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Differentially cleaving peptides as a strategy for controlled drug release in human retinal pigment epithelial cells

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ABSTRACT

Currently, drug delivery to the posterior eye segment relies on intravitreal injections of therapeutics. This approach requires frequent injections and does not guarantee drug delivery to intracellular targets. Controlled release systems and nanoparticles are being investigated to mitigate these challenges but most of these approaches lack translational success to the clinics. In our present study, we report a peptide-based delivery system that utilizes enzyme assisted cleavable linkers to release conjugated cargo within the retinal pigment epithelial (RPE) cells. Peptide linkers with differential cleavage rates were developed and tested in the vitreous humor, RPE cell homogenates and intact RPE cells. Selected peptide linkers were conjugated to cell penetrating peptides and D-peptide cargoes. The peptide-based delivery systems were non-toxic to the RPE cells, chemically stable in porcine vitreous and delivered cargo prototypes (hydrophobic & hydrophilic) to the RPE cells. Importantly, we show quantitatively with LC/MS analytics that the intracellular cargo release is controlled by the sequence of the peptide linker. The controlled cleavage of the peptide linkers is not only a useful strategy for intracellular drug delivery to the RPE targets but might also be useful in utilizing the RPE cells as mediators of drug delivery to intracellular targets and surrounding tissues (such as neural retina and choroid).

1. Introduction

Drug delivery to the posterior segment of the eye is a major challenge. This is due to the unique anatomy and physiological barriers in the eye. Topical eye drop administration does not deliver adequate drug concentrations to the retina whereas systemic administration is hampered by the blood retina-barrier [1]. For these reasons, invasive intravitreal injections are commonly used to deliver Fab-fragments, IgG based antibodies, corticosteroids and soluble receptors in the treatment of the posterior segment of the eye [2–5]. Sub-conjunctival, suprachoroidal, and periocular routes have been proposed as alternatives to intravitreal injections [6–9], but these approaches do not result in effective retinal drug delivery [10]. In the case of the sub-retinal route, the technique is too demanding for wide clinical use [11].

New retinal therapeutics are being widely explored as most retinal diseases are still without effective treatment. Retinal drug discovery includes small molecular drugs, proteins and nucleotide-based gene products. In many cases, the drug targets are located in intracellular compartments [12,13]. For example, pathological changes (protein aggregation, oxidative stress, inflammation) take place within the retinal pigment epithelial cells in diseases such as the dry form of age-related macular degeneration [14–16]. Unfortunately, most nucleotide and peptide-based compounds have poor permeability into the target cells [17,18]. Intracellular drug delivery systems are needed for the development of effective retinal treatments. Nanoparticle based formulations, such as liposomes and polymeric nanoparticles have been used to augment the intracellular delivery of biologicals [19–21] but intracellular drug release from particulate based systems is often poorly controlled [12,22]. Furthermore, access of the nanoparticles to target cell layers in the retina is also problematic, especially in the case of cationic nanoparticles that are usually used for intracellular delivery of DNA and RNA based drugs [23]. These formulations tend to stick to the vitreous or inner limiting membrane of the retina [24,25].

The major challenges in the retinal drug delivery are (a) prolongation of the intravitreal dosing interval; (b) intracellular delivery of peptide and nucleotide based drugs (e.g. oligonucleotides, proteins); (c) replacement of intravitreal injections with less invasive modes of drug delivery. Clinical VEGF-A antagonists (treatment of wet AMD) such as IgG antibodies (bevacizumab), Fab-fragment (ranibizumab), and soluble VEGF receptor (aflibercept) [26–28] are injected intravitreally once a month or bi-monthly. Most other drugs (e.g. small molecules) cannot

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be given as intravitreal injections because they are rapidly eliminated from the posterior eye segment necessitating injections at too short intervals to be clinically feasible [29]. To mitigate such issues, controlled drug delivery systems are very much desired for intravitreal treatments. Currently investigated systems include polymeric implants, microspheres and encapsulated cells [30–33]. Intravitreal delivery of implants and microspheres is invasive and material toxicity hampers the development of many systems [34]. Furthermore, these systems lack the inherent ability to deliver the cargo into the target cells.

We aimed to generate a peptide-based drug delivery system that is based on adequate intracellular access and controlled cargo release within the RPE cells. The delivery system is intended for intravitreal administration. Other possible routes of delivery could be suprachoroidal and periocular. Furthering on the idea of using endogenous components to trigger intracellular release, we have explored enzyme-assisted 'peptide-based cleavable linkers' (PCLs) to control cargo release within the RPE cells. Cathepsin D, a lysosomal enzyme, has relatively high expression levels in RPE cells [35]. Consequently, peptide sequences sensitive to cathepsin D were selected as PCLs. The *N*-terminus of the PCLs was conjugated with cell penetrating peptides (CPPs) and D-pentapeptide cargoes were conjugated at the *C*-terminus. CPPs are charged peptide sequences capable of intracellular delivery of molecular cargo [36,37]. D-Pentapeptide cargoes were chosen for ease of synthesis, resistance to proteolytic cleavage, convenient detection and easy manipulation of the sequence to modify physicochemical properties of the cargo prototypes (hydrophobic/hydrophilic). Controlled drug delivery from the intracellular depots was demonstrated and quantitated with LC/MS. The design and strategy of the approach are depicted in Scheme 1.

2. Materials and methods

2.1. Peptide synthesis

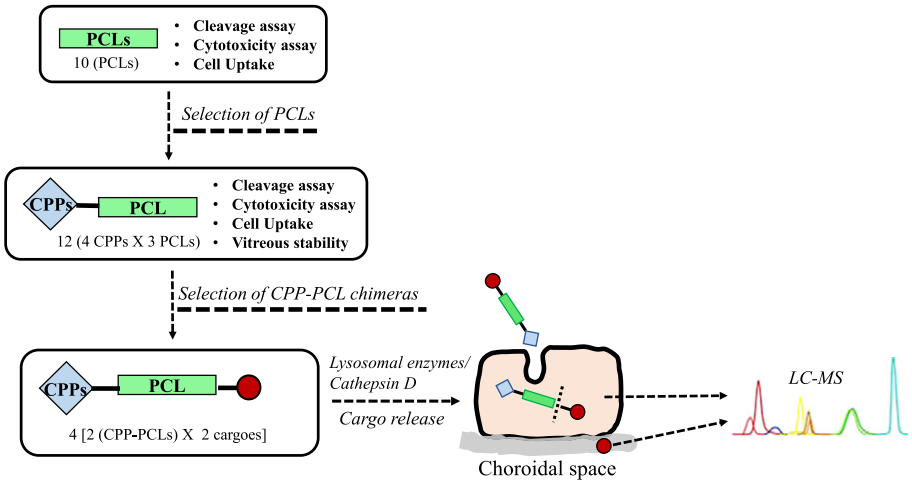
All the PCLs containing both natural and unnatural amino acids (Tables 1, 3); CPP-PCL chimeras (Scheme 2); cargo conjugates (CCs, Table 2) and labeled cargo fragments (Table 2) used in this study were synthesized by Peptide Synthetics-Peptide Protein Research Ltd. (Hampshire, UK). In general, the peptides were synthesized using solid phase peptide synthesis via standard Fmoc chemistry [38] in a 0.1 mmol scale using a Symphony automated peptide synthesizer (Protein Technologies, Arizona). Internally quenched oligopeptide sequences (Scheme 3) contained the fluorescence resonance energy (FRET) pair EDANS/Dabcyl (DABCYL: 4-{[4-(dimethyl amino) phenyl] azo} benzoic acid; EDANS: 5-[(2-aminoethyl) amino] naphthalene-1-sulfonic acid). Spectral properties of EDANS/Dabcyl are shown in Fig. S1 (Supplementary material). For FRET, the donor and acceptor moieties must be in close proximity, typically 10–100 Å. The typical förster radius (R_0) value for EDANS/Dabcyl pair is 33 Å [39]. Considering the axial distance between the alpha carbon atoms of two adjacent residues to be about 3.5 Å; EDANS and Dabcyl moieties were placed 10–13 residues apart in all the oligopeptides (PCLs: Table 1; PCL analogs: Table 3; CPP-PCL chimeras: Scheme 2). The peptides were purified via Prep RP-HPLC using a Varian system comprising of two 210 pumps, a 320 UV dual wavelength detector and Varian star software employing gradients between the mobile phases, water (0.1% TFA) and acetonitrile (0.1% TFA); equipped with a C18 Gemini Axia column (5 μm particle size, 110 Å pore size, 200 mm by 21 mm, Phenomenex). Fractions containing the desired peptide as indicated by UV absorbance were collected manually; analyzed by LC-MS (Agilent 1100 systems equipped with Aeris core shell column, 100A 150 × 4.6 mm) to determine the purity and freeze dried.

2.2. FRET-based peptide cleavage assay

An assay to determine the cleavage rates of the synthesized oligopeptides with FRET pair EDANS/Dabcyl was carried out using either purified enzyme (cathepsin D) or ARPE-19 cell lysate at oligopeptide concentration of 20 μM. The assay was performed in 96-well black plates in 0.1 M sodium acetate buffer pH 4.0 (100 μl). Briefly, ARPE-19 cells were lysed using lysis buffer (Biovision, California, USA) and lysates were centrifuged at 10,000*g* for 10 min at 4 °C and the supernatant was used for the enzymatic assay. Oligopeptide sequences containing the FRET pair were mixed with cell lysate supernatant and the reaction mixture was incubated at 37 °C. The substrate cleavage was monitored by measuring the fluorescence as a function of time (excitation 340 nm; emission 490 nm) using a microplate fluorescence reader (Varioskan Flash, Thermo Scientific). 100% cleavage was assessed based on the fluorescence obtained from an equimolar free concentration of EDANS (the chromophore present in oligopeptides).

2.3. Cell culture

ARPE-19 cells (human retinal pigment epithelial cell line, ATCC CRL-2302) were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Gibco). The cells were cultured in a T-75 flask at 37 °C in a



Scheme 1. Overview of design and experimental set up of the peptide based cargo delivery system.