigms

Bupivacaine formulations were tested for their anesthetic effect over time using several behavioral tests: a) leg-resistance as a measure of motor capacity (22), b) leg-withdrawal to a hot plate (5), and c) a modified vocalization test to hind paw shock (23,24). All animal methods adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985) and received IACUC approval.

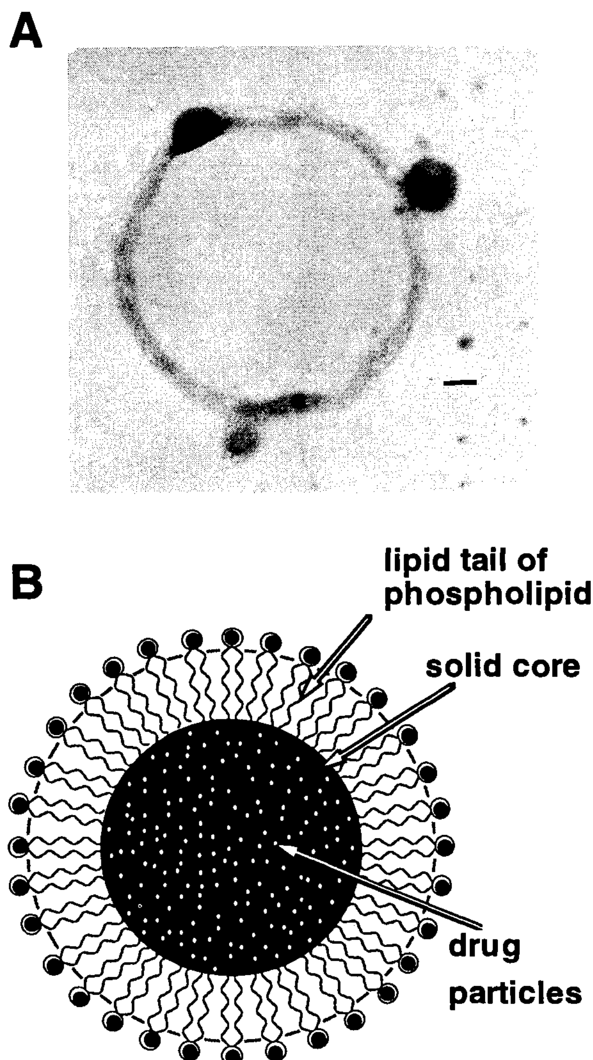


Fig. 1. (A) An T.E.M. photomicrograph of typical bupivacaine-Liposphere formulation (approximate magnification = 55,000×). The scale bar in the bottom right corner is equal to 1 μm. (B) A schematic representation of a Liposphere particle. The bupivacaine Liposphere system consists of solid spheric particles composed of hydrophobic tristearin and bupivacaine free base, entirely covered by a phospholipid surface. The hydrophobic side of the phospholipids are embedded in the surface of the solid hydrophobic core and the hydrophilic side of the phospholipids interfaces with the aqueous solution.

Sciatic Nerve Model

A rat sciatic nerve preparation was modified from a previous investigation to study prolonged local anesthetic blockade from bupivacaine-Liposphere formulations that does not require the induction of hyperalgesia (4,5). Blockade of the rat sciatic nerve is a time honored system for studying local anesthetics. The nerve is easily located for Liposphere administration and local anesthetic effects can be readily detected, including motor, sensory and sympathetic blockade.

Male Sprague-Dawley rats (200–250 g) were surgically prepared for Liposphere administration using a previously described technique (5). In brief, rats were anesthetized with either 50–75 mg/kg pentobarbital or halothane (4% in oxygen for induction and 2% for maintenance) to allow faster recovery for behavioral measurements. Bilateral posterolateral incisions were made in the upper thighs, and the sciatic nerves were visualized with care to avoid direct trauma. Bupivacaine-Lipospheres (0.4 ml) were injected around the nerve on one leg, and no-drug Liposphere formulations (0.4 ml) were injected on the contralateral control leg. The fascia and muscle surrounding the administration site was closed over to partially restrict egress of Lipospheres and the entire wound area was lavaged with 0.5 cc of an antibiotic solution (5000 units/ml penicillin G sodium and 5000 μg/ml streptomycin sulfate). The experimenter performing subsequent behavioral assessments was unaware of which side received the bupivacaine-Lipospheres.

Observed Motor Block

To assess motor block, a 4-point scale based on visual observation was devised: 1) normal appearance, 2) intact dorsiflexion of foot with an impaired ability to splay toes when elevated by the tail, 3) toes and foot remained plantar flexed with no splaying ability, and 4) loss of dorsiflexion, flexion of toes, and impairment of gait. For clarity, partial motor block equals a score of 2 and dense motor block is a score of either 3 or 4 (4,5).

Leg Resistance Motor Test

In addition, a motor test was used that measures the rat's leg resistance against a flat surface (22). The rat is held in the same manner as during leg-withdrawal sensory testing (described below) so that it is positioned to stand on one leg against an electronic balance. The resistance of the rat's leg is measured as the force against the balance in grams. Previous results from related experiments show that a 200–275 g rat exerts about 150–225 grams of force with a normal leg. However, if the leg is showing dense motor block from local anesthetic action, then forces of 30 g to 70 g are expected. This test was used for the 1.6% Liposphere formulations only, so as to enhance the detection of motor blockade by attributing quantitative values to motor function rather than just criteria based observations.

Hot Plate Sensory Test

Sensory blockade was measured by the time required for each rat to withdraw its hind paw from a 56°C plate (IITC Life Science Instruments, Model 35-D, Woodland Hills, CA). The rats were positioned to stand with one hind paw on a hot plate and the other on a room temperature board. Latency to withdraw each hind paw from the hot plate was recorded by alternating paws and allowing at least fifteen sec of recovery between each measurement. If no withdrawal occurred from the hot plate within 15 sec the trial was terminated to prevent injury and the termination time was recorded. Testing ended after five measurements per side and the mean was calculated for each side.

An advantage of the leg withdrawal system described here is that a unilateral hind paw can be tested and compared to the contralateral control leg. Furthermore, this model permits independent motor and sensory testing. Indeed, a rat with complete motor block, scoring 4 on the visual motor block scale,

can still withdraw its paw if sensory blockade is not complete. The adductor muscles joined to the hip, which are innervated by the femoral and obturator nerves, allow leg withdrawal from the hot plate even during sciatic nerve block. In this way, agents that selectively block motor and not sensory function can be detected.

Vocalization Threshold Sensory Test

Sensory blockade was also measured with a vocalization test to transcutaneous electrical stimulation (23,24) that was modified for the rat hind paw. The method of limits was used to find the inflection point of six trials to arrive at the mA vocalization threshold. Specifically, rats were conditioned to vocalize to a small current. During testing, each rat was restrained with cloth wrapping anchored to a rigid plastic container. Vision was obstructed but the nose and mouth remained free. The hind legs were immobilized by tape. A stimulation unit (Grass S44 Stimulator, stimulation rate 0.2 pulses/sec, delay 1 sec, duration 100 msec, 0–120V, and a Grass SIU5 Stimulator Isolation Unit with direct coupling, normal polarity, and output = input) was used to increase current by 0.05 mA increments in a stepwise fashion until the rat vocalized. Because electrical resistance varies in the electrode-hind paw circuit, the amount of current delivered through the rat's hind paw was measured by an oscilloscope (Tektronix 2205 20 mHz) and recorded. Electrodes (approximately 4 mm in diameter), covered with conductive gel, were clamped to the center area of the hind paw in a dorsal-ventral orientation. Three repeatable measurements (±0.1 mA) per foot (6 per animal) established a vocalization threshold for each paw. Before Liposphere administration, rats were habituated to the test until equivalent thresholds (±0.1 mA) were demonstrated between the right and left side for three consecutive days. Daily vocalization thresholds of the experimental paw were compared to the contralateral control paw.

Statistical Analyses

The statistical analyses consisted of the two-tailed Student's t test and the analysis of variance with post-hoc comparisons. A probability value of 0.05 or less was used to reject the null hypothesis and accept the research hypothesis.

RESULTS

Characterization of Liposphere Structure

Lipospheres were examined for particle size, water content, surface phospholipid, analysis of ingredients, and drug loading. The average particle size of several formulations is given in Table I. A bimodal particle size distribution was observed, however, the larger distribution (20–50 μm) amounts to only <7% of total and may be caused by Liposphere material aggregation. The average particle size was 5–15 μm with less than two percent of all particles greater than 50 μm.

Liposphere structure, including an analysis of surface phospholipid content, was determined by macroscopic examination. Lipospheres were determined to be spherical particles (Figure 1A) with over 90% surface phospholipid, much higher than reported for liposomes which are typically at less than 50% surface phospholipid (25). Liposphere analysis is consistent with a spherical monolayer of phospholipid, where the hydrophobic chains of the phospholipids are embedded into the surface of the spherical drug-triglyceride core, illustrated in Figure 1B.

Drug Incorporation into Lipospheres

In using either the melt or solvent formulation techniques, it was found that drug could exist in 4 different regions of the Liposphere (Table II). The melt method is superior to the solvent method in producing Lipospheres with greatest percentage of drug in the solid triglyceride core (72.7% versus 26.9%).

Liposphere *In Vitro* Release

The *in vitro* release study was conducted using large pore dialysis tubing (300,000 MWCO) to minimize tubing effects on the release rate that was apparent with smaller pore tubing (12,000 MWCO). The control solution of bupivacaine (Marcaine-0.75% solution) was completely released (>99%) through the large pore tubing within 3 hr. The drug release from 1.6% and 3.6% bupivacaine loaded Liposphere formulations are shown in Figure 2. Both formulations released drug for 48 hr following first order kinetics ($r^2 = 0.97$). It should be noted that the aqueous vehicle used for release saturates at 4% soluble free drug and that the Liposphere release experiments were designed so that released soluble bupivacaine never went over 0.125%. Therefore, the bupivacaine concentration was less than 2% saturated, far below maximum solubility of free drug. The decrease in drug release rate is due to depletion of drug available for release, *ergo*, first order kinetics ($y = 9.6 + 3.25x - 3.02^{-2x^2}$ for 3.6% bupivacaine-Lipospheres shown in Figure 2).

Liposphere Nerve Block Results

Motor Block

Both 3.6% and 5.6% bupivacaine formulations showed significant levels of motor blockade for 3 days (Figure 3). According to a visual scale, motor function in all these animals

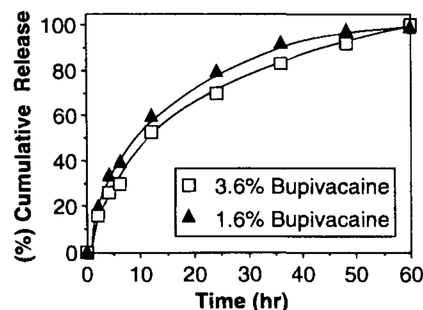


Fig. 2. The graph shown represents the *in vitro* release of bupivacaine from 1.6% and 3.6% loaded formulations (material content given in Table I). One ml of the Liposphere formulation or bupivacaine solution was placed in dialysis tubing (300,000 MWCO), sealed, and placed into 800 ml of phosphate buffer solution at 37°C (pH 7.4). One ml samples were removed at predetermined time points and the bupivacaine concentration was determined by HPLC. Symbols depict the mean of three points, encompassing standard errors of the mean. All Liposphere formulations were sterilized by gamma irradiation (90 rad) for *in vitro* and *in vivo* experiments.

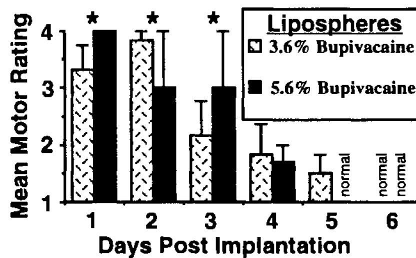


Fig. 3. Graph of motor blockade produced by administration of Lipospheres containing 3.6% and 5.6% bupivacaine free base. The data is represented as means ± SEM. *$p < 0.05$ significance in comparison to contralateral control side (3.6% and 5.6% rats, n = 6 and n = 3, respectively, were pooled for statistical analysis since they were not statistically different from each other).

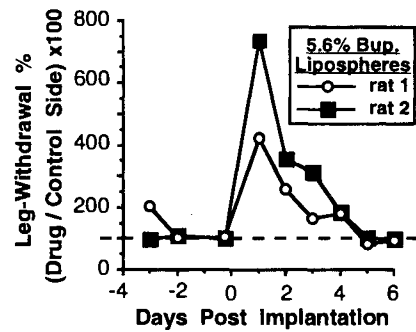


Fig. 5. Graphic depiction of withdrawal latency to a 56°C plate after administration of 5.6% bupivacaine-Liposphere formulations is shown. Observed anesthetic motor blockade (not shown) was dense on day 1, partial on days 2–3, and not present on day 4 through the end of the 14 day observation period.

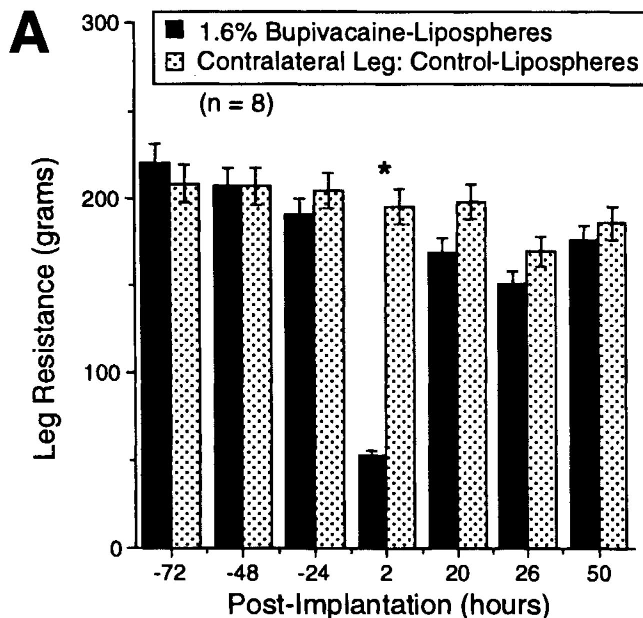
returned to normal by day 6. Using the leg-resistance procedure, 1.6% bupivacaine formulations showed a significant level of motor blockade for 2 hr before returning to control resistance levels by 20 hr (Figure 4A).

Sensory Block (Hot Plate)

The 1.6% bupivacaine-Lipospheres significantly increased leg-withdrawal latencies in hot plate test for 20 hr duration ($p < 0.05$, n = 5) (Figure 4B). No impairments were observed on contralateral control side, administered with an equal mass of Lipospheres without drug. All rats returned to pre-Liposphere and contralateral control latencies by day 2. In two rats, 5.6% bupivacaine-Lipospheres administered to sciatic nerve showed increased latencies to leg-withdrawal to hot plate test 4 days post-application (Figure 5), even after motor function to a visual scale was back to normal by day 3 in both rats (both rats showed motor block for 2 days). Both rats returned to pre-Liposphere and contralateral control latencies by day 5. In comparison, administration of 0.75% bupivacaine-HCl solution (3 ml) to sciatic nerve produces only approximately 2–4 hr of dense to partial local anesthetic block in hot plate and motor observation assessments.

Sensory Block (Vocalization Tests)

Rats were also tested for sensory blockade by using vocalization threshold to electrical stimulation of the hind paw. Figure 6 depicts plots of vocalization threshold to electrical stimulation, expressed as a ratio of the drug side to the contralateral control side from the mean mA required to elicit three reproducible vocalizations. The 3.6% bupivacaine-Liposphere formulations administered to six rats produced a 25–50% increase in vocalization threshold for three days ($p < 0.05$, n = 6). In all rats, hind paw tests returned to the pre-Liposphere level by day 4.



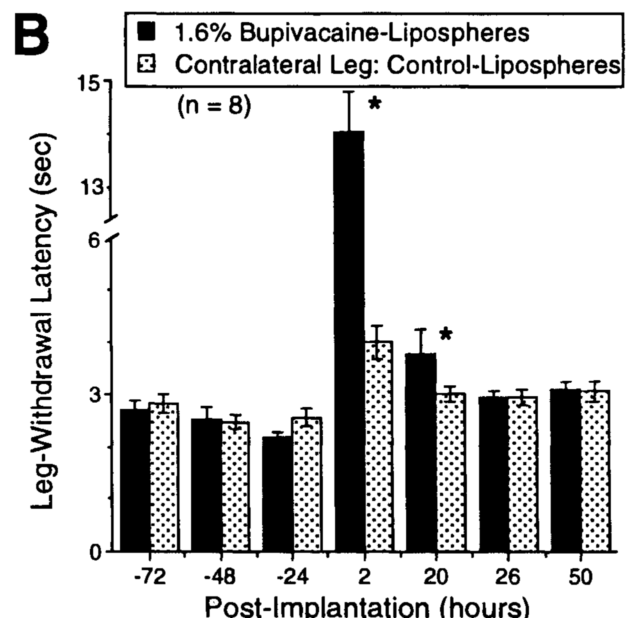


Fig. 4. Graphic depiction of anesthetic blockade observed with 1.6% bupivacaine-Liposphere formulations in motor capacity tests that measure leg resistance (A) and in sensory tests that measure withdrawal latency to a 56°C plate (B) are shown.

Histology of Administration Site

At the conclusion of the behavioral testing (one week post-application), sciatic nerves were removed and fixed in formalin, embedded in paraffin, and examined following hematoxylin-eosin staining. Histologic evaluation by light microscopy revealed no evidence of nerve damage and very little perineural inflammation or foreign body response, however, further safety testing is needed.

DISCUSSION

This study reports that Lipospheres, a type of liposomal matrix, successfully incorporate and release the local anesthetic, bupivacaine, showing first order kinetics over a 2 day period under ideal sink conditions, *in vitro*. This study demonstrates that a) bupivacaine-Liposphere preparations can provide dose-dependent degrees of motor and sensory blockade of rat sciatic nerves for at least 1–3 days, *in vivo*, and b) nerve blockade by these preparations appears entirely safe and reversible. The duration of local anesthetic release from Lipospheres *in vitro* approximated the duration of sensory blockade *in vivo*, and the dose of bupivacaine delivered effects the observed duration of nerve blockade. These findings indicate that bupivacaine-Lipospheres greatly extend the local anesthetic blockade that can be produced by bupivacaine alone, however, further testing is needed to evaluate nerve blockade duration, pharmacokinetics, and toxicity (6,26).

In comparison to a chloroform solvent method, Liposphere formulations produced by the melt method were preferred (Table II). For several drug:tristearin:phosphatidyl choline ratios, the melt method was far superior in incorporating bupivacaine into the solid core. These results also show that drug loading is dependent on the amount of carrier and phospholipid used in the formulation process. Although some drugs may be sensitive to a melt method, it is advantagous over a chloroform solvent in that there is no concern over residual solvent remaining in the Lipospheres which could produce toxic effects.

The *in vitro* release results suggest that the drug dose within the inner triglyceride core can change the amount of drug released without appreciably altering the percentage of released drug under ideal sink conditions (Figure 2). Thus, dosing the Lipospheres to release drug at greater rates with the same cumulative release profile is easily accomplished. For example, *in vivo*, larger bupivacaine dosages in Lipospheres produce longer duration SLAB, indicating that a efficacious dose is being delivered for a longer period. *In vivo*, at a sciatic nerve site, it is also possible that drug release from Lipospheres may be impeded by slow fluid flow, resulting in reduced sink conditions. Therefore, Lipospheres with a higher dose of drug will be affected to a greater degree than lower doses to extend drug release and its action at the nerve site. It is likely that both the physical release characteristics of Lipospheres and the physiological characteristics of the administration site affect drug delivery to the nerve.

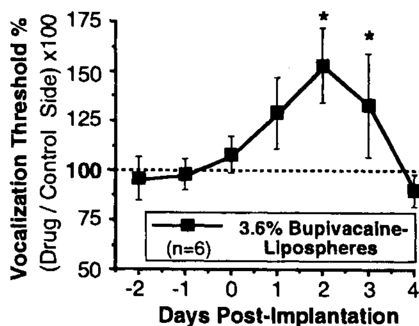


Fig. 6. The results shown in graph depict the effects of Liposphere formulations tested in vocalization threshold-sensory block tests using the method of limits. This graph plots the ratio of experimental over control leg mA. A ratio of 100% is obtained when both experimental and control leg are equal.

The Liposphere particle size allows administration at many sites, including perineural, subcutanèous or intramuscular locations. Indeed, the small particle size of Lipospheres (<20 μm) is hypothesized to be well-tolerated by single cell contact, whereas larger particles (>50 μm) are thought to be much more reactive due to attractive forces (e.g., van der Waals). Since lecithin (i.e., phosphatidyl choline) is a normal constituent of cell membranes, it is hypothesized to be non-immunogenic and a biocompatible material (10). Furthermore, lecithin appears to produce little self-aggregation or cell binding tendencies (27). It will be important, however, to evaluate the safety and efficacy of Lipospheres at all application sites for SLAB.

The action of local anesthetics on peripheral nerves will depend on nerve fiber diameters, the penetration of drug into nerve, the method of delivery, and drug clearance. In bolus injections, central nerve fibers are the last to be blocked and the last to regain function as local anesthetic is cleared from the more peripherally located nerve fibers. When local anesthetic is released in a slow controlled manner and concentrations taper off asymptotically, it is not clear whether central or peripheral nerve fibers will be first to regain function. Therefore, the bupivacaine-Liposphere SLAB system requires investigation of nerve pharmacokinetics to understand the dynamics of drug titers within the nerve. The present investigation demonstrates that the local anesthetic effects from Lipospheres *in vivo* outlast expected release rates determined *in vitro*, under ideal sink conditions. It is possible that perineural tissues absorb bupivacaine (partition coefficient = 130) over time, since it is mostly nonionized and lipid soluble, so that drug titers around the nerve do not decrease until after the Lipospheres are depleted. Moreover, this may be a saturable effect since 5.6% bupivacaine-Lipospheres did not increase the local anesthetic effect duration compared to the 3.6% dosage formulation. It is also possible that perineural tissue absorption reduced the expected drug effect duration of the 1.6% formulation by lowering nerve drug titers below an efficacious dose.

Since sensory blockade measurements are complicated by motor blockade, a method of detecting sensory blockade that is independent of motor responses (i.e., escape) was used. In this regard, vocalization threshold in response to defined transcutaneous electrical stimulation of points on the paw was used as a criterion for measurement of sensory blockade. However, this method has several limitations, including: (1) potential for amount of delivered current to vary, even if skin contact points are held fixed, and (2) potential for current to activate saphenous nerve dermatomes, which will confound sciatic nerve block tests. Nevertheless, vocalization threshold tests showed that a single application of 3.6% bupivacaine-Lipospheres to the sciatic nerve produced 3 days of SLAB (Figure 6).

There are several potential clinical applications for Lipospheres. This drug delivery system may be used for numerous drugs for site-specific pharmacotherapy. Indeed, a single injection of a mixture that time-delivers local anesthetic to produce SLAB has the potential to greatly enhance current pain therapies. In addition, since the timed-release of local anesthetic (or other drugs) in this SLAB system can produce a stable local titer of bupivacaine which is less likely to produce rapid increases in circulating blood levels, it may provide a safer means to produce stronger and longer lasting analgesia than multiple injections. In fact, local anesthetics which are usually injected as an aqueous solution are eventually absorbed from the application site into the general circulation and, therefore, their therapeutic indices, dosages, and frequency of dosage must be strongly considered before administration to a patient (28,29). Site-specific delivery of a local anesthetic, sustained-release formulation could provide a technique to produce more discrete nerve block than has been previously possible.

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