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SOLID LIPID NANOPARTICLES AS PROMISING TOOL FOR INTRAOCULAR TOBRAMYCIN DELIVERY: PHARMACOKINETIC STUDIES ON RABBITS

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Running title: Ocular biodistribution of tobramycin incorporated in Solid Lipid Nanoparticles

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Abstract

Eye drops are widely accepted as formulations for targeting the anterior segment notwithstanding their limitations in term of bioavailability. The unique structure of the eye requires specially-designed formulations able to favour the pharmacokinetic profile of administered drugs, mainly minimizing the influence of ocular barriers. Nanotechnology-based delivery systems lead to significant technological and therapeutical advantages in ophthalmic therapy.

The aim of the present study is to determine whether tobramycin as ion-pair incorporated in mucoadhesive Solid Lipid Nanoparticles (SLN) reachs the inner parts of the eye favouring drug activity.

After technological characterization of the tobramycin entrapped SLN formulation (Tobra-SLN), a pharmacokinetic study in rabbits after topical instillation and intravenous administration of the formulation has been carried out. In addition, the intracellular activity of Tobra-SLN formulation against phagocytosed *Pseudomonas aeruginosa* was investigated.

The SLN were spherical in shape, showed a hydrodynamic diameter of about 80 nm, a negative zeta potential (-25.7 mV) with a polydispersity index of 0.15, representative of a colloidal dispersion with high quality, characterized by an unimodal relatively narrow size distribution. As demonstrated by FTIR and DSC, tobramycin ion-pair could be concentrated into lipid inner core of SLN, without interaction with the stearic acid, thus promoting a slow and constant drug release profile in the dissolution medium.

Surprisingly, the drug concentration was significantly higher in all ocular tissues after ocular and intravenous administration of Tobra-SLN formulation with respect to reference formulations and only Tobra-SLN allowed the penetration of drug into retina. Furthermore, the use of Tobra-SLN resulted in both higher intraphagocytic antibiotic concentrations in polymorphonuclear granulocytes and greater bactericidal activity against intracellular *Pseudomonas aeruginosa*, probably due to the ability of Tobra-SLN to penetrate either into phagocytic cells, or alternatively to cross bacterial barrier.

The present study broaden the knowledge on the use of SLN as carriers for ocular drug delivery to the posterior chamber and might open new avenues for treatment of ocular infections, representing a strategy to overcome the microbial resistance.

1. Introduction

The eye is considered a complex and delicate organ of the human body that represents a very challenging issue in drug delivery. Eye drops are widely accepted as formulations for targeting the anterior segment notwithstanding their limitations in term of bioavailability for extensive pre-corneal loss due to blinking, rapid washout by tearing, drainage through the naso-lacrimal duct, non-productive absorption [1-4]. Furthermore, eye drops capability to treat vitreoretinal diseases, such as bacterial infections, endophthalmitis, cytomegalovirus retinitis (CMV), uveitis, proliferative vitreoretinopathy (PVR), diabetic retinopathy, age-related macular degeneration (AMD) is worthless because of poor drug penetration in the posterior segment [1, 4-6]. The main challenges in ocular therapy are represented by the reduction of precorneal drainage of the instilled formulations and the overcoming of the protective barriers of eye to reach a sufficient drug concentrations at the site of action. The unique structure of the eye requires specially-designed carriers able to optimise the pharmacokinetic behaviour of topically administered drugs, mainly minimizing the influence of ocular barriers [1, 3, 5-9]. The corneal layers, in particular the epithelium and stroma, are considered the major barriers for absorption of topically applied drugs, which should have amphipathic nature, in order to permeate through the lipophilic/hydrophilic layers. In addition, the sclera, mainly consisting of collagen fibers and proteoglycans embedded in an extracellular matrix, is considered to be comparable in permeability to the corneal stroma. The permeability of drug molecules across the sclera is inversely proportional to the molecular radius and to the charge of the drug molecule: positively charged molecules exhibit scarce permeability, presumably for their binding to the negatively charged proteoglycan matrix [9-11].

The systemic route represents a successful treatment for ocular diseases involving the anterior segment or the ocular surface, but it is not suitable to treat vitreoretinal diseases. In fact, the outer and inner blood-retinal barriers, formed respectively by retinal pigment epithelium and retinal vascular endothelium, inhibit the intraocular penetration of drugs when applied sistemically [3, 12, 13].

Nanotechnology-based delivery systems (nanocapsules, microemulsions, liposomes, nanomicelles and lipid nanoparticles) lead to significant technological and therapeutical advantages in ophthalmic therapy, as increase of precorneal drug retention time, sustained drug release, reduction in administration frequency, reduced drug toxicity, targeted delivery to specific eye tissues. Furthermore, micro/nanocarriers can be generally administered as dispersion in the form of eye drops without causing blurred vision and irritation [7, 8, 13-16]. All these aspects cause an improvement in bioavailability of drugs and in the patient compliance. Among all nanocarriers, lipid carriers, in particular SLN, were extensively investigated as promising delivery systems for hydrophilic or lipophilic ophthalmic drugs [8, 17-23]. Besides being a droppable preparation and improving ocular bioavailability of drugs, lipid nanocarriers have the advantage to reduce both the leakage of drugs encapsulated, as for liposomes, and the instability during storage typical of emulsions, polymeric nanoparticles and liposomes.

In previous works, we showed that solid SLN modified the pharmacokinetic parameters and the distribution of several incorporated drugs in the biological tissues, increasing their passage through barriers such as the blood brain barrier [24-26]. In detail, we observed that a SLN dispersion containing the antibacterial tobramycin significantly enhanced the drug bioavailability in the aqueous humor [27] and that pilocarpine incorporated in SLN tripled the miotic effect of the drug in comparison with the commercial formulation [28]. Recent reports referred on the positive behaviour of the association between the drug-entrapped SLN carriers prepared with different lipidic components and innovative “in situ” gel forming vehicles [29] and on the improved

efficacy of SLN by incorporating hyaluronic acid and protamine to obtain a versatile carrier for gene therapy [30].

Even if in the last ten years SLN have been studied extensively and several manuscripts reported the advantage obtained by their use as delivery systems for many ophthalmic drugs (antiinflamatory, antiglaucoma, antiibacterial, antifungal), natural extracts, antioxidant molecules and gene therapy [9, 20, 21, 30-35], in vivo studies are almost rare, while they should be helpful to establish the real therapeutical potential of SLN as ocular drug delivery.

The aim of the present study is to broaden the knowledge on the ocular delivery of tobramycin on rabbits by determining whether tobramycin incorporated in a mucoadhesive SLN dispersion is able to reach the inner parts of the eye. Hence, a pharmacokinetic study in rabbits after ocular instillation and intraveneous administration of the SLN has been carried out. Since the entrance of antimicrobial agents into phagocytic cells in association with cellular bactericidal mechanisms is a prerequisite for their activity, we also investigated the intracellular activity of tobramycin in comparison with SLN formulations against phagocytosed *Pseudomonas aeruginosa*, which is responsible for numerous eye infections [36-37], in order to demostrated all their possible benefits. Furthermore, the physical and chemical stability over the time of drug-loaded SLN has been investigated.

2. Materials and methods

2.1. Materials

Stearic acid and 1-fluoro-2,6 dinitrobenzene were from Fluka (Buchs, Switzerland); Epikuron 200 (soya phosphatidylcholine 95%) was kindly provided by Cargill (Hamburg, Germany); tobramycin base, mucin from porcine stomach type III, type VII alkaline phosphatase and fluorescamine were from Sigma Chemical Co. (Missouri, USA), taurocholate sodium salt was kindly provided by PCA (Basaluzzo, Italy). Sodium hexadecylphosphate was prepared as

indicated by Brown [38]. The commercial eye drops Tobral® (0.3% of tobramycin, Alcon Italia S.p.A., Italy) was used as control. The other chemicals were of analytical grade.

2.2. Animals

All experiments were carried out on male New Zealand albino rabbits of 2.8-3.5 Kg (Pampaloni Rabbitry, Fauglia, Italy). The animals were housed in singly standard cages in a light-controlled room (10 h dark/14 h light cycle) at 19 ± 1°C and 50 ± 5% RH and were given a standard pellet diet and water *ad libitum*. During the experiments the rabbits were placed in restraining boxes but their eyes movements were not restricted. The rabbits were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, following a protocol approved by the Ethical-Scientific Committee of the University of Pisa and under veterinary supervision.

Eight groups of animals, each of three rabbits, were used for the different treatments.

2.3. Preparation of Solid Lipid Nanoparticles incorporating tobramycin (Tobra-SLN)

Solid lipid nanoparticles containing the tobramycin as complex (Tobra-SLN) were prepared, as tuned previously by us, from o/w microemulsions consisting of stearic acid as internal phase (0.70 mmol), Epikuron 200 as surfactant (0.14 mmol), sodium taurocholate as cosurfactant (0.72 mmol), and deionized water as continuous phase (110.10 mmol). The drug was added to the microemulsion as ion-pair complex with hexadecylphosphate in a 1:2 tobramycin:hexadecylphosphate molar ratio to increase the lipophilicity of tobramycin. The final amount of tobramycin complex was 0.05 mmol [27]. The ion-pair complex favored partition of tobramycin in the oil phase of the microemulsion and was prepared using a co-precipitation method with hexadecylphosphoric acid as tobramycin counter ion [39, 40]. Tobra-SLN aqueous dispersions were obtained by dispersing warm o/w microemulsion in cold water at a 1:10 microemulsion:water (v/v) ratio under mechanical stirring. Dispersions were washed three times

by diaultrafiltration with TCF2 system (Amicon, Danvers, USA) using a Diaflo YM 100 membrane (cutoff 100,000 Daltons) to remove the majority of the cosurfactant used to obtain the microemulsion. After the purification, Tobra-SLN dispersion was freeze-dried and subsequently dispersed to obtain the required final concentration of tobramycin of 2.0 mg/mL. The quantitative determination of tobramycin incorporated in Tobra-SLN was carried out on a freeze-dried sample of Tobra-SLN dispersion after dissolution in methanol [27].

To perform the biological studies on rabbits, the tobramycin-loaded SLN formulation was packaged in vials and sterilized by autoclaving at 121°C (2 bar) for 15 minutes.

2.4. Preparation of tobramycin reference solution (Tobra-SOL)

Tobramycin reference solution was prepared in physiological solution (0.9% w/w of sodium chloride) at a concentration of 2.0 mg/mL and sterilized by microfiltration (0.22 μm, Millex-GS syringe filter unit, Merck Millipore, Germany) under laminar flow cup before each *in vivo* experiment.

2.5. Quantitative determination of tobramycin

The amount of tobramycin incorporated in Tobra-SLN was determined in accordance with analitycal protocols previously applied by us, by spectrophotometric method, using a DU 730 spectrophotometer (Beckman Coulter, Italy), on an exactly weighed sample of freeze-dried Tobra-SLN dissolved in methanol before the derivatization reaction with fluorescamine according to Cavalli et al. [27]

Quantitative determination of tobramycin in the aqueous samples of *in vitro* release study was carried out by an indirect spectrophotometric method (λ_{ex}= 390 nm) using fluorescamine as derivatizing agent according to Tekkeli et al. [41].

Tobramycin in the samples of aqueous humor, vitreous humor and retina was quantified by a reverse-phase HPLC method (25-27, 42] with a UV detector (λ= 350 nm) using 1-fluoro 2,4-

dinitrobenzene to derivatize tobramycin. The mobile phase based on acetonitrile:phosphoric acid (5.0 g/L) 70:30 v/v was pumped in HPLC systems (Shimadzu, Italy) consisted of a LC-10 AC pump, 20 μL loop injector (Reodyne, Sigma-Aldrich, Italy) and a C-R6A recorder. The column was a Simmetry C-18 column (250 nm x 4.6 mm, Waters). The biological samples were prepared as described elsewhere [23]. Briefly, tissue samples were thawed and sonicated for 30 min after adding sterile phospate buffer solution pH=7.4, 66.7 mM (PBS). A 100 μL portion was added to 1.0 mL of methanol, sonicated for 15 min and maintained for 30 min at 65°C. The mixture was diluted with 0.9 mL of PBS, vortexed for 1 min and finally centrifuged at 5000 g for 10 min. 180 μL were then transferred to a vial containing 80 μl of 1-fluoro-2,4-dinitrobenzene prepared in methanol (180 mg/mL), 60 μL of 0.1 M borate buffer (pH 9.3) and 680 μl of methanol. The vial was vortexed for 1 min, heated to 80°C for 45 min and cooled to room temperature before HPLC analysis. A linear calibration curve with a good correlation coefficient (r^2 = 0.9980) was obtained in the concentration range 50-600 ng/mL^{-1}

2.6. Characterization of solid lipid nanoparticles

The mean diameter, polydispersity index and zeta potential of Tobra-SLN dispersion were determined by photo correlation spectroscopy (PCS) using a 90 Plus instrument (Brookhaven, New York, USA). For mean diameter and polydispersity index the measurements were performed at a fixed angle of 90° and a temperature of 25°C. Both SLN water dispersions were diluted 1:30 v/v before analysis and each batch was analyzed ten times [43]. For zeta potential determination, samples of SLN were diluted with KCl 0.1 mM and placed in the electrophoretic cell, where an electric field of 15.24 V/cm was applied. Each sample was analyzed at least in triplicate. The measured electrophoretic mobility was converted into zeta potential using the Smoluchowsky equation [44].

The evaluation of the interaction between tobramycin and lipid component of SLN was determined on samples of pure drug, stearic acid, ion-complex and Tobra-SLN, using FTIR

analysis. The spectra were recorded on a Perkin Elmer 2000 instrument in the range between 400 and 4000 cm^{-1}.

2.7. Transmission electron microscopy analysis

Transmission electron microscopy (TEM) analysis was performed using a Philips CM10 instrument (Eindoven, Holland). For this purpose SLN aqueous dispersions were diluted 1:40, stained with a 2% v/v aqueous solution of osmium tetraoxide and sprayed on a copper grid.

2.8. Thermal analysis of freeze-dried SLN

Differential Scanning Calorimetry (DSC) analysis was performed using a DSC/7 differential scanning calorimeter (Perkin-Elmer, Connecticut, USA) equipped with a TAC 7 /DX instrument controller. The instrument was calibrated with indium for melting point and heat of fusion. A heating rate of 20°C/min was used in the range of 25-240°C. About 5 mg of freeze-dried samples of SLN were taken in the standard aluminum sample pans for analysis and an empty pan was used as reference in each case. Analyses were performed under nitrogen purge; triple runs were carried out on each sample.

2.9. *In vitro* release kinetic studies

Exactly weight freeze-dried samples of Tobra-SLN were mixed with 66.7 mM phosphate buffer (pH 7.4) to obtain a dispersion with drug concentrations of 0.43 mM that was placed in the donor compartment of a multi-compartment rotating cell. An aqueous solution of tobramycin was used at the same concentration and pH as control. A hydrophilic dialysis membrane (cut-off 12000, Dialysis tubing, Sigma Chem. Co., Missouri, USA) was used to separate the receptor compartment containing phosphate buffer at the same concentration and pH. Each experiment lasted two hours and at fixed times the receptor buffer was completely withdrawn and replaced with fresh buffer. The withdrawn solutions were then analyzed by HPLC to determine the amount of tobramycin.

The tobramycin release data were analyzed by a relationship proposed by Ritger and Peppas [45] and using Prism software (GraphPad Software Inc., San Diego, CA, USA).

2.10. Physical and chemical stability of drug-loaded SLN over time

The stability of Tobra-SLN dispersion packaged in vials, sterilized by autoclaving and maintained at 4 °C, was evaluated over time analyzing their average diameters and polydispersity index by PCS after 1, 24, 48 hours and after 6, 12 and 24 months. At the same times the drug concentration of tobramycin in SLN was evaluated by HPLC .

2.11. In vitro determination of Tobra-SLN interaction with mucin

Tobra-SLN mucoadhesion capacity was determined *in vitro,* evaluating the interaction with porcine mucin [46].

The interaction was studied by incubation of mucin and SLN (1:4 weight ratio) in PBS at pH = 7.4. The incubation was carried out under stirring for 1 hour at temperature of 37°C. Then, the system was centrifuged and the supernatant was used for the measurement of the free mucin content, evaluating the trasmittance value using a DUO Beckman specctrophotometer.

The amount of mucin interacting with the nanoparticles was calculated from a calibration curve. For this purpose, a series of mucin aqueous solutions with different concentrations (0.025, 0.05, 0.1, 0.2 and 0.5 mg/mL) were prepared. The amount of the mucin adsorbed on the nanoparticles was determined as a difference between its initial mucin concentration and the concentration found in the supernatant.

2.12. In vivo experiments: intravenous administration of the formulations

Four different groups of animals, each of three rabbits, were treated by intravenous injection in the ear marginal vein with a) Tobra-SLN and b) Tobra-SOL (reference solution) formulations, both at a dose of 2 mg/Kg. After 1 and 3 hours the rabbits were euthanized with an intravenous

dose of sodium pentobarbital (Pentothal sodium, Farmaceutici Gellini, Aprilia, Italy) and about 100 μL of aqueous humor were aspired from the anterior chamber of each eye and transferred into microtubes before HPLC analysis. Then, the eyes were enucleated, washed with saline solution and dissected to isolate and harvest individual tissues, including vitreous humor and retina. All the samples were stored at −20°C until quantitative analysis.

2.12. In vivo experiments: topical ocular administration of the formulations

Fifthy microliters of Tobra-SLN, corresponding to 0.15 mg of tobramycin, was administered in the lower conjunctival sac of both eyes of each rabbit. The commercial product, Tobral® , used as control, was administered at the same dose at a different groups of animals. At intervals of 1 and 3 hours the rabbits were euthanized with an intravenous dose of sodium pentobarbital (Pentothal sodium, Farmaceutici Gellini, Aprilia, Italy), the eyes enucleated and the tissues (vitreous and retina) were collected and stored at −20 °C until quantitative analysis.

2.14. Antimicrobial activity and effects on polymorphonuclear granulocyte (PMN) functions of Tobra-SOL and Tobra-SLN

The antimicrobal activity of tobramycin solution and Tobra-SLN were evaluated measuring the Minimal Inhibiting Concentrations (MICs) against a clinical isolate of *P. aeruginosa* by the standardized dilution method in Mueller-Hinton broth (Unipath, Milan, Italy) with an inoculum of $2x10^7$ cfu/mL.

PMNs were separated from lithium heparinized venous blood [47] using Ficoll-Paque (Pharmacia S.p.A., Italy). Using trypan blue testing, the viability of PMNs was determined to be above 95%. PMNs were suspended in RPMI 1640 medium with 10% fetal calf serum. The interval between PMNs harvest and the start of experiments was below 30 min. The effects of tobramycin solution and Tobra-SLN on either the phagocytosis of radio-labeled *P. aeruginosa* [^{3}H-uracil (specific

activity: 1,224.7 GBq/mmol; NEN Life, Italy)] or intracellular bacterial killing by PMNs were investigated by incubating the bacteria ($2x10^7$ cfu/ml) and the phagocytes ($2x10^6$ cells/ml) at 37°C in a shaking water bath for periods of 30, 60, or 90 min in the presence of the MIC of the drug. Antibiotic-free controls were also included. Radioactivity was expressed as the counts per minute/sample. The percentage of phagocytosis at a given sampling time was calculated as follows: percent phagocytosis = [(cpm in PMNs pellet)/(cpm in total bacterial pellet)] x 100 [48, 49]. The PMN killing values were expressed as the Survival Index (SI), which was calculated by adding the number of surviving bacteria at time zero to the number of survivors at time *x*, and dividing by the number of survivors at time zero. According to this formula, if bacterial killing was 100% effective, the SI would be 1.

To differentiate between any separate effect of Tobra-SOL and Tobra-SLN on bacteria and PMNs, the experiments were conducted after exposing bacteria and PMNs separately to the antibiotics (1xMIC) for 1.0 h, before they were incubated together [50]. After withdrawal of the drug, pre-exposed bacteria were added to PMNs and bacteria to pre-exposed PMNs. A control system was assayed in parallel with no antibiotic. The phagocytic and bactericidal activities of PMNs were determined as described above.

Each test was performed in triplicate; the results were compared with those obtained with the control systems and are expressed as means and standard errors of the means (SE) for ten separate experiments.

2.15. Statistical analysis

The statistical difference between treatments were evaluated using the Analysis of Variance (ANOVA) test. The evaluation included calculation of the means and standard error and group comparison using the Dunnet test. The confidence level was set at 95% and $P \leq 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characterization of Tobra-SLN

The average diameter, the polydispersity index (PI) and zeta potential (ζ) of tobramycin loaded SLN are reported in Table 1.

The SLN formulation showed a hydrodynamic diameter of 80.01 ± 11.10 nm with a PI value of 0.155 (± 0.03), representative of a colloidal dispersion with high quality, characterized by an unimodal relatively narrow size distribution In fact, even if PI values ≤ 0.1 characterize the highest quality of a colloidal dispersion, many researchers indicate PI values ≤ 0.3 as ideal index for an uniform system [51]. Interestingly, the slight increase of about 4 % w/w of the amount of cosurfactant in comparison with the previous formulation [24] produced small, homogenous and physically stable nanodispersion. Indeed, the negative zeta potential value (ζ = -25.7 ± 0.20 mV) was sufficiently high to favour a high degree of repulsion among similary charged SLN particles and to guarantees the physical stability of the colloidal dispersion [51]. The standardization of the size of SLN as small nanoparticles of 80.01 ± 11.0 nm seems promising to favour their penetration into ocular structures (vitreous and retina).

Morphological analysis of Tobra-SLN in aqueous dispersions, detected by TEM analysis, proved that most of the nanoparticles were spherical in shape reaching several tens nanometers in their size, according to the measurements performed by dynamic light scattering. TEM photomicrograph for a Tobra-SLN sample is reported in Figure 1.

The interactions among the different components of the Tobra-SLN formulation detected by FTIR analysis, are shown in Figure 2, where the spectrum of Tobra-SLN was compared with that of acid stearic and tobramycin as ion-pair. The FTIR analysis of tobramycin showed absorption bands in the range between 1600-1300 cm^{-1} that could be attributed to NH group, CH2 scissoring, OH bending and a strong absorption bands around 1000 cm^{-1} corresponding to C-O and C-N bonds. These last absorption bands were absent in the spectrum of Tobra-SLN, that, on the

contrary, was characterized by typical peaks of stearic acid. In fact, in both spectra of stearic acid and Tobra-SLN were detected absorption bands around 2800-3000 cm^{-1} and 1700 cm^{-1} corresponding to OH and C=O stretching frequency.

Therefore, it is plausible that tobramycin ion-pair is dispersed into the lipid component of the lipid nanoparticle without interaction with the stearic acid.

Thermal analysis was used to investigate the crystal structure of tobramycin in SLN: the results of DSC analysis are shown in Figure 3. The thermogram of tobramycin revealed a series of endothermic peaks characterized by the fusion of metastable form and recrystallization with a final melting peak at 217 °C. They were completely absent in the curve of drug loaded SLN, where appeared exclusively the endotermic peak at 69.25 °C corresponding to the fusion of stearic acid, the main component of nanoparticle matrix. Indeed, the presence of the drug, surfactant and cosurfactant in SLN decreased the fusion temperature in comparison with pure stearic acid, as previously shown.

This thermal behaviour suggests that the drug was completly solubilized inside the matrix of the SLN and it was not able to crystallize, in agreement with previous results with other formulations [52, 53] and it may be related to the preparation methods consisting in a rapid quenching of a melt liquid. Additionally, tobramycin was still present in the amorphous form or molecularly dispersed in SLN after 24 months from the preparation (data not shown). The increased solubility of tobramycin in the lipophilic matrix, represent a positive aspect from the point of view of biopharmaceutical performance of the preparation as discussed elsewhere [54] and could affect the drug release kinetic.

The quantitative analysis of tobramycin incorporated in freeze-dried SLN proved that the concentration of tobramycin in SLN was 2.5 % w/w (see Table 1). The loading capacity might be suitable for obtaining the 0.3 % drug concentration currently used in therapeutic treatment with commercial eye drops.

A large amount of mucin was absorbed on the Tobra-SLN surface. Indeed, the mucin binding efficiency was about 87 %. The mucin absorption was confrmed by a marked decrease of the zeta potential value (data not shown).

Therefore, morphological and physico-chemical properties of the solid lipid nanoparticles (average particle size, polydispersity index and composition) made them suitable as release systems for ophthalmic administration. Interestingly,our group has previously shown that SLN improved the drug passage across anatomical barriers, such as the blood brain barrier, and could be used to prolong the release of several drugs [24, 27].

3.2. *In vitro* release kinetics

The in vitro drug release profiles at pH 7.4 of drug from the Tobra-SLN formulation is shown in Figure 4. The analysis of the drug release showed a fast diffusion of the drug from the aqueous solution (Tobra-Sol) and a controlled release rate from Tobra-SLN. The drug release mechanism was studied using the semi-empirical power law relationship proposed by Ritger and Peppas [45], $Mt/M\infty = Kt^n$, where $Mt/M\infty$ is the fraction of drug released at each time point (t), k a kinetic constant relative to the properties of the matrix and n is the release exponent, indicative of the mechanism of drug release. The data related to SLN formulations fitted well into pseudo-zero order release kinetics (n = 0.96, R^2 = 0.996) with a cumulative percentage of tobramycin released after 2 h of about 24% w/w with no initial burst effect. On the contrary, after two hours from the beginning of the experiment, the percentage of tobramycin recovered in the receiving compartment for the reference aqueous solution (Tobra-SOL) were approximately 80% w/w and the release kinetic followed a diffusive mechanism (n = 0.58; R^2 = 0.981).

The difference in kinetic behaviour and in percentage of drug released is probably related to different lipophilicities of drug in the preparation. As well as tobramycin has a strong hydrophilic feature, incorporating it in SLN as an ion-pair with hexadecyl phosphate to increase its lipophilicity, accordingly to our previous study on pilocarpine [55], produced a promising

sustained-release ocular formulation. The lipophilic nature of tobramycin ion pair and its affinity for the lipophilic component is also demonstrated by the absence of a burst effect in the early stage of the in vitro release study, highlighted in the literature for many types of SLN. The burst effect is often attributed to the presence of drug on the surface of the SLN, where the lipid concentration is lower and the concentration of the surfactant is higher [34, 56] In our case the tobramycin could be concentrated into lipid inner matrix of SLN, thus promoting its slow and constant release in the dissolution medium that could also occur in vivo after ocular or intravenous admistration.

3.3. Physical and chemical stability of drug-loaded SLN over time

The results of the stability for Tobra-SLN formulation as aqueous dispersion stored at 4°C are reported in Table 2, where the main technological parameters (average diameter, PI (ζ), and incorporated drug) are reported *vs* time. The preparation showed good stability up to 12 months with no aggregation or precipitation phenomena, variations in zeta potential values or in amount of the drug encapsulated into the nanoparticles.

Unexpectely, their average diameters and PI values confirmed the presence of an increase in SLN dimensions (about 30 %) after 24 months (Table 2): the average diameters of Tobra-SLN increased from 80 nm to 106 nm. However, the slightly increase of PI, ranging from 0.15 to 0.18 and the decreasing of ζ up to -22.8 mV might not represent a significative instability of the formulation. In fact, the amount of drug incorporated into Tobra-SLN formulation did not change after 24 months at 4°C.

3.4. In vivo experiments: intravenous and ocular administration of the formulations

As expected, Tobra-SLN formulation was well tolerated without any symptoms of ocular inflammation and irritation in the different eye tissues. This behaviour agreed with experimental data reported in our previous pubblication [27].

The concentration of tobramycin in the biological tissues (vitreous humour and retina) 1.0 and 3.0 hours after ocular or intravenous administration of the two formulations under study is summarized in the Figures 5 - 7 where the concentration of Tobramycin in the different tissues collected from rabbits eye has been reported *vs*. time. The aqueous humor data published in our previously paper are reported as a comparison [27].

Surprisingly, the drug concentration was significantly higher in all ocular tissues (aqueous humor and vitreous humor and retina) after instillation and intravenous administration of Tobra-SLN formulation with respect to reference experimental formulations either commercial eye drop (Tobral®) or Tobra-Sol. Furthermore, regardless of the route of administration, exclusively the SLN formulation allowed an appreciable penetration of tobramycin into posterior segment of the eye. In the case of the solution, tobramycin was undetectable in the vitreous humor at 3 hours and in the retina at the time 1 hour and 3 hours, although the dose administered was relatively high. Ocular administration of Tobra-SLN produced a concentration of tobramycin in the aqueous humour two-fold and five-fold ($P<0.01$) higher compared to those obtained for Tobral® commercial preparation after 1 hour and 3 hours respectively. Any traces of tobramycin was detected into vitreous humour and retina after instillation of Tobral®, while considerable amounts of the drug were found into vitreous at both the selected times (30.6 μg/mL and 20.2 μg/mL at 1 and 3 hours, respectively) after SLN administration.

The drug recovery into retina was of 4.74 μg/g 1 hour after instillation of Tobra-SLN and about ten-fold lower after 3 hours (0.571 μg/g) as reported in Figure 7.

A similar result was obtained after intravenous administration: indeed 1 and 3 hours after injection of Tobra-SLN the concentrations of drug in the aqueous humor were about ten times higher than

those obtained with the reference solution (Tobra-Sol) and a significant drug concentration was detected at the same times into vitreous humour. The result achieved analysing the retina was especially interesting, since Tobla-SLN formulation allowed a high accumulation of drug in the retina after 1 hour and its amount was more than 3 times higher than that obtained by instillation (17.2 vs 4.74 μg/g).

These data confirm our previous results and emphasize the suitability of Tobra-SLN for an improvement of ocular drug bioavailability by exploiting drug passage through anatomical barriers and prolonging drug release. These results seem in agreement with the hypothesis that SLN could represent a versatile ocular drug delivery for the treatment of anterior and posterior chamber pathologies both when topically or/and sistemically applied. Numerous researchers demostrated throught ex vivo permeation studies performed on rabbits cornea [20, 23, 31, 57] that the cornea does not represent a substantial barrier to permeation of drug loaded in SLN . In fact, SLN for their lipophilic nature, favour drug permeation through the highly hydrophobic corneal ephitelium and this behaviour, in our case, could be supported by the considerable interaction with mucin, strictly depending by the capability of the nanometric size carrier (80 nm) to remain entrapped among the mucopolysaccharide chains of mucin available in the precorneal area. Furthermore, it cannot be excluded that the transcleral route might contribute to drug absorption in vitreous humour through the periocular route analougously to literature data [21, 23, 57].

In the light of this hypothesis, also the costant drug release rate, confirmed by *in vitro* data without any burst effect, could be a positive factor able to increase the ocular drug bioavailability. Indeed, the constant and sustained drug release from the lipid matrix over time ensure the maintaining of a constant drug concentration gradient and consequently the presence of a drug reservoir available for corneal and eventually scleral diffusion.

Considering the above mentioned aspects, our new pharmacokinetic data after topical instillation of Tobramycin entrapped SLN allow to hypothesize that both trans-corneal diffusion followed by

entry into vitreous and subsequent distribution to ocular tissues (trans-vitreous route) and probably drug diffusion around the sclera followed by trans-scleral absorption (periocular route) might be the mechanisms to achieve the posterior segment of the eye [58].

On the other hand, several recently published studies describe the capability of sistemically administered nanosystems as SLN [59 61] to overcome important physiological barriers. The higher amount of Tobramycin in the retina after systemic administration of the Tobra-SLN formulation with respect to ocular instillation, could be ascribable to the presence of the high vasculature of choroid that, with its fenestrated choriocapillaris, determines a rapid equilibration of drug molecules present in the bloodstream with the extravascular space. Through this route and by-passing the blood–retinal barrier, which restrict the movement of the substances to the intraocular spaces, the achievement of therapeutic levels of drug in the intraocular tissues might be reached [3, 62, 63].

3.5. Antimicrobial activity and effects of tobramycin and Tobra-SLN on polymorphonuclear granulocyte (PMN) functions

The MIC of tobramycin solution (Tobra-Sol) for the clinical strain of *P. aeruginosa* was found to be 4.0 μg/ml, whereas that for Tobra-SLN it was 2.0 μg/ml. PMN viability remained unchanged throughout the experiments. Table 3 shows the *in vitro* effects of tobramycin solution and Tobra-SLN on phagocytosis and killing of intracellular bacteria by PMNs. The presence of 1x MIC of tobramycin solution and Tobra-SLN exerted little effect on the human granulocyte phagocytosis, resulting in a slightly increased percentage of ingested Pseudomonas, in comparison with controls (Table 3; columns A, A1). Incubation of PMNs containing intracellular *P. aeruginosa* in either an antibiotic-free control culture or in the presence of tobramycin enabled the bacteria to increase in number grow: SI values were >2, which is compatible with intracellular survival (Column D). Conversely, Tobra-SLN added to PMNs after phagocytosis had occurred significantly enhanced

intracellular microbicidal activity against ingested Pseudomonas: during the 90-min period the intracellular bacterial load was reduced, achieving 53-70% of killing ($P<0.01$; Table 3; Column D1).

To investigate the direct effect of the two formulations on the phagocyte functions, bacteria and human PMNs were pre-incubated for 1h with 1xMIC of drug. After withdrawal of the antibiotic, bacterial uptake and microbicidal activity were determined. Tobramycin pre-treatment of the bacteria during their growth phase had no effect on phagocytosis itself (Column B). Conversely Tobra-SLN pre-treatment of bacteria led to a significant increase in the phagocytic capability of PMNs, after 30 min of incubation ($P<0.05$; Column B1). Pre-treatment of *P. aeruginosa* for 1h with 1xMIC of drug markedly increased the microbicidal activity of PMNs throughout the entire 90 min period of observation. However, much higher percentages of tobramycin-pretreated Pseudomonas were killed by the PMNs compared with their Tobra-SLN-treated counterparts: 75-69-54% versus 48-32-16% ($P < 0.01$) (Column E, E1).

Pre-exposure of the PMNs to 1xMIC of tobramycin solution and Tobra-SLN for 60 min did not affect their phagocytic capacity: the Pseudomonas were phagocytosed by PMNs at a rate that was similar to that in the controls (Column C, C1). In contrast, an enhanced bactericidal effect by PMNs occurred with Tobra-SLN-pretreated PMNs, leading to a significant decrease in SIs at all three time-points compared with controls and tobramycin-pretreated PMNs ($P < 0.01$; Column F, F1).

The efficacy of an antibiotic in treating bacterial infections depends upon the interaction of bacterium, drug and phagocytes [47-49]. Current trends require the use of antibiotics that combine good antimicrobial properties with the capacity to act in concert with the immune system, in a way that potentiates the host's defense mechanisms. Aminoglycosides penetrate little into cells and the high doses required carry a risk of drug toxicity. The use of Tobra-SLN may result in both higher intraphagocytic antibiotic concentrations in PMNs and greater bactericidal activity against intracellular *P. aeruginosa,* in comparison with the use of free tobramycin. This marked

bactericidal efficacy is probably related to the ability of Tobra-SLN to penetrate either into phagocytic cells, or alternatively to cross bacterial barriers such as the outer membrane, in its active form, without undergoing enzymatic attack. These data suggest that incorporation of tobramycin into delivery systems could be essential to obtain a superior therapeutic effect

These findings confirm the results reported in literature: it has been recently demostrated that the complexation of antimicrobial agents with amphiphilic moieties was able to maintain, and in particular condition to increase the antibacterial activity of the free drugs [64].

4. Conclusion

Current treatment of ocular infections is primarily based on the topical administration of appropriate anti-infective agents. Aminoglycosidic antibiotics, in particular tobramycin, are often prescribed to eradicate external ocular infections, because of its marked activity against *Pseudomonas aeruginosa*.

The success of antibacterial therapy depends on several features of the antibiotics, as well as on the ophthalmic vehicle. In this paper, ocular instillation of Tobra-SLN into the rabbit eye determined high concentration of tobramycin in both vitreous humor and retina, while any amount of tobramycin was found in these ocular tissues after administration of commercial Tobral® eye drops. In addition, similar results were obtained after intraveneous injection of Tobra-SLN.

In conclusion, the present study demostrates that the incorporation of tobramycin into small, homogeneous and mucoadhesive SLN increases drug penetration into eye structures, maintaining sufficiently high levels of drug in the aqueous humour, vitreous humor and retina, not only after parenteral but also after ocular administration.

To our knowledge, these findings on the use of a stable SLN formulation as a sustained drug carrier for ocular delivery to the posterior chamber might open new avenues for ocular infections also considering that nanocarriers might represent a strategy to overcome the microbial resistance and to ameliorate the patient compliance.

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The article is dedicated to loved memory of Prof. Maria Rosa Gasco, who inspired, discussed and sustained the work.

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Formulation	Average diameter nm (± SD)	Polydispersity Index	Zeta Potential mV(± SD)	Incorporated drug (% w/w ± SD)
Tobra-SLN	80.01 (±11.10)	0.15 (± 0.03)	- 25.70 (± 2.00)	2.50 (±0.18)

Table 1. Technological characteristic of the Tobra-SLN formulation: average diameter, polydispersity index, zeta potential and amount of Tobramycin incorporated in freeze-dryied SLN (Means ± SD, n=10).

Technological parameters	Time (months)			
	0	6	12	24
Average diameter nm (± SD)	80.01 (± 11.10)	83.01 (± 14.10)	80.10 (± 17.03)	120.10 (± 18.04)
Polydispersity Index	0.15 (± 0.03)	0.15 (± 0.06)	0.15 (± 0.04)	0.18 (± 0.05)
Zeta potential (mV± SD)	- 25.7 (± 2.0)	- 25.9 (± 2.0)	- 24.5 (± 1.7)	- 22.8 (± 0.6)
Incorporated drug (% w/w)	2.50 (±0.18)	2.49 (±0.16)	2.48 (±0.19)	2.50 (±0.15)

Table 2 - Technological characteristic of the Tobra-SLN formulation: average diameter, polydispersity index, zeta potential and amount of Tobramycin incorporated into liophilized SLN during stability study, (Means ± SD, n=10).

	% Phagocytosis				Survival Index (%)			
Time (min)	**Controls**	**A**	**B**	**C**	**Controls**	**D**	**E**	**F**
		Tobra-Sol					**Tobra-Sol**	
30	8.3 ± 0.3	9.42 ± 0.1	8.4 ± 0.2	7.88 ± 0.15	> 2	> 2	1.25[b] ± 0.07 (75%[c])	> 2
60	7.8 ± 0.37	7.8 ± 0.03	7.5 ± 0.04	7.61 ± 0.02	> 2	> 2	1.31[b] ± 0.09 (69%)	> 2
90	4.8 ± 0.2	7.1 ± 0.34	5.0 ± 0.3	5.3 ± 0.06	> 2	> 2	1.46[b] ± 0.12 (54%)	> 2
		A_1	**B_1**	**C_1**		**D_1**	**E_1**	**F_1**
		Tobra-SLN					**Tobra-SLN**	
30	8.3 ± 0.3	9.97 ± 0.05	12.8[a] ± 0.9	8.15 ± 0.18	> 2	1.47[b] ± 0.12 (53%)	1.52[b] ± 0.10 (48%)	1.60[b]± 0.06 (40%)
60	7.8 ±0.37	6.02 ± 0.4	8.7 ± 0.09	7.85 ± 0.09	> 2	1.37[b] ± 0.11 (63%)	1.68[b] ± 0.09 (32%)	1.68[b] ± 0.03 (32%)
90	4.8 ± 0.2	6.4 ± 0.26	6.97[a]± 0.05	4.9 ± 0.25	> 2	1.3[b] ± 0.05 (70%)	1.84[b] ± 0.09 (16%)	1.7[b]± 0.15(30%)

Table 3. Effect of 1x MIC of tobramycin and Tobra-SLN on human PMN phagocytosis and intracellular killing of *P.aeruginosa* (Means ± SE)

A,A_1, D, D_1: addition of pseudomonas and 1xMIC of tobramycin or Tobra-SLN to PMNs; **B,B_1, E,E_1**: addition of bacteria to PMNs following 1h pre-exposure of pseudomonas to 1xMIC of tobramycin or Tobra-SLN; **C,C_1, F,F_1**: addition of bacteria to PMNs following 1h pre-exposure of phagocytes to 1xMIC of tobramycin or Tobra-SLN

[a] Significantly different (P<0.05) from the controls

[b] Significantly different (P<0.01) from the controls

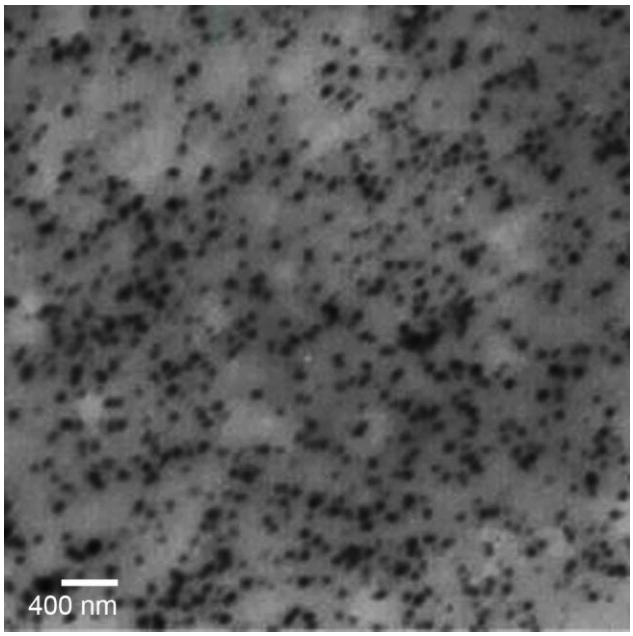


Figure 1. Trasmission electron microphotograph of Tobra-SLN ..

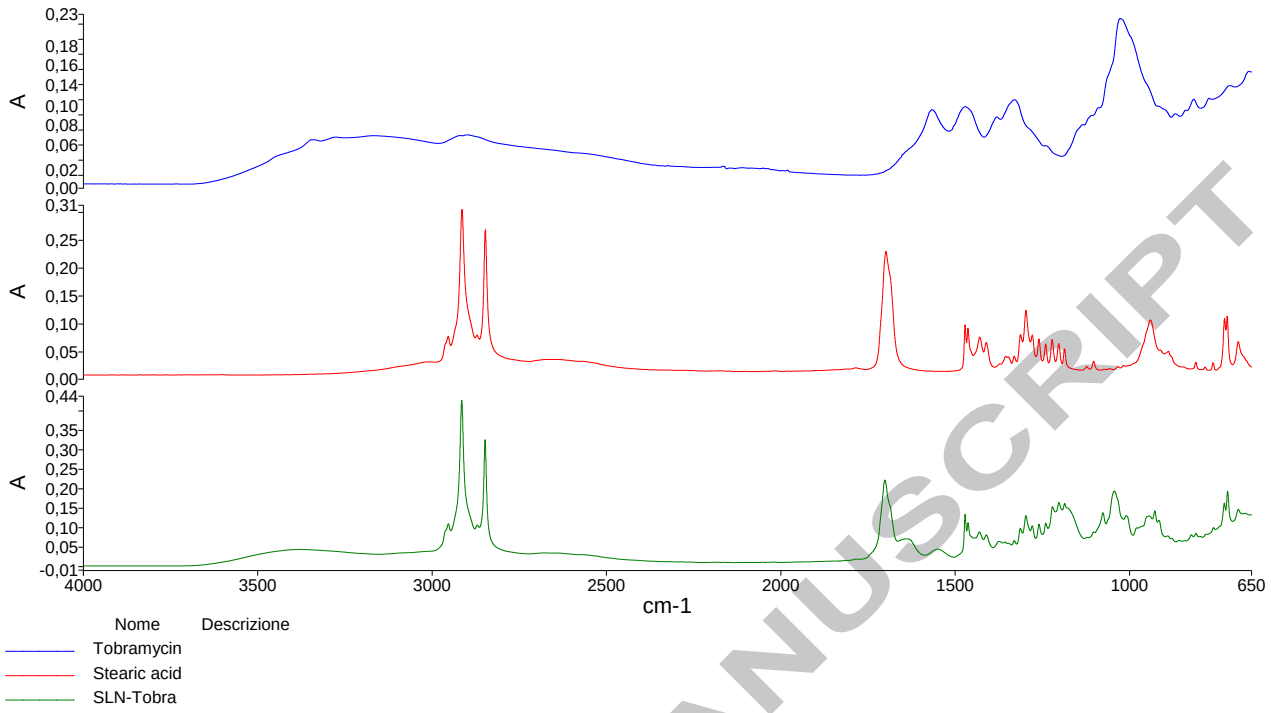


Figure 2 - FTIR spectroscopy analysis of Tobramycin as ion-pair, stearic acid and Tobra-SLN formulation

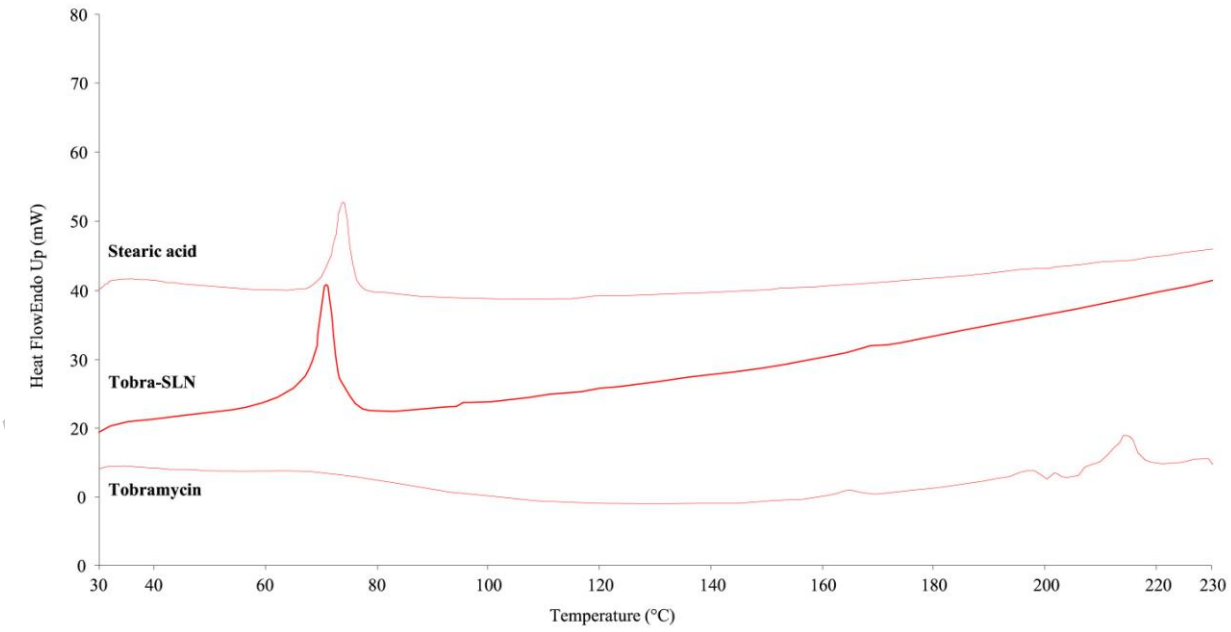


Figure 3. DSC thermograms of Tobramycin, and Tobra-SLN and stearic acid

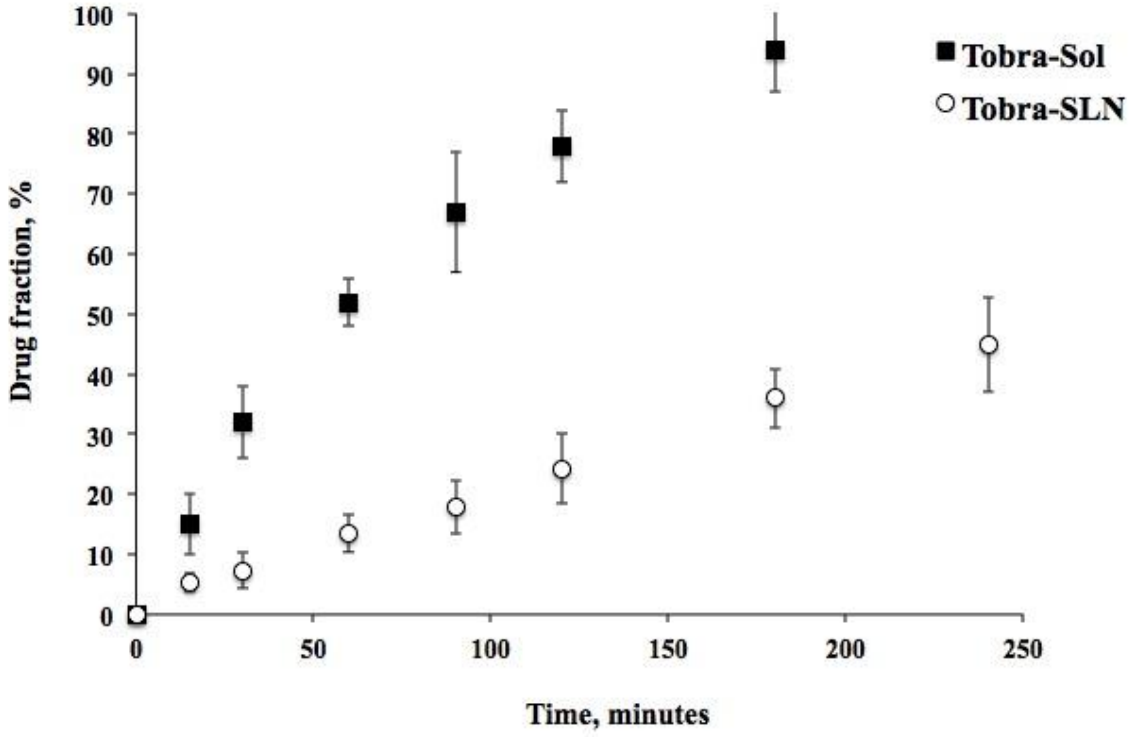


Figure 4. *In vitro* release profiles of Tobramycin from the Tobra-SLN formulation in pH = 7.4 phosphate buffer (Means ± SD, n=6)

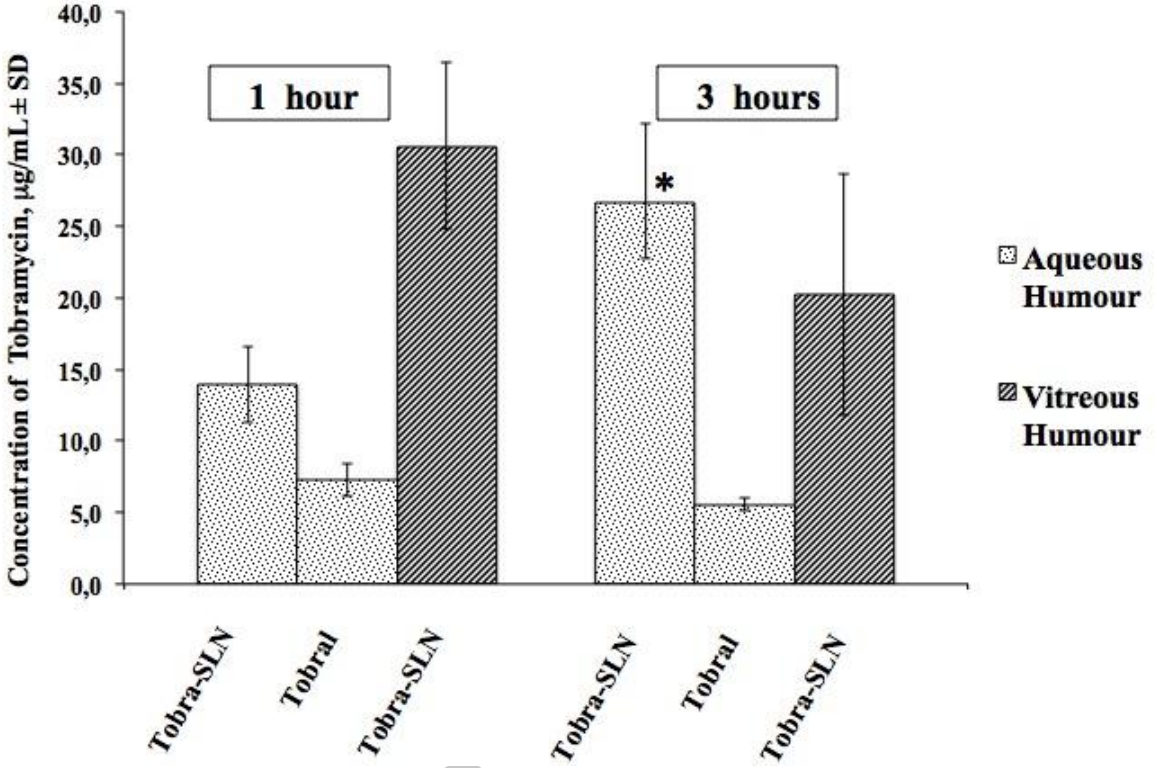


Figure 5.. Tobramycin concentrations in the aqueous humour and vitreous humor 1 hour and 3 hours after ocular administration of the formulations under study. The values are expressed as means ± SD.

* Significantly different (P<0.01) from the Tobral®

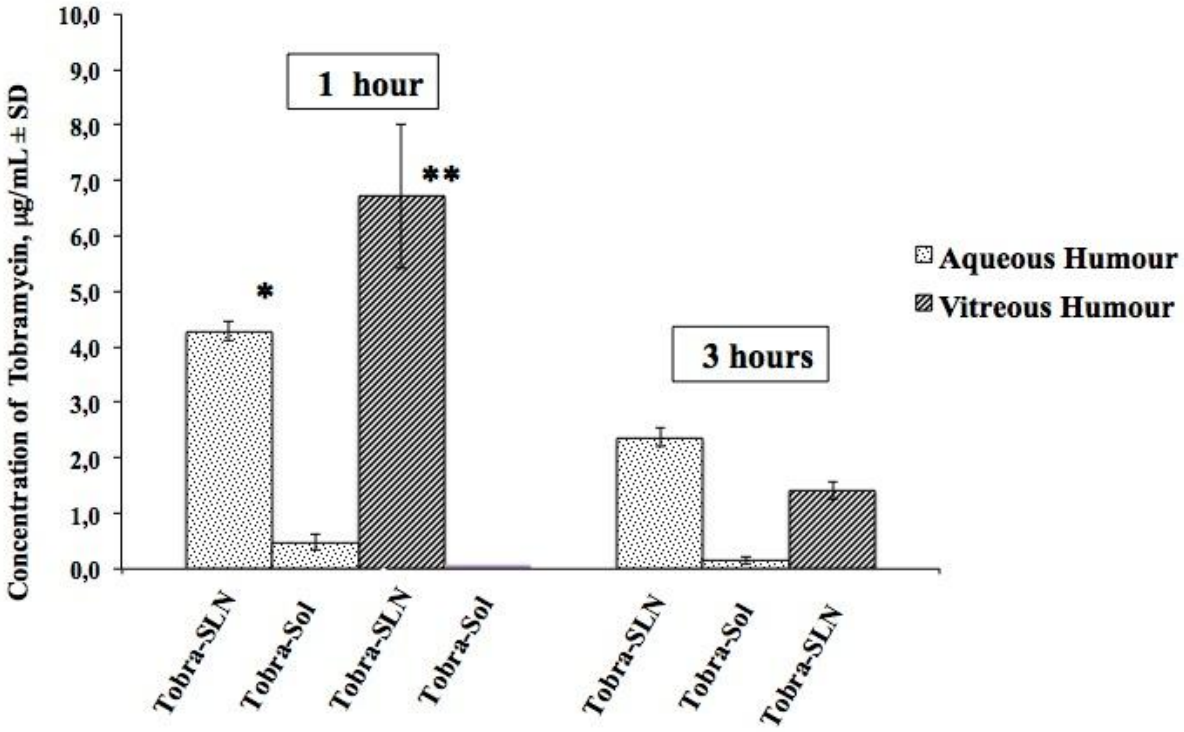


Figure 6. Tobramycin concentrations in aqueous humour and vitreous humor 1 hour and 3 hours after intravenous administration of the formulations under study. The values are expressed as means ± SD..

*Significantly different (P<0.01) from the Tobra-Sol (reference solution);

**Significantly different (P<0.02) from the Tobra-Sol.

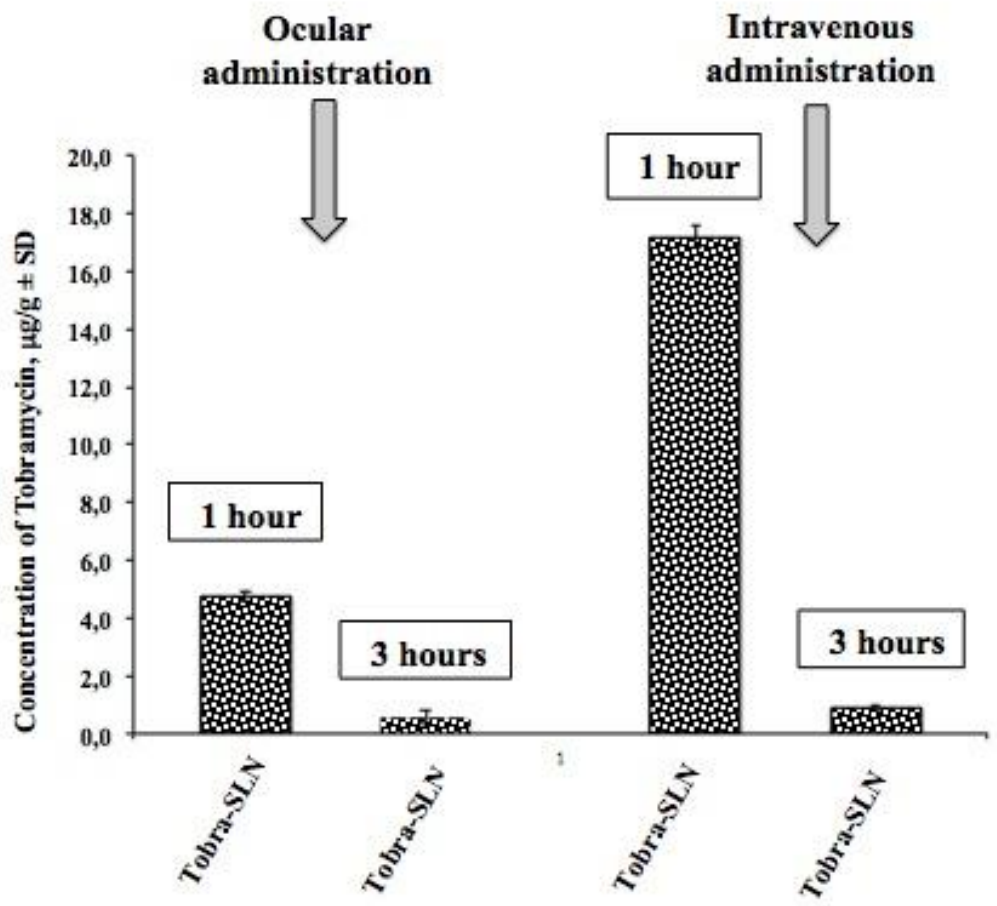
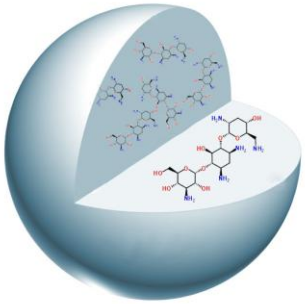


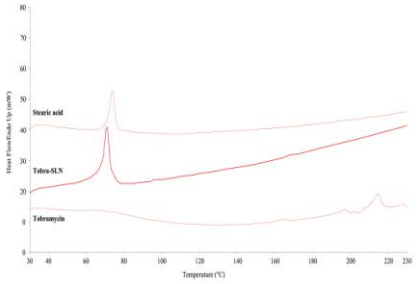
Figure 7. Tobramycin concentrations into the retina 1 hour and 3 hours after intravenous administration of the formulations under study. The values are expressed as means ± SD).

Graphical abstract

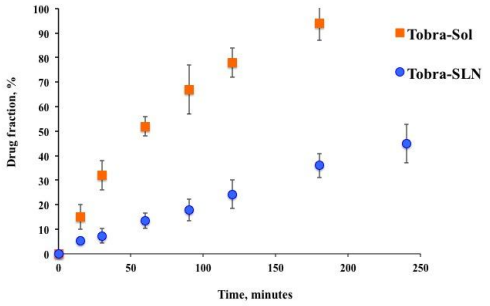


Average diameter nm (± SD)	Polydispersity Index	Zeta Potential mV(± SD)	Incorporated drug (% w/w ± SD)
80.01 (±11.10)	0.15 (± 0.03)	- 25.70 (± 2.00)	2.50 (±0.18)

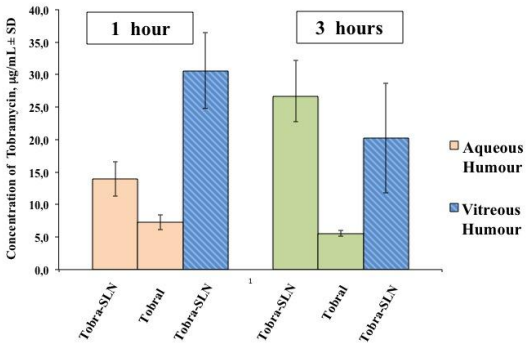
DSC



In vitro DRUG RELEASE



TOPICAL



TOPICAL

SYSTEMIC