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Research paper

Novel inulin-based mucoadhesive micelles loaded with corticosteroids as potential transcorneal permeation enhancers

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A B S T R A C T

In this work a new copolymer of inulin (INU) derivatized with ethylendiamine (EDA) and retinoic acid (RA), named INU-EDA-RA, was synthetized, characterized and employed to produce micelles as carriers for topical administration of corticosteroids for the potential treatment of diseases of posterior eye segment. Spectroscopic analysis confirmed a molar derivatization degree of 11.30 and 4.30% in EDA and RA, respectively. INU-EDA-RA micelles are capable of strong mucoadhesive interactions which result time-independent and stable over time but concentration depending. Moreover micelles are able to encapsulate efficiently from 3 to 13% (w/w) of lipophilic drugs, as dexamethasone, triamcinolone and triamcinolone acetonide. Drug loaded micelles are stable for three months when stored as freeze-dried powders and able to release high amount of drug when compared to drug dissolution profiles from suspensions. Moreover, drug loaded micelles are compatible with different ocular cell lines that are also able to internalize fluorescent micelles. Finally, drug loaded micelles enhance drug fluxes and permeability coefficients across corneal epithelial cells, thus reducing drug loss due to retention inside the cells.

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1. Introduction

Vascular degenerative disorders of the posterior eye segment, such as age-related macular degeneration (ARMD), diabetic retinopathy (DR) and diabetic macular edema (DME), are the main cause of visual impairment and blindness worldwide [1–3].

Today, intravitreal injections of vascular endothelial growth factor (VEGF) inhibitors are the standard of care for patients with retinal neovascularization. The "intravitreal operative medication" (IVOM) is the main technique used because it yields effective local drug concentrations while markedly reducing systemic side effects. However, IVOM is a painful and invasive procedure that must be performed under sterile conditions. Moreover, intraocular injections increase the risk of haemorrhages, endophthalmitis, retinal detachment, cataracts and other complications [4]. In addition, anti-VEGF drugs only bind to the synthesized VEGF and inhibit its action, while corticosteroids exert a complex and multi-factorial anti-inflammatory, anti-edematous and anti-angiogenetic mechanism of action. In particular, they decrease VEGF synthesis by destabilizing VEGF mRNA, inhibit cellular proliferation, stabilize the blood-retinal barrier, increase the production of tight junctional proteins, regulate the expression and distribution of ion channels and water channels in retinal glial Müller cells and improve retinal oxygenation. To summarize, corticosteroids improve vision by re-establishing the blood retinal barrier, thereby reducing exudation and lessening interstitial edema [5–7]. As a consequence, during the past 10 years, corticosteroid therapy emerged as a promising treatment for the main degenerative pathologies of the retina. However, it is necessary to found appropriate ocular delivery systems able to release in efficacious manner corticosteroids after a topical application that is the most widely accepted route for ocular delivery due to its non-invasive nature, easy administration and patient compatibility. Unfortunately, only 2–7% of drugs administered topically result bioavailable due to the innate defensive mechanisms of the eye like naso-lacrimal drainage, absorption into the systemic circulation, very low capacity of human cul-de-sac, winking, basal and reflex tearing, tear dilution and metabolism of drugs by tear enzymes.

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The use of nanocarriers could be a useful strategy for topical ocular drug delivery, for their capacity to protect the encapsulated molecules while facilitating their transport to the different compartments of the eye. Furthermore, mucoadhesive polymer based nanosystems could reduce the drainage rate thus improving ocular bioavailability. Thus different ocular drug delivery systems based on nanotechnology could be promising for the treatment of chronic ocular diseases requiring frequent drug administrations [8–11]. In particular, polymeric micelles are self-assembling colloidal systems obtained by amphiphilic copolymers with distinct hydrophobic and hydrophilic segments. The polymer self-assembles to form micelles in aqueous media, wherein water insoluble segments form the core and hydrophilic segments form the corona. This hydrophobic core allows an effortless encapsulation of several poorly water soluble therapeutic agents, such as corticosteroids. Moreover, polymeric micelles seem to be very promising in ocular drug delivery for many peculiarities, including their ability to act as absorption promoters which might improve drug permeability across ocular epithelia [13,14].

Thus, the aim of this work was to produce novel polymeric micelles to increase corticosteroid water solubility and retention time on the ocular surface as well as to enhance drug absorption through the corneal layer.

In this paper, inulin, a natural occurring polysaccharide, was chosen as a starting polymer because of its biocompatibility and chemical versatility [15–19]. Inulin was functionalized with ethylendiamine (EDA) and retinoic acid (RA), to introduce reactive groups able also to interact with mucin and to give an appropriate hydrophobic chain, respectively. This new copolymer, named INU-EDA-RA, was then used to prepare polymeric micelles encapsulating corticosteroids (dexamethasone, triamcinolone and triamcinolone acetonide). Micelles were characterized in order to investigate their potential as effective ocular drug delivery systems administered topically. In particular, mucoadhesive properties, drug loading and drug release as well as stability were evaluated. In addition, *in vitro* experiments were performed on primary human and murine ocular cells, in order to evaluate their biocompatibility and to study the cellular uptake. Finally, great attention has been given in the assessment of *in vitro* transcorneal permeation using transwell support systems.

2. Materials and methods

2.1. Materials

All solvents and chemicals were of analytical grade and were used without further purification. Inulin (INU), 4-bis-nitrophenyl carbonate (BNPC), Ethylendiamine (EDA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC**.**HCl), N-hydroxysuccinimide (NHS), retinoic acid (RA), Triethylamine (TEA), Dulbecco's phosphate buffer saline (DPBS; composition: KCl 0.2 g/l; KH_2PO_4 0.2 g/l; NaCl 8.2 g/l; Na_2HPO_4 anhydrous 1.15 g/l), dexamethasone (DEX), triamcinolone (T), triamcinolone acetonide (TA), Sephadex G 15 and G 25, pyrene, mucine type III (from porcine stomach), poly acrylic acid (PAA) and Sodium Hydrogen Carbonate were purchased by Sigma-Aldrich. Hydrochloric acid 2 N and sodium hydroxide 0.1 N were purchased by VWR-international. AlexaFluor-NHS_{488} was purchased by Life Technologies.

HEPES buffer solution pH 7.4 simulating ocular fluid was prepared by dissolving HEPES (5.96 g) and NaCl (9 g) in 1 L of bidistilled water and adjusting pH to 7.4 with NaOH 5 M.

Human Corneal Epithelial Cells (HCEpiC) were purchased by Sciencells. Keratinocyte Serum Free Basal Medium and its supplements Bovine Pituitary Extract (BPE) and recombinant human Epithelial Growth Factor (EGF) were purchased by Gibco.

Human Retinal Pigment Epithelial Cells (HRPE), Retinal Pigment Epithelial Cell Basal Medium and its growth supplements L-glutamine, Fetal Bovine Serum (FBS), Basal Fibroblast Growth Factor (FGF-B) and Gentamicin Sulfate-Amphotericin B (GA-1000) were purchased by Lonza.

Murine Primary Cells (Corneal Epithelial cells and Retinal Pigment Epithelial Cells) were obtained through murine eye dissection and enzymatic treatment. Murine corneal epithelial cells were isolated by 12 U/mL Dispase II and 0.25% Trypsin treatments (Sigma-Aldrich). After, the cells were cultured in DMEM-F12 medium supplemented with 10% FBS, 10 ng/mL Epithelial Growth Factor, 2 mM L-glutammine, 100 U/mL Penicillin and 100 μg/mL Streptomycin (Sigma-Aldrich), at 37 °C in 5% CO_2. Murine Retinal pigment epithelial cells were derived from RPE dissociation using a mix of recombinant collagenases (1.7 U/mL ColG and 9.5 U/mL ColH, Abiel s.r.l) and Trypsin treatment (Sigma-Aldrich). The cells were cultured in MEM-alpha modified medium supplemented with 10% FBS, 2 mM L-glutammine, 100 U/mL Penicillin, 100 μg/mL Streptomycin, N1 supplement, non essential ammino acids, 250 mg/L Taurine, 20 μg/L Hydrocortisone and 0.013 μg/L Triiodothyronin (Sigma-Aldrich), at 37 °C and 5% CO_2.

2.2. Methods

2.2.1. Synthesis and characterization of INU-EDA-RA copolymer

INU-EDA-RA copolymer was synthetized by a multistep procedure. First of all, INU-EDA was synthetized by microwaves assisted activation of INU hydroxyl groups (via 4-BNPC) and then reaction with a large excess of EDA, as reported in a previously published article [20]. Secondly INU-EDA was additionally functionalized with retinoic acid (RA). RA (78 mg; 0.26 mmol) was dissolved in DMF_a (6 ml) and then NHS (45 mg; 0.39 mmol), EDC**.**HCl (75 mg; 0.39 mmol) and TEA (35 μl; 0.25 mmol) were added in order to activate the carboxyl group of RA. The reaction was carried out at 25 °C for 4 h, in the dark. Afterwards, activated RA was added to INU-EDA (300 mg; 1.75 mmol) previously dissolved in DMF_a (12 ml) and the reaction mixture was kept at 25 °C overnight (~18 h) under continuous stirring in the dark. Subsequently, the crude product was precipitated in diethyl ether/dichloromethane mixture 2:1 (v/v) and the obtained powder was then washed three times in diethyl ether/dichloromethane mixture 2:1 (v/v) in order to remove the excess of RA not reacted. After that, the obtained solid product was solubilized in bidistilled water and then purified by dialysis (SpectraPore RC; cut off: 1 kDa).

Yield 85%, referred to the starting INU-EDA.

INU-EDA and INU-EDA-RA derivatives were characterized by spectroscopic analyses, such as ^{1}H NMR, UV–VIS and ATR-FTIR measurements.

^{1}H NMR spectra were recorded using a Bruker Avance II 300 spectrometer operating at 300.12 MHz.

UV–VIS spectra were recorded on a 2401 PC Shimadzu Recording Spectrophotometer UV. Data were collected in the 600–200 nm spectral range. INU-EDA and INU-EDA-RA aqueous solutions were analysed at a fixed concentration (0.1 mg/ml) lower than INU-EDA-RA Critical Aggregation Concentration value (see below).

ATR-FTIR spectra were recorded on a Fourier Transform Infrared Spectrometer (FTIR) (Bruker Alpha instrument) equipped with a ATR unit for surface analysis. Spectra were collected by accumulation of 32 scans in the 4000–600 cm^{-1} spectral range and rationed to the appropriate background spectra.

2.2.2. Determination of critical aggregation concentration (CAC)

The self-assembling ability of INU-EDA-RA was evaluated by a RF-5301 PC Shimadzu spectrofluorimeter in bidistilled water, DPBS

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