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# Cholesterol-poly(ethylene) glycol nanocarriers for the transscleral delivery of sirolimus

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ABSTRACT

The aim of this study was to prepare and characterize cholesterol-poly(ethylene) glycol (chol-PEG) nanocarriers of two different molecular weights (1 and 5 kDa) and to determine their effect on the transscleral retention and permeation of a lipophilic multi-therapeutic agent, sirolimus (rapamycin), with potential application in angiogenic and immunogenic ocular diseases. Sirolimus-containing nanocarriers were prepared using the thin-film hydration method and characterized for their physicochemical properties including size, drug entrapment (EE) and loading (DL) efficiencies, stability, surface charge, morphology, critical micelle concentration (CMC) and thermal properties. Ussing chambers were used to determine the retention and permeability of sirolimus-containing nanocarriers in porcine sclera followed by ultrastructural tissue examination. Sirolimus-containing nanocarriers had an average size of 11.7 nm (chol-PEG 1 kDa) and 13.8 nm (chol-PEG 5 kDa) and zeta potentials of 0.41 and −1.05, respectively. Both nanocarriers had similar transscleral permeabilities (chol-PEG 1 kDa $6.44 \times 10^{-7}$ and 5 kDa $6.16 \times 10^{-7}$ $cm^2\ s^{-1}$), and very high scleral retention compared with a free solution of sirolimus (chol-PEG 1 kDa 16.9 μg/g; chol-PEG 5 kDa 7.48 μg/g; free sirolimus 0.57 μg/g). The DL (EE) for chol-PEG 1 and 5 kDa were 2.93% (77.4%) and 3.10% (81.6%), respectively. The CMC values for the nanocarriers were similar to those previously reported in literature ($3.85 \times 10^{-7}$ M for chol-PEG 1 kDa; $4.26 \times 10^{-7}$ M for chol-PEG 5 kDa). In conclusion, chol-PEG nanocarriers successfully loaded sirolimus and resulted in scleral permeation and high retention, which shows potential utility for the topical delivery of lipophilic ocular drugs.



## 1. Introduction

Sirolimus (rapamycin) has been shown to exhibit antifungal, immunosuppressive, cytostatic and anti-angiogenic activities, with potential use in the treatment of multiple anterior and posterior segment eye diseases (Guba et al., 2002; Houchens et al., 1983; Martel et al., 1977). However, like many potential therapeutic agents, it has very poor water solubility (2.6 μg/mL) and is therefore not ideally suited for topical delivery (Simamora et al., 2001). One method of solubilising such an agent is by dissolving it in organic solvents such as dimethyl sulfoxide (DMSO) or methanol, and then diluting the solution in phosphate buffered saline (PBS) (Cooper et al., 2012). However, this is not suitable for topical ocular delivery as exposure to such solvents could result in ocular irritation, toxicity and scleral oedema (Conquet et al., 1977; Cruysberg et al., 2005). Alternative attempts to solubilise and deliver sirolimus for ocular delivery include the use of liposomes, cyclodextrin solutions and microemulsions. However, these formulations showed very poor or no ocular permeation (Buech et al., 2007). This may be attributable to their large particle size (100–165 nm), as studies have shown that trans-ocular permeation is greatly limited by particle size (Nagarwal et al., 2009; Pade and Stavchansky, 1997). Particle permeation across the retina is also size dependent, with smaller particles (20 nm) showing greater permeation and better

*Abbreviations:* chol-PEG, cholesterol-poly(ethylene) glycol nanocarriers; CMC, critical micelle concentration; DL, drug loading efficiency; DLS, dynamic light scattering; DMSO, dimethyl sulfoxide; DSC, differential scanning calorimetry; EE, entrapment efficiency; LDV, laser Doppler velocimetry; PBS, phosphate buffered saline; RP-HPLC, reversed-phase high performance liquid chromatography; SEM, scanning electron microscopy; TEM, transmission electron microscopy; XRPD, X-ray powder diffraction.

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uptake in ARPE-19 cells, as compared to larger particles, up to 2 μm in size (Kim et al., 2009; Amrite and Kompella, 2007). Thus, the preparation of our drug-loaded nanocarriers with sizes less than 20 nm is expected to enhance permeation across ocular tissue.

Cholesterol has often been used to prepare liposomes. Once modified, it offers several advantages for topical ocular administration including enhanced stability and bioavailability (Hathout et al., 2007; Lasic, 1993), but it does not circumvent the issue of large particle size. As cholesterol is hydrophobic in nature, modification with a hydrophilic polymer such as poly(ethylene) glycol (PEG), would result in the formation of micelles, which could be used to encapsulate the drug. These amphiphilic nanocarriers have several advantages including small size (typically less than 50 nm versus 200 nm for liposomes), prolonged drug release, enhanced stability and bioavailability, and passive and site-specific targeting (Aliabadi et al., 2008; Lin et al., 2003).

Our aim was therefore to prepare and characterize sirolimus-loaded nanocarriers using cholesterol-poly(ethylene) glycol (chol-PEG) polymers and to assess their effect on transscleral permeation and drug retention. To our knowledge, this is the first study to assess cholesterol-PEG nanocarriers for the topical ocular delivery of sirolimus.

## 2. Materials and methods

### 2.1. Materials

The following materials were used as received and were analytically pure: sirolimus (sirolimus, LC Laboratories, Woburn, MA), cholesterol-PEG (chol-PEG, molecular weight: 1 kDa and 5 kDa, Grobbendonk, Belgium), absolute ethanol 99.6% (Haymans Ltd, Essex, UK), chloroform (VWR, International, Lutterworth, UK), paraformaldehyde (97%, Alfa Aesar, Ward Hill, MA) and sodium hydroxide (99% pellets, BDH, Poole, UK). The following materials were obtained from Sigma–Aldrich (MO, USA): pyrene (≥99%), trifluoroacetic acid (TFA, ≥99), PBS and DMSO. High performance liquid chromatography (HPLC) grade deionised water (used throughout the study) and acetonitrile were obtained from Fisher (Leicestershire, UK).

### 2.2. Preparation of blank and sirolimus-loaded nanocarriers

Nanocarriers were prepared using the thin-film hydration method as described in previous studies, with modifications (Wei et al., 2009; Zhang et al., 1996). Briefly, 200 mg of chol-PEG (1 or 5 kDa, with or without 8 mg of sirolimus) was dissolved in 10 mL of absolute ethanol and 1 mL of chloroform in a round bottom flask. The polymer-to-drug ratio was selected from preliminary studies (results not shown) as the optimised ratio with the highest drug entrapment efficiency. The solvents were removed using rotary evaporation (Hei-VAP Advantage Rotary Evaporator, Heidolph, Schwabach, Germany) set at 150 rpm, 80 °C and 0 bar pressure (KNF Laboport, KNF Neuberger, Freiburg, Germany) for 20 min to obtain a thin film. The system was then dried to remove any residual solvent. The resultant thin film was hydrated with 10 mL of water (preheated at 55 °C) and mixed thoroughly on a 55 °C water bath (Buchi B480, Sweden) for 5 min. This solution was then bath sonicated (Ultrawave bath sonicator, Ultrawave Ltd, Cardiff, UK) for 10 min and left to settle at room temperature for a further 10 min. The solution was then passed through a sterile 0.22 μm filter (Millex-MP, Millipore, Carrigtwohill, Ireland) to remove any unentrapped insoluble drug. The resultant filtrate was used for further analysis (both as a solution and as a lyophilised powder). Samples were protected from light throughout the preparation and in all of the analytical procedures.

### 2.3. Characterization of nanocarriers

#### 2.3.1. Particle size and charge (zeta potential)

A Zetasizer (Malvern Nano ZS, Malvern Instruments Ltd, Worcestershire, UK) was used to analyse the mean particle size and zeta potential (or charge) of the nanocarriers using dynamic light scattering (DLS) and laser Doppler velocimetry (LDV), respectively.

#### 2.3.2. Stability of drug-loaded nanocarriers

The stability of the sirolimus-loaded nanocarriers was determined by analyzing the particle size and the amount of drug remaining in the nanocarriers over time using reversed-phase high performance liquid chromatography (RP-HPLC), as described by Ribeiro et al. (2012) with modifications. Briefly, samples were analysed at room temperature at baseline, then agitated, re-filtered and analysed again at days 7 and 21.

#### 2.3.3. Nanocarrier morphology

Nanocarrier morphology was analysed using a FEI CM 120 Bio Twin transmission electron microscope (TEM, Philips Electron Optics BV, Netherlands). Approximately 40 μL of the preparation was placed on a copper grid with a nitrocellulose covering and negatively stained with 1% uranyl acetate.

#### 2.3.4. Drug loading and drug entrapment efficiency

The amount of sirolimus that was incorporated in the nanocarriers, and had retained and permeated across the sclera, was determined using RP-HPLC. Acetonitrile (ACN) was used to disrupt the nanocarriers and solubilise the drug for analysis. The drug loading (DL) and entrapment efficiency (EE) were calculated using Equations (1) and (2) below:

$$\mathrm{DL}\ (\%) = \frac{\text{Amount of drug}}{\text{Amount of polymer} + \text{drug}} \times 100 \quad (1)$$

$$\mathrm{EE}\ (\%) = \frac{\text{Measured drug loading}}{\text{Theoretical drug loading}} \times 100 \quad (2)$$

#### 2.3.5. Critical micelle concentration

The critical micelle concentration (CMC) was determined using a fluorescence spectrometer (Perkin Elmer precisely LS55 luminescence spectrometer, Wellesley, USA) with pyrene as the fluorescence probe. Samples were serially diluted with pyrene at a concentration of $6 \times 10^{-7}$ M and the fluorescent intensity for each concentration measured at an emission of 331 nm and an excitation of 334 nm. At the CMC, where the amphiphilic nanocarriers begin to form, the pyrene partitions preferentially towards their hydrophobic core, causing an increase in the fluorescence intensity. This change presents as a large change in the first and third highest emission peaks ($I_1$ and $I_3$ respectively) of pyrene's emission spectra. The CMC value was taken at the point of intersection from the two tangents drawn at high and low concentrations from the plot of the intensity ratio ($I_1/I_3$) against log of the micellar concentration (Kalyanasundaram and Thomas, 1977).

### 2.4. Thermal properties

#### 2.4.1. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed on the samples to characterize their thermal properties and observe the interactions between the drug and the nanocarriers (Q2000 module, TA Instruments, New Castle, DE). Lyophilized preparations were sealed in aluminium hermetic pans (TA Instruments,