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Sustained Release and Permeation of Timolol from Surface-Modified Solid Lipid Nanoparticles through Bioengineered Human Cornea

A. A. Attama
Institut für Pharmazeutische Technologie, Technische Universität Carolo-Wilhelmina zu Braunschweig, Braunschweig, Germany, and Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka Enugu, Nigeria

S. Reichl and C. C. Müller-Goymann
Institut für Pharmazeutische Technologie, Technische Universität Carolo-Wilhelmina zu Braunschweig, Braunschweig, Germany

ABSTRACT

Purpose: The aim of the study was to formulate and evaluate surface-modified solid lipid nanoparticles sustained delivery system of timolol hydrogen maleate, a prototype ocular drug using a human cornea construct. *Materials and Methods:* Surface-modified solid lipid nanoparticles containing timolol with and without phospholipid were formulated by melt emulsification with high-pressure homogenization and characterized by particle size, wide-angle X-ray diffraction, encapsulation efficiency, and *in vitro* drug release. Drug transport studies through cornea bioengineered from human donor cornea cells were carried out using a modified Franz diffusion cell and drug concentration analyzed by high-performance liquid chromatography. *Results:* Results show that surface-modified solid lipid nanoparticles possessed very small particles (42.9 ± 0.3 nm, 47.2 ± 0.3 nm, 42.7 ± 0.7 nm, and 37.7 ± 0.3 nm, respectively for SM-SLN 1, SM-SLN 2, SM-SLN 3, and SM-SLN 4) with low polydispersity indices, increased encapsulation efficiency (>44%), and sustained *in vitro* release compared with unmodified lipid nanoparticles whose particles were greater than 160 nm. Permeation of timolol hydrogen maleate from the surface-modified lipid nanoparticles across the cornea construct was sustained compared with timolol hydrogen maleate solution in distilled water. *Conclusions:* Surface-modified solid lipid nanoparticles could provide an efficient way of improving ocular bioavailability of timolol hydrogen maleate.

Keywords: bioengineered cornea; permeation; surface-modified solid lipid nanoparticle; sustained release; timolol hydrogen maleate

INTRODUCTION

Pharmaceutical nanoparticles encompass a wide variety of materials for numerous applications. Delivery of drugs to the tear film is routinely done with eye drops, which are well accepted and, for most

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Correspondence: A. A. Attama, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka Enugu 410001, Nigeria. E-mail: aaattama@yahoo.com or anthony.attama@unn.edu.ng

patients, easy to use. However, attainment of an optimal drug concentration at the site of action is a major problem. Poor bioavailability of drugs from ocular drug delivery systems is mainly due to the pre-corneal loss factors, which include tear dynamics, non-productive absorption, transient residence time in the cul-de-sac, and relative impermeability of the corneal epithelial membrane.[1,2] Non-productive absorption from eye drops could lead to serious side effects as is the case with timolol.[3] For instance, sufficient timolol may be absorbed from the eye to cause serious adverse effects on the heart and airways in susceptible individuals, and timolol may interact with orally administered verapamil and increase the risk of heart block.[3] Development of an alternative to solution-type timolol eye drops that would provide its sustained and controlled delivery is a major challenge. This informed our reason for embarking on this research. Timolol hydrogen maleate is a water soluble drug that ordinarily would find it difficult to penetrate ocular membrane, and formulation as a solid lipid nanoparticle would ensure the conferment of some hydrophobic characters needed for permeation through the corneal barrier and sustained release effect as a result of formulation strategies and adhesive properties of solid lipid nanopartricles (SLN).[4,5] Timolol is a nonselective agent, with no local anesthetic activity. It has excellent ocular hypotensive effects when administered topically in the eye and has an elimination half-life of 4–5 hr.[3] It is usually formulated as a solution, exposing it to the problems of solution-type eye drops, which this research set out to address. In this study, surface-modified solid lipid nanoparticles (SM-SLN) containing timolol hydrogen maleate were formulated with a natural fat obtained from *Theobroma cacao* and a phospholipid and evaluated for sustained ocular delivery potential using bioengineered human cornea.

MATERIALS AND METHODS

The materials include Phospholipon 90G® (Phospholipid GmbH, Germany), timolol hydrogen maleate (Dr. Mann Pharma, Germany), thimerosal (Synochem, Germany), sorbitol and polysorbate 80 (Tween 80®) (Across Organics, Germany), theobroma oil (Caesar & Loretz, Germany) and bidistilled water. Cell cultures used were provided by Nestec (Nestec Ltd, Nestlé Research Centre, Lausanne, Switzerland) and Cornea Bank of MHH Hannover. Stromal fibroblasts were isolated and cultivated from human corneal scleral rings (Cornea Bank, Hannover, Germany) in accordance with ethical regulations.

Formulation of SLN

SLN with a gradient of timolol hydrogen maleate (THM) with and without Phospholipon 90G® were formulated by melt emulsification (Ultra-Turrax, IKA, Germany) with high pressure homogenization (EmulsiFlex-C5, Avestin, Canada) as earlier described.[6,7] Surface-modified SLN (SM-SLN) was prepared with a lipid matrix composed of 30% phospholipid in theobroma oil, a homolipid, which has been optimized for use as efficient lipid matrix for drug delivery.[8] SM-SLN contained 0.5, 1.0, and 1.5% timolol hydrogen maleate (SM-SLN-1, SM-SLN-2, and SM-SLN-3, respectively) and no timolol hydrogen maleate (SM-SLN). SLNs containing theobroma oil alone as the lipid matrix with 1% timolol hydrogen maleate (SLN-5) and without timolol hydrogen maleate (SLN-6) were similarly prepared for comparison.

Characterization of SLN

Particle Size

The particle size of the SLN was determined by phase angle light scattering (PALS) using Zetasizer Nano Series (Nano-ZS, Malvern Instruments, England). Samples were diluted with double-distilled filtered water before measurement.

Wide-Angle X-ray Diffraction

Wide-angle X-ray studies were done on the formulated SLN as earlier described[6] using an X-ray generator (PW3040/60 X'Pert PRO, PANalytical, The Netherlands) connected to a copper anode, which delivered X-ray of wavelength $\lambda = 0.1542$ nm at a voltage of 40 kV and an anode current of 25 mA. The SLN samples were filled into capillaries and WAXD measurements were taken with a goniometer (MPD-System, PANalytical). The interlayer spacing d was calculated from the scattering angle θ using Bragg's equation.

Encapsulation Efficiency (EE%)

The EE% of the SLN was determined indirectly after centrifugation in a membrane concentrator (Vivaspin 6 MCO 5000, Vivascience AG, Hannover, Germany) for 90 min at 9000 × g and 5°C in a Beckman Coulter Allegra™ 64R centrifuge (Beckman Coulter, Palo Alto, CA, USA). The drug concentration in the aqueous continuous phase was determined by HPLC. The drug content of the nanoparticles was thereafter calculated considering the initial amount of drug loaded.

In Vitro Drug Release

In vitro release studies were carried out in a modified Franz diffusion cell (FD-C)[9] over 6 hr at a temperature of 37 ± 1°C. The diffusion barrier was a siliconized Spectrapore® membrane MWCO 6000–8000 (Spectrum Laboratories Inc., The Netherlands). In each case, a finite dose of the SLN was introduced into the donor compartment and the open ends of the apparatus sealed with Parafilm® to prevent evaporation. Isotonic phosphate buffer (pH 7.4) was used as the acceptor and was stirred with a magnetic stirrer at 600 rpm during the experiment. Aliquots of 1 ml of the acceptor phase were withdrawn at intervals and the withdrawn samples analyzed for drug content by HPLC. Sink condition was maintained throughout the release period. Data obtained in triplicate were analyzed graphically.

Transmission Electron Microscopy

A transmission electron microscope (Leo 922, D-Oberkochen, Germany) was used to study the SLN morphology as earlier described for unloaded SLN.[6]

Human Cornea Construct (HCC) and Drug Permeation

HCC was bio-engineered step by step in Transwell® cell culture inserts as previously described[7] using immortalized human corneal endothelial cells (HENC), stromal fibroblasts, and epithelial cells (CEPI 17 CL 4). Drug transport studies through bio-engineered HCC were carried out using a Franz diffusion cell. Briefly, the modified FD-C consists of a donor compartment and an acceptor compartment. The donor compartment was filled with the SLN formulation from each batch, whereas the acceptor compartment contained phosphate buffer (pH 7.4). The barrier (HCC) was sandwiched on top of a polycarbonate membrane (3.0 μm pore size) between the two compartments. Sampling was done at predetermined time intervals up to 6 hr from the acceptor compartment, and volume of acceptor was kept constant throughout the experiment with fresh buffer. The whole apparatus was kept at 37°C in a water bath while the acceptor solution was stirred with a magnetic stirring bar. Permeation studies were performed in triplicates.

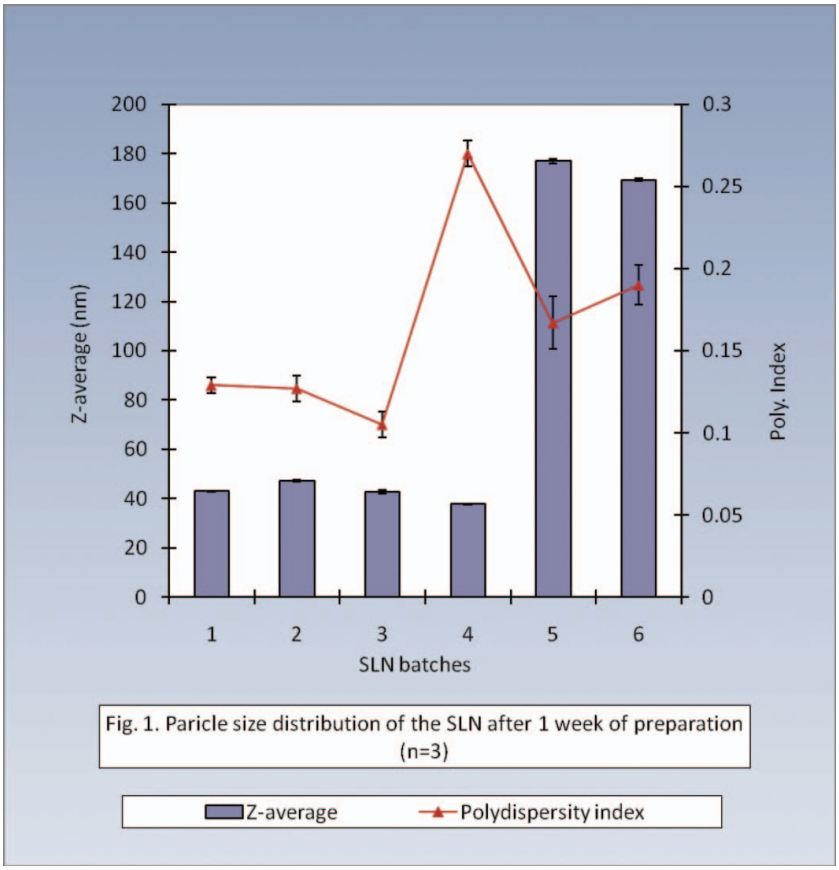

Figure 1. Particle size distribution of the SNL after 1 week of preparation ($n = 3$).

The permeation parameters of timolol hydrogen maleate from the SLN were calculated by plotting the amounts of drug permeated through HCC ($\mu g/cm^2$) versus time (min). The steady-state flux (J) values across HCC were evaluated from the linear ascents of the permeation graphs by means of the relationship:

$$J = \frac{dQ}{Adt}(\mu g/(cm^2 s)) \quad (1)$$

where Q indicates the quantity of substance crossing HCC, A is the area of HCC exposed, and t is the time of exposure. The permeation coefficient P in each case was calculated from Equation (2).

$$P = \frac{J}{C_o}(cm/s) \quad (2)$$

where C_o represents the initial drug concentration in the donor compartment.

HPLC Methodology

An HPLC system, Waters 486, 515, 717plus (Waters, Eschborn, Germany) was used with data analysis by Waters Millenium 32 Chromatography Manager Software. Solvent system consisted of acetonitrile/buffer (triethylammonium (0.5) and distilled water (500), pH 2.9) (62.5:437.5), and injection volume of 20 μl, flow rate of 1.1 ml/min; retention time of 3.8 min, and detection was done at 295 nm ($r^2 = 0.9988$; 20 – 70 μg/ml).

Data and Statistical Analysis

All experiments were performed in replicates ($n = 3$) for validity of statistical analysis. Results were expressed as mean ± SD. ANOVA and Student t-tests were performed on the data sets generated using Origin for Windows®. Differences were considered significant for p values < 0.05.

RESULTS AND DISCUSSION

Figure 1 shows the particle size distribution of the SM-SLN, where nano-sized particles with low standard deviations (42.9 ± 0.3 nm, 47.2 ± 0.3 nm, 42.7 ± 0.7 nm, and 37.7 ± 0.3 nm, respectively, for SM-SLN 1, SM-SLN 2, SM-SLN 3, and SM-SLN 4) and low polydispersity indices are presented. The low polydispersity

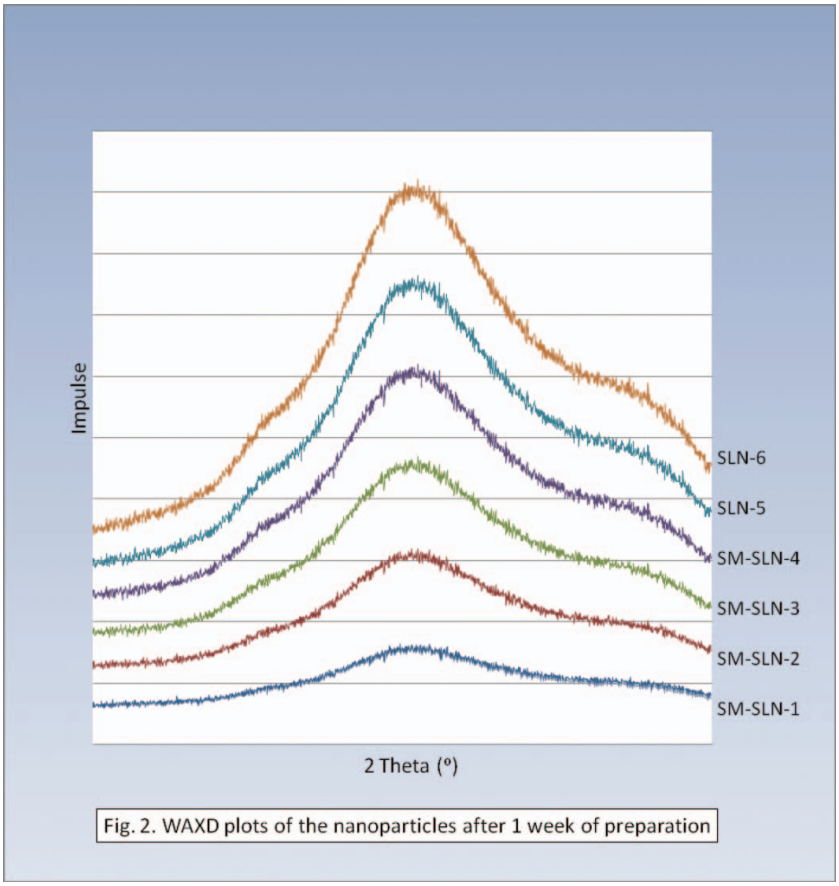

Figure 2. WAXD plots of the nanoparticles after 1 week of preparation.

indices of the particles indicate narrow size distribution. SM-SLNs (SM-SLN-1, SM-SLN-2, SM-SLN-3, and SM-SLN-4) possessed low sizes compared with unmodified SLNs (SLN-5 and SLN-6). For ocular administration, irritation and tear washout may occur on administration of large-sized particles, since smaller particles are better tolerated.[2] Particulate drug delivery systems like nanoparticles and microparticles are better tolerated by patients than larger particles and, hence, microspheres and nanospheres represent very comfortable prolonged action ophthalmic drug delivery systems[2] compared with ointments or coarse suspensions. Modification stabilizes particles and increases its drug holding capacity as shown by the higher EE% of the former compared with the latter. Encapsulation efficiency of greater than 44% was recorded for all the batches of SM-SLN formulated compared with unmodified SLN (36%) ($p < 0.05$) confirming superior drug holding capacity of SM-SLN. Figure 2 shows the WAXD plots for the nanoparticles, indicating recrystallization of the lipid particles had not yet completed after 1 week.

Figure 3 shows *in vitro* drug release across an artificial membrane (MCO 5000–8000) into phosphate buffer (pH 7.4), while Figure 4 shows permeation profile of timolol across the regenerated human cornea (HCC). *In vitro* drug release across artificial membrane and permeation profiles across regenerated human cornea indicate sustained release and permeation of timolol. This study was carried out using SM-SLN only, as other *in vitro* properties of SLN without phospholipid such as bigger particle size and high polydispersity indices, which suggest the presence of wide size

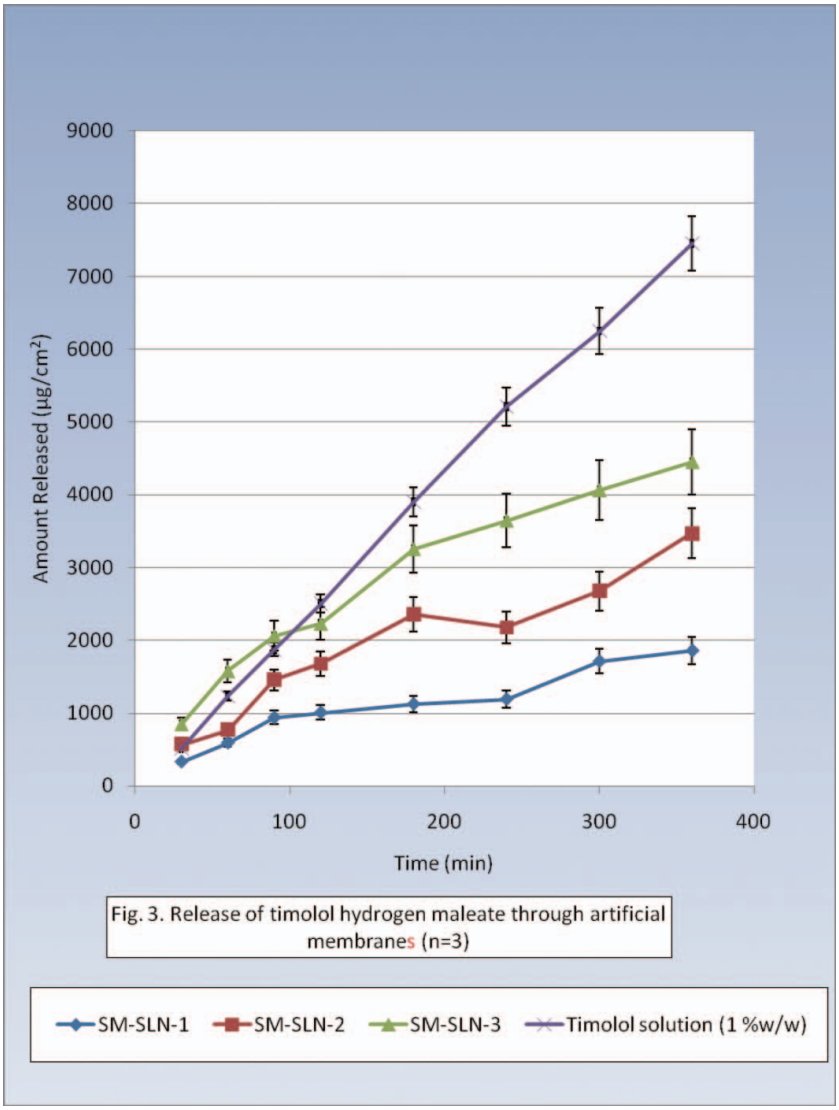

Figure 3. Release of timolol hydrogen maleate through artificial membranes ($n = 3$).

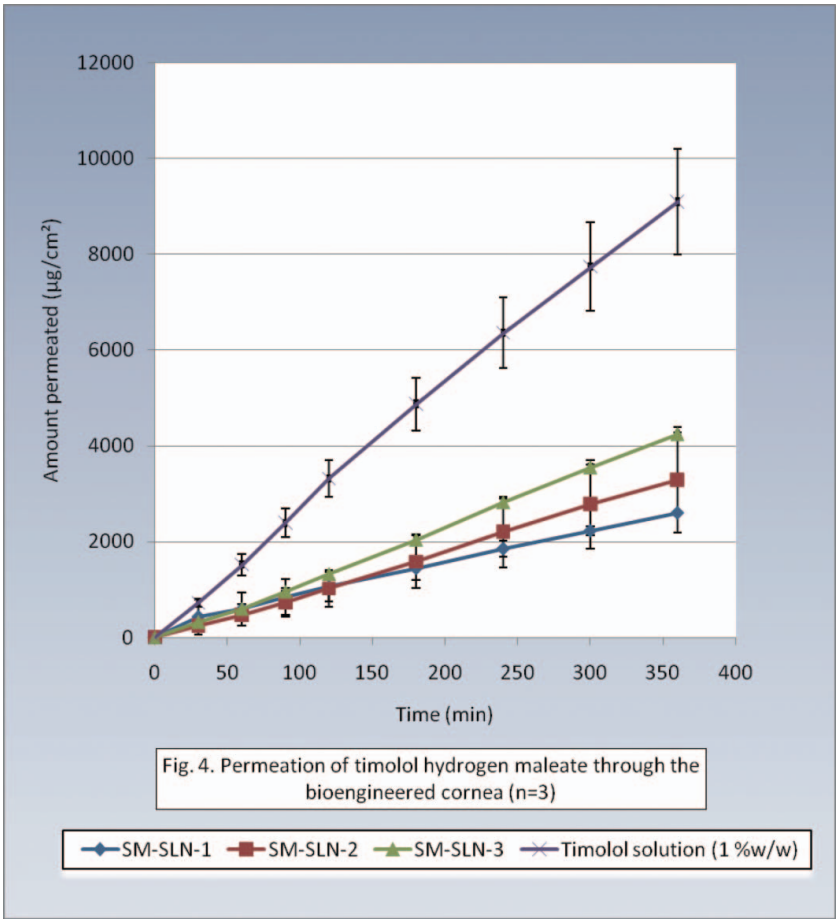

Figure 4. Permeation of timolol hydrogen maleate through the bioengineered cornea ($n = 3$).

distribution, were excluded from further studies. Particle size, particle size distribution, and stability are a major issue considered by formulation scientists when formulating dispersed systems, especially those intended for ocular administration. Smaller particles are better tolerated. *In vitro* release showed typical capacity-limited release, i.e., increase in release with increase in drug loading. Burst effect was not observed, indicating better control of the release process due to the formulation strategy adopted. Burst effect release pattern usually occurs with unmodified SLN compared with SM-SLNs, due to the usually high amount of unencapsulated or surface-attached drug.[7] Controlled release of THM was achieved here because of the structure formed at the surface, which possibly ensured drug release by leaching, diffusion, or gradual erosion, or a combination of these processes. Permeation coefficients were obtained from the permeation fluxes evaluated from Equation (1). The permeation coefficients obtained were 2.31×10^{-5}, 1.56×10^{-5}, 1.33×10^{-5}, and 4.27×10^{-5} cm/sec for SM-SLN-1, SM-SLN-2, SM-SLN-3, and 1% w/w timolol solution, respectively. The permeation coefficients recorded for the SM-SLNs were high and sustained, considering their different drug loads compared with the permeation of the free THM solution, which was about two to three times higher than the permeation of the THM from SM-SLNs, and this proved the formulations effectively permeated through the constructs. There was less resistance to the permeation of the free timolol solution by the construct, and thus sustained permeation of the drug was not significantly achieved ($p < 0.05$) compared with the surface-modified lipid nanoparticles. Permeation studies were done using the drug loaded SM-SLNs only. These SM-SLNs developed for ocular delivery of THM contained a biocompatible lipid and pharmaceutically acceptable mobile surfactant. Thus, the issue of toxicity and safety with respect to future clinical applications will not present any problem.

Figure 5 shows a typical TEM of SM-SLN-1 showing crystalline edges of SM-SLN (see arrows). Contrary to WAXD, TEM was done after 1 month of preparation,

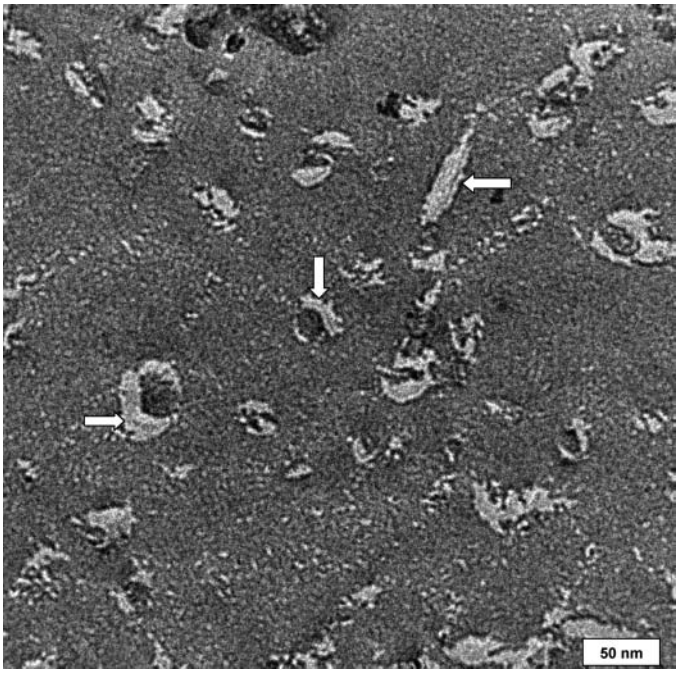

Figure 5. Typical transmission electron micrograph of SM-SLN-1.

and crystalline edges indicate complete recrystallization, confirming the solid nature of the SM-SLN. Studying the recrystallization pattern of SLN is very important because previously encapsulated drug could be expelled during subsequent recrystallization and transformation to a more stable form for a lipid that undergoes polymorphism. Although theobroma oil used in this study undergoes polymorphism, addition of the phospholipid has been shown to modify the rcrystallization pattern and its polymorphic transition such that the included drug would not be expelled on complete recrystallization because the ultimate crystallinity remains low despite its solid nature,[6,8] as depicted in Figure 5. Recrystallization was not yet complete at 1 week (Fig. 2).

After topical ocular application, drugs may be absorbed into the eye through the corneal or conjunctival and scleral routes, with conjunctiva being a conduit for drug clearance into the systemic circulation.[10] Conjunctival and scleral routes are important mostly for very hydrophilic and large molecules that are not able to penetrate through the corneal barrier.[11] SM-SLN formulation of timolol, which showed high *in vitro* ocular transport, would offer two advantages. Encapsulation of timolol into the lipophilic particle would facilitate transport through the corneal route, and the particulate nature of the formulation would ensure adherence to the surrounding membranes, preventing tear washout and providing sustained release of timolol, as SLN are highly adhesive.[4] Although *in vivo* experiments were not carried out, HCC employed in this study has been shown in our laboratory to correlate well with *ex vivo* studies using human donor corneas.[12] Bioengineering human cornea tissues from donor cornea cells has been reported to eliminate species-related problems while avoiding animal experiments.[13] Corneal equivalents have been observed to show tight junctions and desmosomes in the flattened apical cell layer[14] and resemble intact cornea with morphologically identifiable desmosomes, tight junctions, microvilli, and cell layers with apical flat cells. HCC also has other advantages in drug delivery compared with intact animals such as cost effectiveness and accessibility.

CONCLUSION

Higher encapsulation efficiency was obtained with surface-modified SLN compared with unmodified SLN, and high and sustained permeation were also achieved with this novel SM-SLN formulation compared with timolol solution. This indicates this SM-SLN could be an effective ocular drug delivery system for timolol.

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Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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