Antibacterial Activity of Contact Lenses Bearing Surface-Immobilized Layers of Intact Liposomes Loaded with Levofloxacin

ANNE DANION,[1,2] ISABELLE ARSENAULT,[1,2] PATRICK VERMETTE[1,2]

[1]Laboratoire de Bioingénierie et de Biophysique de l'Université de Sherbrooke, Department of Chemical Engineering, Université de Sherbrooke, 2500, blvd de l'Université, Sherbrooke, Québec, Canada, J1K 2R1

[2]Research Centre on Aging, Institut universitaire de gériatrie de Sherbrooke, 1036, rue Belvédère Sud, Sherbrooke, Québec, Canada, J1H 4C4

Received 21 August 2006; revised 22 October 2006; accepted 1 December 2006

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.20871

ABSTRACT: *In vitro* methods to evaluate antibacterial activity were used with contact lenses bearing levofloxacin-loaded liposomes developed for the prevention and treatment of bacterial ocular infections such as keratitis. Levofloxacin was incorporated into liposomes before these intact liposomes were immobilized onto the surfaces of soft contact lenses using a multilayer immobilization strategy. The release of levofloxacin from contact lenses bearing 2, 5, and 10 layers of liposomes into a saline buffer at 37°C was monitored by fluorescence. The levofloxacin release, as a function of time, was described by a mechanism taking into account two independent first-order kinetic models. The total release of levofloxacin from the contact lenses was completed within 6 days. The release of levofloxacin from contact lenses bearing 10 layers of liposomes and subsequently soaked overnight in a levofloxacin solution was also studied and compare to that of dried contact lenses without any chemical modification rehydrated in a levofloxacin solution. The antibacterial activity of the liposome-coated contact lenses against *Staphylococcus aureus* was evaluated by measuring (i) the diameters of the inhibition zone on an agar plate and (ii) the optical density using a broth assay. The liposome-coated lenses showed an antibacterial activity both on agar and in broth following 24 h. When initial bacteria inocula were equal or below 10^6 CFU/mL, all the bacteria were inhibited within 2 h. When using initial bacteria inocula of 10^8 CFU/mL, an initial burst release provided by soaking the liposomal lenses was required for the first hours to inhibit bacteria growth. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:2350–2363, 2007

Keywords: immobilized layers of intact liposomes; contact lens; kinetic of antibiotic release; *Staphylococcus aureus*; biomaterials; targeted drug delivery; coating; controlled release; *in vitro* models

INTRODUCTION

The cost of blindness: In 2003, the direct cost to the Canadian government for just the federal disability tax exemption and disability payments for people with vision loss is conservatively estimated at $2 billion per year.[1] In addition to being a public health problem, blindness and visual impairment have important socio-economic implications.[2] It has been estimated that in the USA, if all the avoidable blindness in persons under 20 and working-age adults were prevented, a potential saving of US $1 billion per year would accrue to the federal budget.[3] The 2002 USA market for prescription ophthalmic drugs was

Correspondence to: Patrick Vermette (Telephone: +1-819-821-8000x62826; Fax: +1-819-821-7955; E-mail: Patrick.Vermette@USherbrooke.ca)

Journal of Pharmaceutical Sciences, Vol. 96, 2350–2363 (2007)
© 2007 Wiley-Liss, Inc. and the American Pharmacists Association

WILEY InterScience® DISCOVER SOMETHING GREAT

$2.8 billion, and, rising at an average annual growth rate of 8.6%, will reach $4.5 billion in 2007. Products within the ocular infection treatment segments accounted for $500 million in 2002. Many ophthalmic diseases, such as viral and bacterial infections, chronic conditions, and corneal graft rejection are treated by topical drug administration to the ocular surface.[4–7] Most ophthalmic drugs are applied in solution form to the ocular surface.[6] A major problem with this approach is limited drug uptake, since the drug solution is quickly washed away by tearing action.[6] Because of the rapid clearance, an ophthalmic drug has to be administered several times a day, which reduces patient compliance, and can be quite uncomfortable for the patient, as some drugs cause blurred vision for hours after application.[4]

Ocular infectious diseases: Defects in the tear film, chemical or foreign body trauma, allergic hypersensitivity reactions, and overuse of contact lenses, as well as complications after laser *in situ* keratomileusis, can result in injury to the ocular surface and predispose the cornea to infection.[8,9]

Because of its high incidence and potential complications, bacterial keratitis is one of the most threatening ocular infections. *Pseudomonas aeruginosa* and *Staphylococcus aureus* frequently cause severe keratitis that may lead to progressive destruction of the corneal epithelium and stroma.[10,11] Infectious keratitis due to these organisms often causes corneal scarring, corneal perforation, and blindness if aggressive and appropriate therapy is not promptly initiated.[12,13]

For successful therapy of bacterial keratitis, the drug must be able to attain rapidly high concentrations at the site of infection. Since the cornea is not vascularized, it is not readily permeated by systemically administered drugs, which are therefore generally not used for the treatment of keratitis.[12] On the other hand, topical treatment may fail to achieve therapeutically active drug levels in the cornea, as continuous tear flow reduces the bioavailability of topically applied antibiotics and the corneal epithelium acts as a barrier against drug penetration. For this reason, standard treatment of severe bacterial keratitis requires administration at frequent intervals (every 15–60 min for 48–72 h) of eye-drops often containing fortified solutions of fluoroquinolones (more concentrated than commercially available solutions) or multiple antibiotics, usually a cephalosporin and an aminoglycoside.[12,14–16] However, this regimen not only is disruptive to the patient and usually necessitates hospitalization, but it has also been associated with *in vitro* toxicity to the corneal epithelium.[17,18] Efforts are now directed to testing new antimicrobials that better permeate the cornea and to developing delivery systems capable of prolonging the contact time between antibiotics and the corneal tissue, thereby potentially enhancing intra-corneal delivery of ophthalmic medication.

Antibiotic resistant infectious diseases impose a significant burden on society, and surpass $30 billion in annual direct treatment costs in the USA alone. The high cost stems from several key factors: (1) Individuals infected with drug resistant organisms usually have a poor prognosis and are more likely to require hospitalization and (2) drug-resistant infections acquired in hospitals nearly triple the cost of hospital stays. The cost of antibiotic resistance is expected to grow larger as medical experts anticipate record increases of antibacterial resistance in the next decade.[19–22]

Pharmaceutical companies have exhausted the arsenal of known antibiotic classes. Currently, the launch of new antibacterial products usually includes stronger dose formulations of old antibiotic classes. This results in the development of more antibiotic resistant strains and increased resistance of current strains. New classes of antibiotics and delivery systems that would limit the use of massive concentration of antibiotics are needed. Two major ocular pathogens that have demonstrated widespread antibiotic resistance are *S. aureus* and *Pseudomonas aeruginosa*.[19–22]

Eye-drops are the conventional dosage forms that account for 90% of currently accessible ophthalmic formulations.[23] Despite the excellent acceptance by patients, one of the major problems encountered is rapid precorneal drug loss.[24] The value that drug delivery adds can improve safety, efficacy, convenience, and patient compliance. Standard delivery of drugs results in burst of medication at the time of dosing, followed by a rapid loss of the drug.[25] The development of drug delivery systems is essential to achieve long-term release, and polymer technology has made such delivery systems available. It is also important to have available a system that delivers drugs locally, lowering the overall dose needed to achieve a therapeutic concentration. This makes medication more effective with lower side effects.[23,24,26] Several types of ophthalmic drug delivery systems have been proposed to provide a sustained release over time, and these include hydrogels,[27] cyclodextrins,[28] collagen shields and contact

lenses,[29] used either alone or loaded with therapeutic agents, and colloidal systems suspended in a liquid or ointment carrier.[30–32] However, most hydrogels offer only moderate to marginal improvement of ocular drug bioavailability and can cause blurred vision.[23,27] Soft contact lenses have become a valuable tool in the management of many ophthalmic disorders.[29,33,34] Contact lenses can be loaded with medications by presoaking them in a medication solution for therapeutic applications. However, contact lenses only presoaked in medications provide a marginal mean of delivery because therapeutics freely dispersed within the contact lens structure are rapidly released (i.e., burst-release), often leading to increased topical drug side effects and toxicity reactions.[35]

Drug delivery by injectable liposomes is well known in the pharmaceutical industry. Liposome suspensions of various compositions have been developed to enhance sustained release of medications in the front of the eye,[30,36,37] but one of the major problems in such ocular applications is limited drug uptake because liposome suspensions are quickly washed away by tearing action. Recently, a group has proposed to disperse dimyristoylphosphatidylcholine (DMPC) liposomes into the contact lens material.[38,39] However, the procedure suggested in this study requires the use of radicals for the polymerization of the contact lens matrix, which cannot be used with drug sensitive to radicals.

Because many polymers cannot be loaded with diffusible drugs owing to insufficient solubility of the drug into the polymer or an inadequate diffusion rate of the drug through and out of the polymeric materials that constitute the biomedical device, we recently proposed to deliver such drugs by loading them into liposomes and binding the intact liposomes onto the surface of devices.[40–42] The objectives of the present study were to investigate the antibacterial activity of contact lenses bearing surface-immobilized layers of intact liposomes loaded with levofloxacin. Levofloxacin was chosen as a model drug for its broad antibacterial spectrum against both Gram-positive and Gram-negative bacteria[43] and its commercial availability in a relatively pure form (and without additives) without any commercial constraint. *S. aureus* was selected to determine the antibacterial activity of the device because it is known to be a significant cause of keratitis.[44] First, the kinetics release of levofloxacin from a liposome-coated contact lens was studied. Then, the antibacterial activity was tested both using agar and broth assays.

MATERIALS AND METHODS

Materials

Contact lenses (Hioxifilcon B, Opti-Gel 45G, Opti-Centre, Sherbrooke, QC, Canada) were kindly provided by Robert Mercure from Opti-centre and used as substrates for surface immobilization of intact liposomes. These lenses were readily available to us with no commercial restriction.

Hexane (ACS grade) was purchased from ACP (Montréal, QC, Canada). Levofloxacin (98% purity, #28266), disuccimidylcarbonate (DSC, technical grade, #225827), anhydrous acetonitrile (CH_3CN, 99.9% purity, #271004), *N*-hydroxysuccinimide (NHS, #H-7377), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES, #H-3375, 99.5%), *t*-octylphenoxypolyethoxyethanol (Triton X-100, #T-9284), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, #E-1769) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Poly(ethylenimine) (PEI, 70 kDa MW, #00618) was obtained from Polysciences, Inc., (Warrington, PA). Müeller–Hinton broth (MHB, #B11443), tryptic soy broth (TSB, DF370173), agar (DF0054176), bacto agar (DF0140010), Brucelle broth, sodium chloride (NaCl, ACS grade), sodium sulfate (Na_2SO_4, ACS grade), chloroform (HPLC-grade), and ethanol (HPLC grade) were purchased from Fisher Scientific (Ottawa, Ontario, Canada). 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (>99%, #850365), cholesterol (CHOL) (>99%, #700000), *N*-[ω-(biotinoylamino)poly(ethylene glycol) 2000]-1,2-distearoyl-*sn*-glycero-3- phosphoethanolamine (DSPE-PEG (2000)-Biotin) (>96%, #890129) were obtained from Avanti Polar Lipids Inc., (Alabaster, AL). Biotin-PEG-CO_2-NHS (NHS-PEG-Biotin, #0H4M0F02) was purchased from Nektar Therapeutics (Huntsville, AL).

NeutrAvidin™ (ImmunoPure® NeutrAvidin™ biotin-binding protein, #31000) was obtained from Pierce (Rockford, IL). NeutrAvidin is a modified avidin with low nonspecific binding properties and does not contain carbohydrates, thus eliminating the potential of binding to lectins (information obtained from Pierce).

S. aureus (ATCC 29213) was used in this study and grown aerobically at 37°C on tryptic soy agar. Isolates were frozen at −80°C in 1.5% bacto agar

and 25% Brucelle broth containing 15% glycerol, with two subcultures made before the organisms were tested.

Methods

Preparation of Liposomes

The solution of antibiotics used to prepare the liposome suspensions contained 100 mg/mL (270 mM) of levofloxacin in a 150 mM sodium chloride buffer (pH adjusted at 6.8 by 2 M HCl) and a NaCl concentration readjusted subsequently, using the Advanced™ Osmometer (Model 3250, Advanced Instruments Inc., Norwood, MA), to obtain a levofloxacin solution with an osmolarity of 290 mOsm. The solubility of levofloxacin is strongly pH dependent and is maximal at pH 6.7. Milli-Q® gradient water (Millipore Canada, Nepean, Ontario, Canada) with a resistivity of not less than 18.2 MΩ · cm was used to prepare the antibiotic solutions. The levofloxacin solution was filtered using sterile 0.22 μm filters (Millex® GP, Millipore, Cork, Ireland) before use in the preparation of the liposome suspensions.

Unilamellar vesicles (ULVs)[45] were prepared by first mixing in a round-bottom flask DSPC, cholesterol, and DSPC-PEG(2000)-Biotin (2:1:5 mol ratios) in chloroform. Approximately 144 μmol of lipids were deposited from 2 mL chloroform solution to form a thin film on the interior surface of a 50 mL round-bottom flask by rotary evaporation under a pressure of ~13332 Pa for 4 h. Following addition of levofloxacin solution, lipids were hydrated in the dark above 65°C. The multilamellar vesicle (MLV) suspension thus produced was subjected to 10 freeze-thaw cycles involving quenching in dry ice and acetone, followed by immersion in a 65°C water-bath. Unilamellar vesicles were finally produced by extrusion through 100 nm pore polycarbonate Avestin® track-etch membranes using the Avestin Liposofast (Avestin Inc., Ottawa, Ontario, Canada) operated at 65°C. Separation of the levofloxacin-containing vesicles from non-entrapped levofloxacin was achieved by gel chromatography, which involved passage through a 2.5×25 cm column of Sephadex™ G-50 Fine (Amersham Pharmacia Biotech, Québec, QC, Canada). The column was eluted at room temperature with a NaCl buffer. The total lipid concentration of the liposome suspension collected at the column outlet was adjusted to the desired concentration using a NaCl buffer.

Immobilization of Liposomes

Immobilization and detailed surface characterization of liposomes onto contact lens surfaces were previously described.[42] Briefly, each contact lens was sonicated in 4 mL of anhydrous acetonitrile for 15 min to remove any trace of contaminants. Each contact lens was then immersed in 3 mL of a DSC solution for 1 h under vigorous shaking. After this step, the lenses were rinsed in acetonitrile and immersed in a 3 mg/mL solution of polyethylenimine (PEI) in water with the pH adjusted to 7.4 with 1 M HCl. The reaction was allowed to proceed overnight under vigorous shaking. To remove any noncovalently adsorbed PEI, the contact lenses were then rinsed overnight under vigorous shaking in a 150 mM NaCl solution with the solution changed twice. The lenses were finally soaked overnight in water with the solution changed twice prior to further use.

PEI-coated lenses were immersed in a 1 mg/mL solution of NHS-PEG-Biotin under cloud point conditions (170 mg/mL of Na_2SO_4 were added to the solution of NHS-PEG-Biotin to form aggregates of PEG). 0.7 mg/mL of EDC and NHS was added to the NHS-PEG-Biotin solution during the coupling procedure to reactivate the ester groups that could be potentially hydrolyzed. The reaction was allowed to proceed overnight at room temperature under vigorous shaking. To remove any noncovalently attached NHS-PEG-Biotin, the contact lenses were then rinsed overnight under vigorous shaking in a 150 mM NaCl solution with the solution changed twice. The lenses were finally soaked overnight in water with the solution changed twice.

Next, PEG-Biotin-coated contact lenses were immersed in a 50 μg/mL solution of NeutrAvidin in 10 mM HEPES buffer at pH 7.4.[46] The reaction was allowed to proceed overnight at room temperature. To remove any proteins not linked to PEG-biotin (i.e., NeutrAvidin molecules that can be loosely adsorbed onto the PEG layer), samples were rinsed overnight in a 10 mM HEPES solution with the solution changed twice.

Immobilization of liposomes was performed by incubating the NeutrAvidin-coated lenses in a 1 mg/mL (total lipid concentration) biotinylated-liposome suspension made of DSPC:CHOL:DSPE-PEG(2000)-Biotin (2:1:5 mol%) for 1 h. NeutrAvidin-coated contact lenses were immersed in 3 mL of the biotinylated-liposome suspension. To remove loosely adsorbed liposomes, the samples were rinsed for 1 h in the HEPES buffer. The buffer

solution was changed three times. Multi-layers of liposomes were fabricated by adding, after the attachment of the first liposome layer, more NeutrAvidin, which can add to biotins on the solution side of the liposomes present, following which, more liposomes can be added to bind onto the NeutrAvidin molecules, and so forth. Contact lenses bearing one layer of surface-immobilized biotinylated liposomes were immersed in a 50 μg/mL solution of NeutrAvidin in 10 mM HEPES buffer at pH 7.4 for 30 min. They were rinsed for 1 h in the HEPES buffer with the solution changed three times. A next layer of liposomes was then added to the surfaces by incubating them in the 1 mg/mL biotinylated-liposome suspension.

Contact lenses were sterilized by a 5 min soaking in 70% ethanol just before liposome attachment, the next steps were done in a laminar flow cabinet under sterile conditions. Ethanol incubated lenses were thoroughly rinsed overnight in sterile water with the water solution changed several times to remove any trace of ethanol.

Some contact lenses bearing 10 layers of liposomes were soaked overnight in a 5 mg/mL levofloxacin solution (a concentration corresponding to that of commercial eye-drops) to investigate the influence of a burst of release of antibiotics at the beginning of the infection (i.e., to inhibit the maximum of bacteria in cases where bacteria concentration would be high) followed by a sustained release provided by the liposome layers. Dried contact lenses without liposome layers were also rehydrated overnight in the same antibiotics solution to compare the two systems.

In Vitro Release of Levofloxacin

Liposomes containing levofloxacin were immobilized onto NeutrAvidin-coated contact lenses and the release of levofloxacin from liposomes was monitored over time using a Bio-Tek Synergy HT well-plate reader (Bio-Tek Instruments, Winooski, VE). Then, 3 mL of the appropriate medium (either a saline solution (150 mM NaCl at pH 7.4) or a solution of 0.5% w/v Triton X-100 made with water) was added to each vial containing a contact lens bearing immobilized liposome layers and incubated at 37°C. Triton X-100 was used to disrupt the liposomes to measure the total concentration of levofloxacin encapsulated within the surface-bound liposomes on the lenses. Triton X-100 instantaneously disrupts the vesicles and liberates their contents. At periodic intervals, 200 μL of the incubating solution was withdrawn from the vial containing the lenses and transferred into 96-well plates for fluorescence readings. After the fluorescence measurement, the 200 μL incubating solution withdrawn for the fluorescence measurement were returned to the vial containing the lens and mixed thoroughly. The fluorescence signal was monitored at 460 nm (excitation at 310 nm and emission at 460 nm). Before the release experiment, a calibration curve was done to correlate the levofloxacin concentration to the fluorescence readings. Each experiment was done in triplicate. The levofloxacin release over time was expressed using Eq. 1:

$$\text{Fraction of levofloxacin remaining in vesicles} = 1 - F/F_{\mathrm{T}} \quad (1)$$

where F is the fluorescence at 460 nm measured at any time during the experiment, and F_{T} is the total levofloxacin fluorescence at 460 nm determined after disruption of the vesicles with Triton X-100.

A model of levofloxacin release over time and the parameters of the model were determined using the Nelder–Mead simplex algorithm.

Antibacterial Activity Assays

Antibacterial activity of levofloxacin-loaded liposomes covalently bounded onto contact lens against *S. aureus* (ATCC 29213) was determined using both agar and broth assays.

Agar Assay. In studies using agar, the antibacterial activity of the modified contact lenses was assessed by a diffusion test on Müeller–Hinton agar culture plates (100 mm in diameter, 15 mm in height, Fisher). Before the tests, 5 mm diameter disc samples were cut into each tested contact lens. The bacterial inoculum was prepared according to NCCLS standards (Kirby–Bauer).[47] A sterile cotton swab was dipped into the bacterial inoculum broth suspension and excess fluid was removed by rotating the swab several times against the wall of the vessel. The inoculum was streaked evenly in three planes onto the surface of the agar. Then, one 5 mm diameter disc sample bearing levofloxacin-loaded liposomes was placed at the center of the right part of each plate and gently pressed down to ensure contact. The same procedure was employed with one 5 mm diameter

disc control of a contact lens bearing empty liposomes: this one was placed at the center of the left part of the plate. The diameters of the inhibition zone were measured after incubating the plates for 24 h at 37°C. Each study was performed in triplicate.

Broth Assay. In studies using broth, a contact lens bearing liposomes loaded with levofloxacin was immersed into a Müeller–Hinton broth (3 mL) in a culture tube inoculated with either 1×10^8 or 1×10^6 or 1×10^4 CFU/mL. The initial optical density of each tube inoculated with 1×10^8 CFU/mL was approximately 0.100 at 650 nm. The inocula of 1×10^6 and 1×10^4 CFU/mL were obtained by successive dilutions of the initial inoculum of 1×10^8 CFU/mL. Then, it was placed in a 37°C incubator for 48 h. The antibacterial activity of levofloxacin released from the contact lenses was determined by measuring the optical density of the broth using a spectrophotometer (Novaspec II, Pharmacia Biotech, Cambridge, England) at 650 nm, while that of clear broth was used as a blank. Positive controls with *S. Aureus* (1×10^8, 1×10^6, and 1×10^4 CFU/mL) inoculated in the broth without contact lenses were used. Each study was performed in triplicate.

RESULTS AND DISCUSSION

Kinetics of Levofloxacin Release

The release kinetics of levofloxacin from contact lenses coated with layers of stable liposomes loaded with levofloxacin is shown in Figures 1 and 2. On the molecular scale, the course of the release of levofloxacin may be complex, but the form of the empirical rate law shown in Figure 2 suggests that the particular path via which the release of levofloxacin takes place follows first order release models. Overall, this suggests that levofloxacin was released by diffusion rather than disruption of the liposomes. To compare the release kinetics of each lens, the collected data were analyzed by exponential functions.

Figure 2 shows the experimental data as well as the mathematical models correlating the progression of the levofloxacin release from the different modified lenses. The experimental data

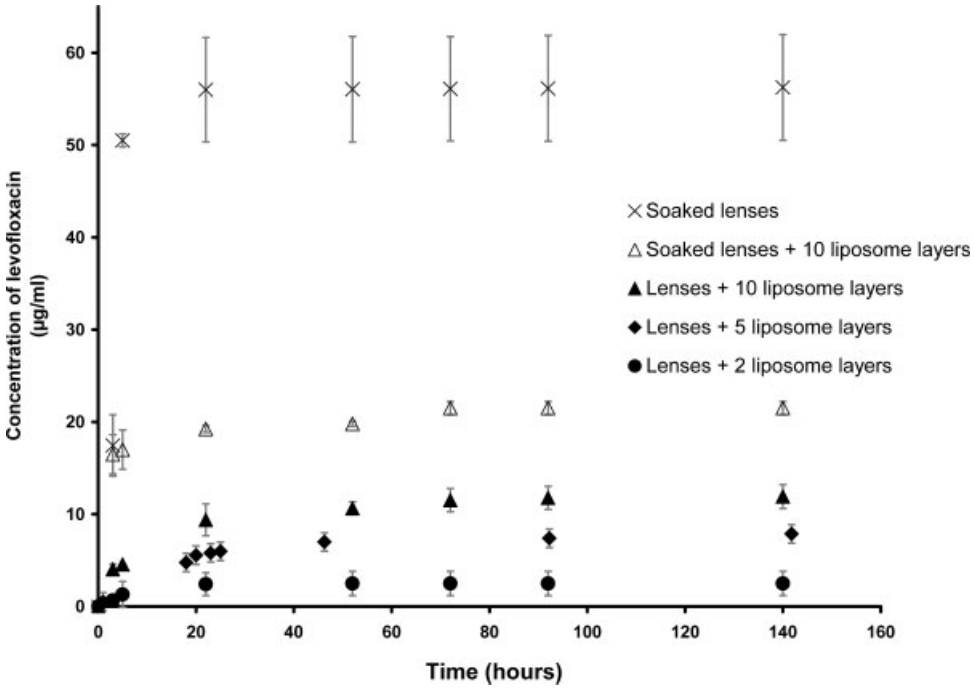

Figure 1. Concentration of levofloxacin released at 37°C by 2, 5, 10 layers of liposomes immobilized on contact lens surfaces, by contact lenses soaked overnight in a solution of levofloxacin (5 mg/mL) and by lenses bearing 10 layers of liposomes followed by an overnight soaking in a solution of levofloxacin (5 mg/mL). Error bars correspond to standard deviations.

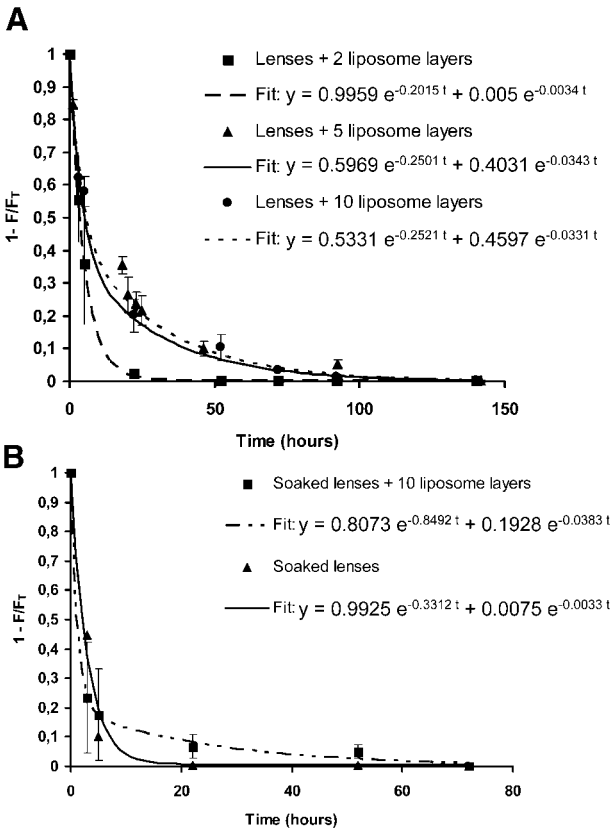

Figure 2. (A) Fraction of levofloxacin remaining at 37°C in 2, 5, 10 layers of liposomes immobilized on contact lens surfaces. (B) Fraction of levofloxacin remaining at 37°C in contact lenses soaked overnight in a solution of levofloxacin (5 mg/mL) and in 10 layers of liposomes immobilized on a contact lens, which had been soaked in a solution of levofloxacin (5 mg/mL). Error bars correspond to standard deviations.

depicted in Figure 2 were in good agreement with the nonlinear correlations shown in Figure 2 and solved by the Nelder–Mead simplex algorithm. A clear tendency was exhibited by the levofloxacin release. The overall levofloxacin release from contact lenses bearing layers of stable liposomes appeared to be a combination of the following mechanisms represented by two-independent first-order kinetics (see Tab. 1 for a resume).

First, following incubation in the buffer solution, the amount of levofloxacin that transferred from the layers of liposomes to the buffer solution exhibited a fast release rate. For lenses bearing two layers of liposomes, this rapid, dynamic behavior (as illustrated by the first term of the fits shown in Fig. 2) was associated to a mass transport phenomenon with a mass transfer coefficient (K_c) of 0.2015 h^{-1}, which can be viewed as a first-order system with a time constant ($1/K_c$) of 4.96 h. This time constant corresponds to the time of incubation of the modified lenses in the buffer solution to release 63.2% of 99.59% of the total liposome-encapsulated levofloxacin. This first term clearly shows that most levofloxacin encapsulated in the immobilized liposomes is released fairly rapidly. For two layers of surface-bound liposomes, it is in good agreement with our precedent study with liposomes loaded with carboxyfluorescein.[42] For lenses bearing 5 and 10 layers of liposomes, the first terms of the fits shown in Figure 2 corresponding to a fast release were similar and were associated to mass transfer coefficients (K_c) of 0.2501 h^{-1} and 0.2521 h^{-1}, respectively, which can be viewed as first-order systems with time constants ($1/K_c$) of 3.99 and 3.97 h, respectively. These time constants correspond to the time of

Table 1. The Overall Levofloxacin Release from Contact Lenses Bearing Layers of Stable Liposomes Can be Modeled by a Combination of Two-Independent First-Order Kinetics

Modified Lenses	$1/K_c$ (hours) Time to Release 63.2% of the Total Liposome-Encapsulated Levofloxacin	Percentage of the Total Liposome-Encapsulated Levofloxacin
Lenses + 2 layers of liposomes	1st first-order model: **4.96**	**99.59**
	2nd first-order model: **294.1**	**0.41**
Lenses + 5 layers of liposomes	1st first-order model: **3.99**	**59.69**
	2nd first-order model: **29.2**	**40.31**
Lenses + 10 layers of liposomes	1st first-order model: **3.97**	**53.31**
	2nd first-order model: **30.2**	**45.97**
Soaked lenses + 10 layers of liposomes	1st first-order model: **1.2**	**80.73**
	2nd first-order model: **26.1**	**19.27**
Soaked lenses	1st first-order model: **3.02**	**99.25**
	2nd first-order model: **303.03**	**0.75**

incubation of the modified lenses in the buffer solution to release 63.2% of 59.69% and 53.31% of the total liposome-encapsulated levofloxacin, respectively, for these two systems. This analysis revealed that the contribution of the fast release rate of levofloxacin over the total release of levofloxacin was smaller for lenses bearing 5 and 10 liposome layers than for those bearing only 2 layers.

For contact lenses bearing 10 layers of liposomes and those bearing no liposome both soaked overnight in a solution of levofloxacin at 5 mg/mL, the first terms of the fits shown in Figure 2 associated with a fast release rate corresponded to mass transfer coefficients (K_c) of 0.8492 and 0.3312 h^{-1}, respectively, which can be viewed as first-order systems with time constants ($1/K_c$) of 1.2 and 3 h, respectively. These time constants also correspond to the time of incubation of the lenses in the buffer solution to release 63.2% of 80.73% and 99.25% of the total loaded levofloxacin, respectively, for these two lenses. Although these lenses can load larger amount of levofloxacin than nonsoaked lenses bearing only liposome layers (see Fig. 1), this analysis reveals that soaked contact lenses (with or without liposome layers) show faster release of their total loaded levofloxacin than nonsoaked lenses bearing only liposome layers. Lenses bearing no liposome and soaked in a levofloxacin solution show a burst release, corresponding to more than 99% of their total loaded medication in ca. 3 h.

Second, following this initial fast release rate, a second and much slower levofloxacin release took place with the lenses bearing 5 and 10 liposome layers. This slower dynamics was well correlated by the second terms of the fits presented in Figure 2. The mass transfer coefficients determined for the second terms of the fits for lenses bearing 5 and 10 liposome layers were in fact equivalent to a transport process with time constants of 29.2 and 30.2 h, respectively. The mass transfer coefficient determined for the second term of the fit for lenses bearing 10 liposome layers and subsequently soaked in a levofloxacin solution was equivalent to a transport process with a time constant of 26.1 h. These values were in sharp contrast to the ones determined for the first terms of the fits. As shown in Figure 2, the response of the second term was slower, as the release of levofloxacin increased very slowly over time. Figure 2 also clearly shows that the progression of the levofloxacin release over time expressed by the first term of the fits obviously overtakes the progression of the second-term for the levofloxacin-soaked lenses and for the lenses bearing two layers of levofloxacin-loaded liposomes. Nevertheless, by combining the two models, the dynamics of the overall nonlinear correlation perfectly matched that of the experimental data monitored by fluorescence measurements.

In summary, the mechanisms that drive levofloxacin release as a function of the time of incubation in a buffer solution at 37°C were shown to be divided into two steps. First, considering the direct exposure of the outermost liposome layer, the levofloxacin release was almost instantaneous, and almost completed following a short period of time for the lenses bearing two layers of liposomes and the soaked lenses bearing no liposome. This assumption was supported by the small time constant obtained from the first term of the nonlinear mathematical model. Upon addition of more liposome layers on the surfaces of the contact lenses, it can be hypothesized that these layers of liposomes became a *de facto* filter medium for the diffusing levofloxacin. The deposited liposomes in fact created an additional resistance to the drug diffusion. The liposome layers can be seen as a bulky mass of vesicles, among which appeared to run small channels that allowed a restrictive molecular movement. This behavior was supported by the large time constants shown in the second term of the empirical correlation. Upon addition of more liposome layers, not only the total amount of levofloxacin was increased but also the macromolecular mobility across the liposome layers became increasingly limited. This results in an increase contribution of the slower release rate over that of the faster release rate in the overall release of levofloxacin in function of the incubation time.

For the contact lenses bearing 10 layers of liposomes and subsequently soaked in levofloxacin solution, they were soaked after liposome immobilization, so it can be hypothesized that the release rate was fast at the beginning due to the release of adsorbed levofloxacin onto the outermost layer of the surface-bound liposomes. Surface-bound liposomes result in very hydrophilic surfaces, probably owing to the external PEG and phosphatidylcholine molecules available on the liposome surfaces, which can form a well-hydrated layer containing levofloxacin. This could result from a fast release from the outermost liposome layer. This behavior was not observed in the case of lenses bearing layers of liposomes containing

levofloxacin because these lenses were exposed to suspension of liposomes containing levofloxacin but in which the nonentrapped levofloxacin was removed by size exclusion chromatography before liposome attachment. In the case of soaked lenses without liposomes, they were directly hydrated in levofloxacin, so the kinetics corresponds to the release of absorbed molecules with a faster overall release rate of levofloxacin than that observed with lenses bearing liposomes.

In our precedent study on carboxyfluorescein release from five layers of liposomes immobilized on contact lenses,[42] the release rate of carboxyfluorescein at 37°C was lower than that found in our present study. In fact, the concentration of levofloxacin used in this study (270 mM) is three times greater than the concentration of carboxyfluorescein used in the previous study (85 mM): this could explain why the diffusion is faster for the levofloxacin (larger concentration gradient across the liposomal membrane). Moreover, the physicochemical properties are different for the two fluorescent molecules: levofloxacin is three times more soluble than carboxyfluorescein.

The total amount of levofloxacin released from a contact lens (Fig. 1) was estimated to be respectively 8 (±3) μg (2.5 μg/mL in 3 mL of incubating solution) for a contact lens bearing 2 layers of liposomes, 24 (±1) μg for a contact lens bearing 5 layers of liposomes and 40 (±10) μg for a contact lens bearing 10 layers of liposomes, that corresponds to 45 times the required concentration to inhibit the growth of 10^6 colony forming units (CFU)/mL of *S. aureus* ATCC 29213.[43,48]

The quantity of antibiotics released from contact lenses bearing 10 layers of liposomes and subsequently soaked overnight in a 5 mg/mL solution of levofloxacin (Fig. 1) was found to be 65 (±2) μg, which should lead to the inhibition of approximately 0.8×10^8 CFU/mL of *S. aureus* ATCC 29213.[43,48] The quantity of antibiotic released from contact lenses without liposomes and rehydrated in the antibiotic solution was 170 (±20) μg (possible inhibition of approximately 2.1×10^8 CFU/mL) for a contact lens. From this observation, it appears that the surface-bound liposomes hinder to some extent the penetration of the levofloxacin within the contact lens structure.

From these results, it appears that contact lenses bearing 5 or 10 layers of liposomes can maintain a sustained delivery of levofloxacin until 120 h, while the release from contact lenses bearing 2 layers of liposomes is completed in 30 h. However, the total amount of loaded antibiotics could be insufficient if the bacteria are exceeding 50×10^6 CFU/mL.

Contact lenses bearing 10 layers of liposomes soaked overnight in the antibiotic solution could be used to provide an initial burst release of levofloxacin during the first hours of infection followed by a sustained release from the surface-bound liposomes. Even if contact lenses rehydrated in the levofloxacin solution can release a larger amount of antibiotics, the delivery rate from these lenses is fast and not controlled over time.

Before the clinical applicability of these modified lenses can be claimed to treat ocular infections, clinicians would have to identify requirements in term of antibiotic regimen concentration for specific cases before to then select the best lens systems that can meet these identified requirements.

Also, these findings reveal that liposomal lenses would have to be stored in a levofloxacin solution to limit levofloxacin leakage during shipping or storage.

Antibacterial Activity

Agar Assay

The antibacterial activity against *S. aureus* of contact lenses bearing layers of liposomes loaded with levofloxacin was determined using an agar assay and is shown in Figures 3 and 4. All contact lenses bearing layers of liposomes loaded with levofloxacin produced a zone of inhibition when placed in plates overlaid with *S. aureus*, while the control contact lens samples without levofloxacin-loaded liposomes (Fig. 3) showed no inhibition zone, except the part directly placed under the sample (that is probably due to a lack of oxygen). After 24 h incubation and for the next 5 days (Fig. 4), the diameter of inhibition was quite similar for the three samples: lenses bearing 2, 5, and 10 layers of liposomes previously loaded with levofloxacin (Figs. 3A, B and C, respectively). This suggests that the levofloxacin release on agar was equivalent for the three samples during a 5-day period. However, in the release assay in solution (Subsection Kinetics of Levofloxacin Release), the three samples exhibit a different behavior. This is probably because the release and the diffusion of levofloxacin into the agar (solid state) are more difficult than into a solution.

The same experience was conducted on the soaked contact lenses (data not shown): the diameter of inhibition recorded after 24 h

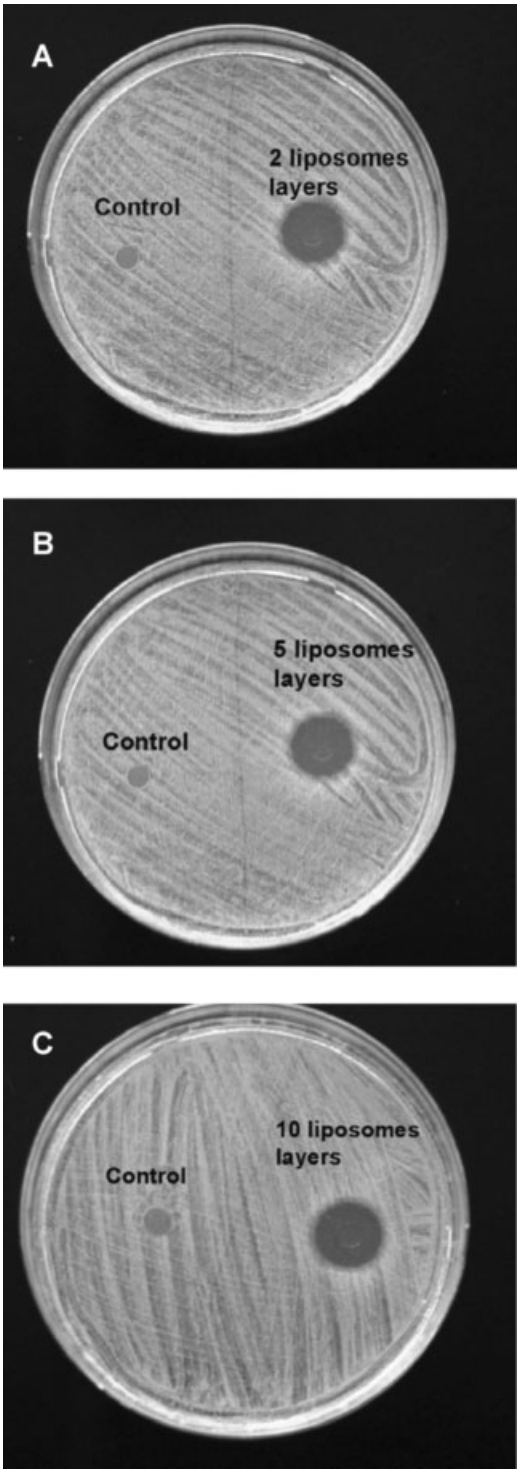

Figure 3. Pictures of the three samples of contact lenses bearing layers of liposomes loaded with levofloxacin and control contact lenses (i.e., bearing layers of "empty" liposomes—those containing no levofloxacin) on culture plates inoculated with *Staphylococcus aureus*: (A) 2 liposomes layers, (B) 5 liposomes layers, and (C) 10 liposomes layers.

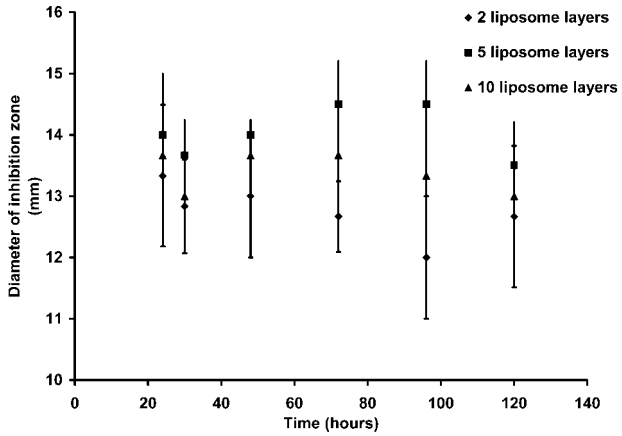

Figure 4. Inhibition zone of *Staphylococcus aureus* in function of the incubation time for contact lenses bearing 2, 5, and 10 levofloxacin-loaded liposomes layers. Error bars correspond to standard deviations.

incubation was similar, 39 (±1) mm, and remains unchanged over 5 days for the contact lenses with 10 layers of surface-bound liposomes and for the contact lenses rehydrated in the levofloxacin solution. This confirms the results obtained with the contact lenses bearing 2, 5, and 10 layers of liposomes: the release on agar is different from that in solution. However, with lenses soaked into a levofloxacin solution, the inhibition diameter was larger than those of liposomal lenses that were not soaked in the antibiotic solution because the amount of levofloxacin was larger for these soaked samples.

Broth Assay

The antibacterial activity of the contact lenses bearing layers of liposomes loaded with levofloxacin was also investigated in broth. Due to their low-loading capacity, the contact lenses with two layers of liposomes were not used in this study. Three different concentrations of *S. aureus* ATCC 29213 (10^4, 10^6, and 10^8 CFU/mL) were inoculated to compare the antibacterial effect of the samples. The results are reported in Figure 5.

Figure 5A shows the antibacterial activity obtained with an initial inoculum of 10^4 CFU/mL. All the tested contact lenses exhibited a complete inhibition of the *S. aureus* growth, whereas the positive control (a contact lens without any treatment immersed into the broth with the inoculum) shows an exponential growth followed by stationary and death phases due to the lack of nutrients in the broth after 24 h.

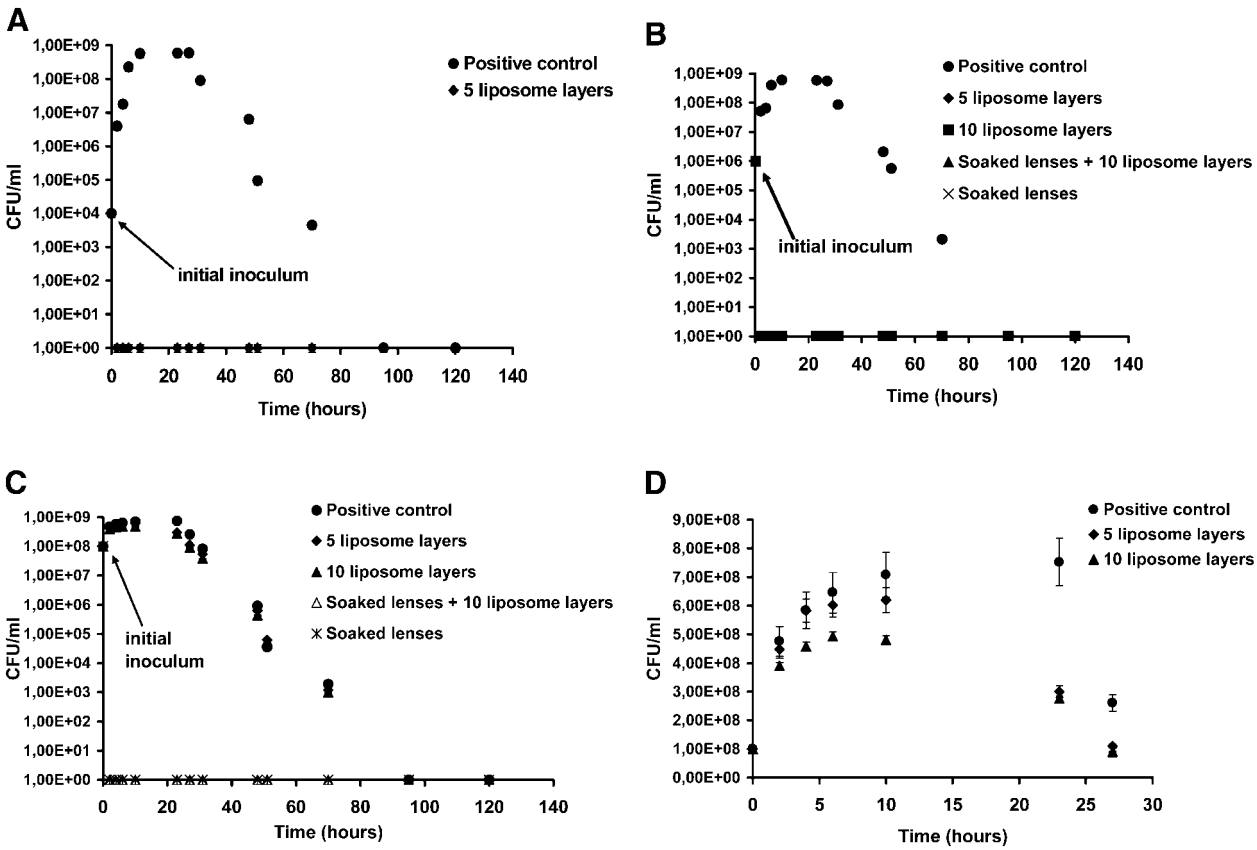

Figure 5. Antibacterial activity of contact lenses bearing 5 and 10 layers of liposomes loaded with levofloxacin, of contact lenses bearing 10 liposomes layers soaked overnight in a 5 mg/mL levofloxacin solution, of dried contact lenses rehydrated in a 5 mg/mL levofloxacin solution and of control contact lenses (bearing layers of "empty" liposomes i.e., containing no levofloxacin) against *Staphylococcus aureus* determined by using a broth assay with an initial inoculum of (A) 10^4 CFU/mL, (B) 10^6 CFU/mL, and (C) 10^8 CFU/mL. (D) Enlargement of the left part of Figure 5C. Error bars correspond to standard deviations.

A similar trend was observed in Figure 5B with an initial inoculum of 10^6 CFU/mL: all the treated contact lenses inhibit the inoculated *S. aureus* within 2 h.

When the bacteria inoculum was higher that is, 10^8 CFU/mL (Fig. 5C), only the soaked-contact lenses (those bearing 10 layers of liposomes and those rehydrated) demonstrated a complete inhibition within 2 h. From Figure 5D, which is the enlarged left part of Figure 5C, it can be seen that the lenses bearing 5 and 10 layers of liposomes progressively developed an antibacterial activity compared to the growth observed in positive controls. The contact lenses bearing 10 layers of liposomes loaded with levofloxacin reveal an antibacterial effect after 4 h and lead to an inhibition of 32% of the *S. aureus* at 10 h. For the contact lenses bearing five layers of liposomes, the antibacterial effect begins after 6 h and reaches 12% of inhibition at 10 h.

These results are in good agreement with the levofloxacin release observed with the kinetics results (Subsection Kinetics of Levofloxacin Release): in fact, the amount of levofloxacin released from the contact lenses bearing 5 and 10 layers of liposomes loaded with levofloxacin was evaluated to be sufficient to inhibit bacteria growth for initial bacterial inocula of 50×10^6 CFU/mL. This explains why all the bacteria were inhibited within 2 h when the inocula did not

exceed 10^6 CFU/mL and why there was a lower antibacterial efficiency when the bacteria inoculum was 10^8 CFU/mL. Then, when the inoculum was between 50×10^6 and 10^8 CFU/mL, the soaked contact lenses are advantageous due to their initial higher release capacity: this is demonstrated in Figure 5C. In the case of a keratitis due to *S. aureus* in a rabbit model, the number of bacteria in a rabbit cornea 10 h postinfection was reported to be approximately 10^7 CFU.[49] However, this is important to keep in mind that these contact lenses are destinated to be used in eyes, and if the release is too fast at the beginning of the wear, a large amount of levofloxacin could be lost and not used to fight the infection. Thus, soaked-contact lenses bearing 10 layers of liposomes loaded with levofloxacin seem to have the advantage to combine a burst release used to inhibit a large amount of bacteria that can be present at the beginning of the infection and necessary to stop the fast exponentially growing bacteria, followed by a sustained release to complete the antibacterial effect.

It was difficult in these experimental conditions to replicate the ocular physiology, for example, by replacing the fluid medium at rates corresponding to tear secretion and elimination. So, all these results have to be confirmed by an *in vivo* study to investigate *in vivo* the kinetics release and the antibacterial efficiency against a *S. aureus* keratitis.

CONCLUSIONS

This study demonstrates that contact lenses bearing surface-immobilized layers of intact liposomes loaded with levofloxacin can provide a sustained release of antibiotics over 6 days. The levofloxacin release, as a function of time, was described by a mechanism taking into account two first-order kinetic models. Using both, agar and broth assays, contact lenses coated with levofloxacin-loaded liposomes showed an antibacterial activity against *S. aureus*.

In vivo studies are needed to confirm these results and to demonstrate that contact lenses bearing surface-immobilized layers of intact liposomes loaded with levofloxacin may constitute a promising approach for controlling drug release to maintain a topical antibacterial activity for a long period of time and, consequently, to decrease the reiteration of antiseptic applications.

ACKNOWLEDGMENTS

We thank F. Malouin for kindly providing the *Staphylococcus aureus* strain. This work was supported by the NSERC through an I2I grant. The contact lenses were kindly supplied by Robert Mercure from the Laboratoires Opti-Centre (Sherbrooke, Québec, Canada).

REFERENCES

1. Maberley D. 2004. The cost of blindness symposium. Toronto, Jan 31-Feb 1.
2. McCarty CA, Mukesh BN, Fu CL, Taylor HR. 1999. The epidemiology of cataract in Australia. Am J Ophthalmol 128:446–465.
3. World Health Organization Report. 1997.
4. Singh K, Bautista RD. editors. 1999. International ophthalmology clinics, Vol. 39. Philadelphia: Lippincott Williams & Wilkins.
5. Smolin G. editor. 1998. International ophthalmology clinics, Vol. 38. Philadelphia: Lippincott Williams & Wilkins.
6. Arffa RC. 1997. Viral diseases. In: Arffa RC, Grayson M, editors. Grayson's diseases of the cornea, 4th ed. St-Louis: Mosby. pp 283–338.
7. Ogawa GS, Hyndiuk RA. 1994. Bacterial keratitis and conjunctivitis—clinical disease. In: Smolin G, Thoft RA, editors. The cornea: Scientific foundations and clinical practice. 3rd ed. Boston: Little, Brown and Company. pp 125–167.
8. Garg P, Bansal AK, Sharma S, Vemuganti GK. 2001. Bilateral infectious keratitis after laser in situ keratomileusis: A case report and review of the literature. Ophthalmology 108:121–125.
9. Liesegang TJ. 1988. Bacterial and fungal keratitis. In: Kaufman HE, Barron BA, McDonald MB, Waltman SR, editors. The cornea. New York: Churchill Livingstone. pp 217–270.
10. Alexandrakis G, Alfonso EC, Miller D. 2000. Shifting trends in bacterial keratitis in south Florida and emerging resistance to fluoroquinolones. Ophthalmology 107:1497–1502.
11. Bourcier T, Thomas F, Borderie V, Chaumeil C, Laroche L. 2003. Bacterial keratitis: Predisposing factors, clinical and microbiological review of 300 cases. Br J Ophthalmol 87:834–838.
12. Callegan MC, O'Callaghan RJ, Hill JM. 1994. Pharmacokinetic considerations in the treatment of bacterial keratitis. Clin Pharmacokinet 27:129–149.
13. Holland SP, Pulido JS, Shires TK, Costerton JW. 1993. Pseudomonas aeruginosa ocular infections. In: Fick RB, editor. Pseudomonas aeruginosa: The opportunist. Boca Raton: CRC Press Inc. pp 160–176.
14. Schaefer F, Bruttin O, Zografos L, Guex-Crosier Y. 2001. Bacterial keratitis: A prospective clinical and

microbiological study. Br J Ophthalmol 85:842–847.
15. Smith A, Pennefather PM, Kaye SB, Hart CA. 2001. Fluoroquinolones—place in ocular therapy. Drugs 61:747–761.
16. Gangopadhyay N, Daniell M, Weih L, Taylor HR. 2000. Fluoroquinolone and fortified antibiotics for treating bacterial corneal ulcers. Br J Ophthalmol 84:378–384.
17. Cutarelli PE, Lass JH, Lazarus HM, Putman SC, Jacobs R. 1991. Topical fluoroquinolones: Antimicrobial activity and in vitro corneal epithelial toxicity. Curr Eye Res 10:557–563.
18. Mallari PL, McCarty DJ, Daniell M, Taylor HR. 2001. Increased incidence of corneal perforation following topical fluoroquinolone treatment for microbial keratitis. Am J Ophthalmol 131:131–133.
19. Goldstein MH, Kowalski RP, Gordon YJ. 1999. Emerging fluoroquinolone resistance in bacterial queratitis. A five-year review. Ophthalmology 106: 1313–1318.
20. Kowalski RP, Pandya AN, Karenchak LM. 2001. An in vitro resistance study of levofloxacin, ciprofloxacin and ofloxacin using keratitis isolates of *Staphylococcus aureus* and Pseudomonas aeruginosa. Ophthalmology 108:1826–1829.
21. Chalita MR, Hofling-Lima AL, Paranhos A, Jr., Schor P, Belfort R, Jr. 2004. Shifting trends in in vitro antibiotic susceptibilities for common ocular isolates during a period of 15 years. Am J Ophthalmol 137:43–51.
22. Marangon FB, Miller D, Muallem MS. 2004. Ciprofloxacin and levofloxacin resistance among methicillin-sensitive *Staphylococcus aureus* isolates from keratitis and conjunctivitis. Am J Ophthalmol 137:453–458.
23. Bourlais CL, Acar L, Zia H, Sado PA, Needham T, Leverge R. 1998. Ophthalmic drug delivery systems. Prog Retin Eye Res 17:33–58.
24. Lang JC. 1995. Ocular drug delivery conventional ocular formulations. Adv Drug Deliv 16:39–43.
25. Segal M. 1991. Patches, pumps and timed release. FDA Consumer Magazine. October 1991. Available at http://www.fda.gov/bbs/topics/CONSUMER/CON00112.html.
26. Nagarsenker MS, Londhe VY, Nadkarni GD. 1990. Preparation and evaluation of liposomal formulations of tropicamide for ocular delivery. Int J Pharm 190:63–71.
27. Malmsten M, editor. 2002. Surfactants and polymers in drug delivery. New York: Marcel Dekker.
28. Loftsson T, Stefansso E. 2002. Cyclodextrins in eye drop formulations: Enhanced topical delivery of corticosteroids to the eye. Acta Ophthalmol Scand 80:144–150.
29. Smollin G, Friedlaender MH. editors. 1991. International ophthalmology clinics, Vol. 31. Philadelphia: Lippincott Williams & Wilkins.
30. Guo LSS, Redemann CT, Radhakrishnan R, Yau-Young A. 1989. Liposomes with enhanced retention on mucosal tissue. US Patent No. 4839175.
31. Reidy JJ, Gebhardt BM, Kaufman HE. 1990. The collagen shield: A new vehicle for delivery of cyclosporin A to the eye. Cornea 9:196–199.
32. Phinney RB, Schwartz SD, Lee DA, Mondino BJ. 1988. Collagen-shield delivery of gentamicin and vancomycin. Arch Ophthalmol 106:1599–1604.
33. Peterson RC, Wolffsohn JS, Nick J, Winterton L, Lally J. 2006. Clinical performance of daily disposable soft contact lenses using sustained release technology. Contact Lens & Anterior Eye 29(3): 127–134.
34. Hiratani H, Mizutani Y, Alvarez-Lorenzo C. 2005. Controlling drug release from imprinted hydrogels by modifying the characteristics of the imprinted cavities. Macromol Biosci 5:728–733.
35. Lesher GA, Gunderson GG. 1993. Continuous drug delivery through the use of disposable contact lenses. Optom Vis Sci 70:1012–1018.
36. Barber RF, Shek PN. 1990. Tear-induced release of liposome-entrapped agents. Int J Pharm 60:219–227.
37. Popescu MC, Weiner AL, Carpenter-Green SS. 1985. Liposome-gel compositions. International Patent No. WO85/03640.
38. Gulsen D, Li CC, Chauhan A. 2005. Dispersion of DMPC liposomes in contact lenses for ophthalmic drug delivery. Current Eye Res 30:1071–1080.
39. Chauhan A, Gulsen D. 2004. Ophthalmic drug delivery system. U.S. Patent No. 0241207.
40. Vermette P, Meagher L, Gagnon E, Griesser HJ, Doillon CJ. 2002. Immobilized liposomes layers for drug delivery applications: Inhibition of angiogenesis. J Control Release 80:179–185.
41. Vermette P, Griesser HJ, Kambouris P, Meagher L. 2004. Characterization of surface-immobilized layers of intact liposomes. Biomacromolecules 5:1496–1502.
42. Danion A, Brochu H, Martin Y, Vermette P. 2006. Fabrication and characterization of contact lenses bearing surface-immobilized layers of intact liposomes. J Biomed Mater Res, Part A, in press.
43. North DS, Fish N, Redington JJ. 1998. Levofloxacin, a second-generation fluoroquinolone. Pharmacotherapy 18:915–935.
44. Callegan MC, Engelberg M, Parke DW, Jett BD, Gilmore MS. 2002. Bacterial endophthalmitis: Epidemiology, therapeutics, and bacterium-host interactions. Clin Microbiol Rev 15:111–124.
45. Hope M, Bally M, Mayer L, Janoff A, Cullis P. 1986. Generation of multilamellar and unilamellar phospholipid vesicles. Chem Phys Lipids 40:89–107.
46. Vermette P, Gengenbach T, Divisekera U, Kambouris PA, Griesser HJ, Meagher L. 2003. Immobilization and surface characterization of NeutrAvidin biotin-binding protein on different

hydrogel interlayers. J Colloid Interface Sci 259: 13–26.
47. National Committee for Clinical Laboratory Standards. 1997. Performance Standards for antimicrobial disk susceptibility tests— 6th ed.: Approved Standard M2-A6. NCCLS, Wayne, PA.
48. Noviello S, Ianniello F, Leone S, Esposito S. 2003. Comparative activity of garenoxacin and other agents by susceptibility and time-kill testing against *Staphylococcus aureus*, Streptococcus pyogenes and respiratory pathogens. J Antimicrob Chemother 52:869–872.
49. Oguz H, Ozbilge H, Oguz E, Gurkan T. 2005. Effectiveness of topical taurolidine versus ciprofloxacin, ofloxacin, and fortified cefazolin in a rabbit *Staphylococcus aureus* keratitis model. Current Eye Res 30:155–161.