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Research Paper

Evaluation of protein drug stability with vitreous humor in a novel *ex-vivo* intraocular model

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ABSTRACT

The stability of protein therapeutics during the residence time in the vitreous humor (VH) is an important consideration for intra ocular treatment and can possibly impact therapeutic efficacy and/or treatment intervals. Unavailability of the reliable *Ex-vivo* intravitreal (ExVit) model to estimate protein stability following IVT has driven the research focus to develop such model which can facilitate protein stability estimation before *in-vivo* experiments. In this manuscript, we have developed and evaluated three ExVit models, namely, ExVit static, semi-dynamic and dynamic. These models were utilized and compared when studying the *in-vitro* stability of model protein formulations under simulated intraocular conditions using porcine vitreous humor (VH). The ExVit static model exhibited significant precipitation and aggregation of proteins, most likely due to pH change occurred in the VH after isolation. The semi-dynamic model assessed was composed of two compartments i.e., VH- and buffer-compartment which has effectively stabilized the pH of the VH and facilitated the migration of VH degradation products. However, some limitations related to investigation of long-term protein stability were also observed with semi-dynamic model. The dynamic model developed, was comprised of three diffusion controlling barriers (two diffusion controlling membranes and a gel-matrix), which allowed modulation of the diffusion rate of macromolecules. The ability of dynamic model to modulate protein retention time in the VH will overcome the challenges faced by the semi-dynamic model such as long-term stability evaluation.

1. Introduction

The breakthrough discovery of Lucentis® has offered significant advancements in the treatment of posterior segment vision threatening disorders such as wet age-related macular degeneration (AMD) and diabetic retinopathy (DR). Further, ocular injectables have been marketed or are currently in clinical development in order to offer alternative superior treatment options. Biologics for ocular treatment are, to date, delivered as intravitreal (IVT) injection which is the most promising way to achieve sufficient therapeutic concentrations of biologics in the vitreous humor (VH) and retina. After IVT injection, these macromolecules (pegaptanib and ranibizumab) are eliminated from the eye primarily via the systemic circulation, resulting in vitreous half-lives of approximately 9–10 days [1–3].

The VH is an aqueous medium composed of water (98–99%) with smaller quantities of positively charged protein such as collagen stabilized with the negatively charged proteoglycans (hyaluronic acid and chondroitin sulfate) [4]. The resulting matrix gives a mechanical support to VH [5]. Although the complete proteome of the human vitreous has not yet been fully characterized, human vitreous is known to contain over 50 proteins in very low concentrations with more than 35 of which are not even detected in plasma [6]. The presence of various proteolytic enzymes such as plasmin and the different members of matrix metalloproteinase family (MMPs) was also reported in the VH [6]. In addition, the VH comprises of traces of urea, creatinine, xanthine and hypoxanthine [5,7,8]. Under pathological conditions the concentration of various proteins in the VH can increase [9–11]. For instance, in diabetic macular edema (DME) elevated levels of PEDF, ApoA-4, ApoA-1, Trip-11, PRBP, and VDBP have been observed [6,10]. Also, during the normal aging process and/or under pathological conditions, the interactions between the collagen and hyaluronic acid deteriorate which eventually results in the liquefaction of the VH [12,13]. The mechanism of age-related irreversible loss of VH consistency is still not fully understood.

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Due to the unique nature of VH (with multiple components), the stability of protein therapeutics during the residence time in the VH is an important consideration and can potentially impact therapeutic efficacy and treatment intervals. Apart from the stability of the protein, it is also of great interest to understand possible interaction with components of the VH that may be of relevance. However, to date, it is difficult to predict the *in-vivo* behavior of protein formulations following IVT administration. In general, intravitreally administered formulations are expected to undergo rapid dilution in the VH with typically not more than 100 μL of volume injected in ~4 mL of the VH (human VH volume) [14]. This is followed by fast clearance of smaller molecular weight (MW) components, such as formulation excipients (due to high diffusivity in VH) compared to large molecules [15–17]. This leaves the active protein exposed to the VH environment, leading to potential interactions with the components of VH. To the best of our knowledge, there are no *in-vitro* models simulating *in-vivo* ocular conditions available which can investigate formulation stability and performance in the VH.

Few groups have reported computational models to study the diffusion of drug and/or excipients in the VH [17,18]. However, these mathematical models only provide limited information about the diffusivity, and do not consider or discuss the stability and interactions of proteins/excipients with the VH components. Additionally, a number of reports describing *in-vivo* and *ex-vivo* transport experiments on the diffusion behavior of small and large molecules in the direction of VH to choroid [19–22] as well as choroid to VH [23] are available. However, *ex-vivo* transport experiments are limited in the duration of the experiment to a few hours only, in order to maintain tissue integrity (retina/choroid). Additionally, it is inconvenient, time consuming and expensive to incorporate *in-vivo*/*ex-vivo* experiments to evaluate stability during various steps of technical development. Furthermore, it is helpful to understand the protein stability following IVT injection as a function of time. Therefore, experimental *in-vitro* models mimicking ocular conditions are of interest to investigate the stability of protein formulations following IVT administration. In this manuscript, we have described and examined three *Ex-vivo* intravitreal (ExVit) models, namely, (a) an ExVit static model, (b) a semi-dynamic model and (c) a dynamic model, to assess protein stability. Three protein molecules, namely, monoclonal antibody 1 (MAb1) and monoclonal antibody 2 (MAb2) and Fab2 fragment (of a full length antibody) (FAb1) were used as model proteins. Different formulations of MAb1 were also examined using these models.

In the current manuscript, the limitations of static and semi-dynamic models have been discussed and the short-comings of these models are addressed by developing the dynamic model.

2. Materials and methods

2.1. Materials

Porcine eyes were procured from a local slaughter-house. Vivaspin 15 (50 kDa MWCO, cat # VS-1532) centrifugal concentrators were purchased from Integra Biolab (Aguadilla, Puerto Rico). FITC-Dextran 4 kDa (Cat # 46944), 40 kDa (Cat # FD40), 70 kDa (Cat # 90718), FITC-BSA (Cat # A9771) and FITC-IgG (Cat # F9636) were purchased from Sigma–Aldrich (St. Louis, USA). Hyaluronic acid (1.4×10^3 kDa) (cat # HA15 M) was acquired from Life Core Biomedical, Inc. (Minnesota, USA). Dialysis membranes with molecular weight cut-off (MWCO) of 50 kDa (cat # 131384) and MWCO of 100 kDa (cat # 131420) were purchased from Spectrum lab (California, USA). All other chemicals utilized in this study were of analytical grade. MAb1 and MAb2 were manufactured in CHO cells and provided by F. Hoffmann-La Roche AG, Basel. FAb1 was produced in *E. coli* and also provided by Roche. MAb1 and FAb1 were formulated in Histidine buffer while MAb2 was formulated in phosphate buffer at optimal pH to provide maximum stability. All formulations contained sugars to adjust tonicity and surfactant to provide additional stability. Different concentrations of MAb1 were prepared by diluting the stock solution with the placebo formulation. In all the experiments, buffer compartment always contained phosphate buffer saline (PBS). Hyaluronic acid, FITC-Dextrans (4, 40, 70 kDa), FITC-BSA and FITC-IgG solutions were prepared in PBS. Further details are provided in respective experimental/method section.

2.2. Methods

2.2.1. Isolation of pig vitreous humor

All the experiments were performed with the VH isolated from porcine eyes. Briefly, eyes were kept on ice bath during the isolation of VH. The eyes were opened by incision, with the dissecting knife and the clear VH was removed using a disposable syringe (without needle). Isolated VH was sterile-filtered using a 0.22 μm filter to remove cellular debris and any microbial contamination. It was stored at −70 °C to avoid any possible metabolic activities until further use. All the experiments were performed according to the ARVO statement for the use of animals in ophthalmic and vision research.

2.2.2. Experimental setup for different ExVit models

2.2.2.1. Static model. The ExVit static model (Fig. 1A) is a relatively simple one-compartment-model, which involves the isolated filtered porcine VH being aseptically transferred in the sterile glass vial. Defined amounts of test protein solutions or test formulations were administered in the VH. Vials were incubated at 37 °C and samples were collected at predetermined time intervals. Samples were analyzed by various analytical techniques such as, size exclusion chromatography (SEC), pH, viscosity, turbidity, osmolality and also microscopy.

2.2.2.2. Semi-dynamic model. The semi-dynamic model (Fig. 1B) is comprised of an internal and external-compartment. The internal-compartment contained 2 mL of filtered porcine VH and the external-compartment comprised of phosphate buffer saline (0.01 M PBS). Both compartments were separated by a membrane with 50 kDa MWCO which was considered due to being close to the retinal exclusion limit (REL) (~70 kDa) [22]. This experimental setup allowed the system to maintain the pH of the VH constant at physiological pH and also eliminated the VH degradation products (with smaller MW than the chosen 50 kDa cutoff) into the PBS reservoir throughout the study period. The devices were capped and tightly sealed with parafilm, and incubated for 24 h at 37 °C, which permitted the conditioning and equilibration of VH. Following 24 h of incubation, test samples (protein solution or various formulations) were administered in the VH-compartment, and devices were sealed and incubated at 37 °C for a predefined time period. Samples were collected from the VH-compartment and PBS-compartment, and analyzed by SEC, pH, viscosity, turbidity, osmolality and also microscopy.

2.2.2.3. Dynamic model. The dynamic model (Fig. 1C) was comprised of three compartments: the VH-compartment, a gel-matrix (GM) compartment and a flow-through (FT) compartment. Here, the FT-compartment was utilized as a buffer reservoir to ensure maintenance of the pH of the VH. The VH-compartment and FT-compartment were separated by the GM-compartment, which acted as a diffusion controlling barrier. Two dialysis membranes were utilized to separate all three compartments. The first