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Research paper

Prediction of intraocular antibody drug stability using *ex-vivo* ocular model

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ABSTRACT

Following intravitreal (IVT) injection, therapeutic proteins get exposed to physiological pH, temperature and components in the vitreous humor (VH) for a significantly long time. Therefore, it is of interest to study the stability of the proteins in the VH. However, the challenge posed by the isolated VH (such as pH shift upon isolation and incubation due to the formation of smaller molecular weight (M_W) degradation products) can result in artefacts when investigating protein stability in relevance for the actual *in vivo* situation. In this current study, an *ex-vivo* intravitreal horizontal stability model (ExVit-HS) has been successfully developed and an assessment of long-term stability of a bi-specific monoclonal antibody (mAb) drug in the isolated VH for 3 months at physiological conditions has been conducted. The stability assessment was performed using various analytical techniques such as microscopy, UV visible for protein content, target binding ELISA, Differential Scanning Calorimetry (DSC), Capillary-electrophoresis-SDS, Size Exclusion (SEC) and Ion-exchange chromatography (IEC) and SPR-Biacore. The results show that the ExVit-HS model was successful in maintaining the VH at physiological conditions and retained a majority of protein in the VH-compartment throughout the study period. The mAb exhibited significantly less fragmentation in the VH relative to the PBS control; however, chemical stability of the mAb was equally compromised in VH and PBS. Interestingly, in the PBS control, mAb showed a rapid linear loss in the binding affinity. The loss in binding was almost 20% higher compared to that in VH after 3 months. The results clearly suggest that the mAb has different degradation kinetics in the VH compared to PBS. These results suggest that it is beneficial to investigate the stability in the VH for drugs intended for IVT injection and that are expected longer residence times in the VH. The studies show that the ExVit-HS model may become a valuable tool for evaluating stability of protein drugs and other molecules following IVT injection.

1. Introduction

The vitreous humor (VH) is a gelatinous material, largely composed of water (98–99%). The remaining constituents (1–2%) are mainly collagen, hyaluronic acid, chondroitin sulfate, proteins and inorganic components such as lactate, xanthine, hypoxanthine, creatinine and urea [1–4]. The positively charged collagen and negatively charged proteoglycans generate a rigid matrix which offers mechanical strength to the VH and helps to maintain the shape of the eye ball. The soluble proteins of VH include albumin, growth factors, matrix metalloproteinase, hyaluronidase, and various other enzymes [5]. Over 50 proteins are reported in the human VH, although, the detailed proteome is yet to be understood. Moreover, out of these identified proteins, around 35 proteins are specific to the VH and not found in the plasma [5]. In the posterior segment, disease conditions such as wet-age related macular degeneration (wAMD), diabetic retinopathy (DR) and diabetic macular edema (DME), show variations in the levels of different proteins of the VH and are extensively reported [6–8]. In addition, increasing age of patients coincides with significant changes in the composition and liquefaction of VH [9,10].

Protein drugs such as ranibizumab (50 kDa) and aflibercept (97 kDa) are routinely used for the treatment of certain back of the eye diseases. These therapeutic proteins are injected intravitreally and eliminated from the VH, mainly, via the systemic circulation with vitreal half-lives of ca 9–10 days [11–13]. Due to the chronic nature of these diseases, relatively short half-life and lim-

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ited intravitreal (IVT) injection volume, multiple doses of these protein drugs have to be administered. Many organizations are exploring multiple ways to reduce the frequency of injection and improve patient compliance by developing high concentration protein formulations and/or sustained delivery systems [14]. Generally, protein formulations are stabilized by many low molecular weight (M_W) excipients such as sugar, surfactant, salt and buffer systems with a pH between 5 and 7 [15]. It is well understood that proteins can be destabilized with a pH change [16,17], increase in temperature [18], and upon dilution of stabilizing excipients [19]. Following an IVT injection of typically 25–100 μL into 4–5 mL of the VH, the formulation is diluted and stabilizing excipients are taken away due to their relatively rapid diffusion and increased elimination from the VH compared to the large M_W proteins. Eventually, active therapeutic protein remains exposed in the VH for an extended period of time, possible up to 1–2 months, at physiological pH, and temperature, and in the absence of stabilizing excipients. In addition, the effects of the VH components on the protein stability are also unknown. Therefore, it becomes very important to understand the fate of the active protein during its presence in the vitreous cavity.

It is very difficult to study the stability of protein within the vitreous cavity using the *in vivo* models, primarily due to lack of analytical techniques and the involvement of a large group of animals. Typically, the *in vivo* set-up only permits stability investigation for limited half-lives (2–3) of therapeutic protein (ca $\leqslant$1 month) after which the concentration of drug in the VH drops significantly making it very difficult to assess physicochemical changes. Therefore, the best alternative is the *ex-vivo* stability study where the protein can be incubated in the isolated VH sufficiently long to assess potency and physicochemical changes. In our previously published manuscript, we have compared three different *ex-vivo* intravitreal (ExVit) models (static, semi-dynamic and dynamic) to study protein stability in the isolated porcine VH, and established a proof-of-concept that the dynamic ExVit model is superior to others [20]. In this study, we have adapted a design of the dynamic ExVit model using horizontal chamber to increase protein retention time in the VH and studied the physicochemical stability of a bi-specific mAb in an isolated VH under physiological conditions (pH 7.4, 37 °C) for 3 months.

2. Materials and methods

2.1. Materials

Porcine eyes were acquired from a local slaughter-house located near Zürich, Switzerland. Custom made side-by-side diffusion chambers were purchased from SES GmbH-Analytical Systems (Bechenheim, Germany). A diffusion controlling membrane, with molecular weight cut-off (MWCO) of 50 kDa (cat # 131384), was procured from Spectrum lab (California, USA). Bispecific mAb (mAb) was manufactured in a CHO cell line and provided by F. Hoffmann-La Roche AG, Basel. MAb was formulated in histidine buffer (20 mM, pH 6.0) containing sugar and surfactant to provide tonicity and stability, respectively.

In all the experiments, phosphate buffer saline (PBS, pH-7.4) was used in the buffer-compartment. All other reagents utilized in these studies were of analytical grade.

2.2. Methods

2.2.1. Isolation of the vitreous humor (VH)

Porcine eyes were opened with the incision placed near the conjunctiva using a dissecting knife and the clear VH was collected with the disposable syringe without a needle. The VH was then sterile-filtered through a 0.22 μm filter to ensure removal of any microbial contamination and cellular debris. The VH was stored (in small aliquots) below −70 °C to avoid possible metabolic activity and degradation or change in VH. Throughout the process of VH isolation, eye balls were kept in an ice-bath. All the experiments were performed according to the Association of Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research.

2.2.2. Experimental set-up for the ExVit-HS model

The ex-vivo intravitreal horizontal stability model (ExVit-HS) is a two compartment model, VH-compartment and buffer-compartment. As demonstrated in Fig. 1, two compartments were separated by a diffusion controlling membrane with MWCO of 50 kDa. The buffer-compartment was always filled with sterile PBS (10 mM, pH 7.4) whereas the VH-compartment was filled with the sterile porcine VH. The devices were sealed with a sterile Teflon® cap and incubated overnight at 37 °C (MaxQ-4000 incubator, Thermo Scientific). Following incubation, PBS from the buffer-compartment was replaced with fresh sterile PBS (pre-incubated at 37 °C). Simultaneously, 50 μL (120 mg/mL) of mAb was injected in the VH-compartment. Devices were sealed and incubated at 37 °C for the defined time intervals i.e., 1 h, week-1, week-2, week-4, week-8 and week-13. The VH-compartment, without mAb, was considered as negative control. Also, to investigate the effect of pH and temperature on the stability of the mAb, the VH-compartment, filled with PBS, containing 50 μL of mAb was used as control. At defined time intervals, samples were aseptically transferred from both the compartments in sterile glass vials. The performance of the model was evaluated by estimating the pH, osmolality and total protein concentration in both VH and buffer-compartments. Samples were further evaluated to investigate physical stability, chemical stability and binding affinity of mAb.

2.2.3. Evaluation of model performance

2.2.3.1. Estimation of pH. The pH was investigated at each time intervals following incubation at 37 °C. Briefly, 50 μL of the samples were collected from the VH- and buffer-compartments, from the controls (VH alone, and buffer + mAb) and test article (VH + mAb). The samples were aseptically transferred in eppendorf tubes and analyzed by calibrated pH meter (827 pH Lab, Metrohm, Switzerland). Results were plotted for the time interval *vs* pH unit.

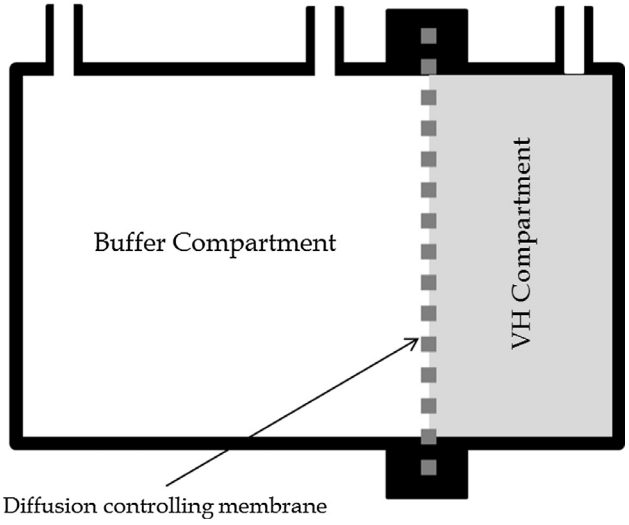

Fig. 1. *Ex-vivo* intravitreal horizontal stability model diagram (ExVit-HS).

2.2.3.2. Concentration of mAb in VH-compartment. Total concentrations of mAb in the VH- and buffer-compartments were estimated by two different analytical methods, UV–visible spectroscopy and Micro-BCA™. For the UV–visible analysis, 5 μL of the samples (controls and test) were placed in NanoDrop™-2000C (Thermo Scientific) and the analysis was performed at 280 nm. In order to achieve true absorbance of the mAb, the absorbance of test samples (VH + mAb) were normalized using the absorbance of a negative control (VH alone) at 280 nm. The concentration of mAb was then calculated from the linearity equation acquired from the standard curve of mAb (50–3000 μg/mL). Results were plotted for the time *vs* amount of mAb. To further confirm the results, the same samples were also analyzed by Micro-BCA™ total protein estimation assay. The analysis was performed as per the protocol suggested by the supplier (Thermo Scientific). The standard curve of mAb was prepared between the range of 1.25–40 μg/mL and the normalized absorbance values were utilized to calculate the total mAb concentration in the VH- and buffer-compartments. Samples were analyzed at 562 nm using a spectra-max 96-well plate reader.

2.2.3.3. Estimation of osmolality. Osmolality of the control VH and test (VH + mAb) were estimated as per the protocol suggested within the European Pharmacopoeia (EP). Before the analysis, the osmometer (based on the principle of freezing point depression (Osmomat 030 3P Cryoscopic Osmometer)) was calibrated in the range of 0–0.500 Osmol/kg. Following calibration, 50 μL of the samples were placed in the osmometer and the osmolality was evaluated. Results were plotted for the time *vs* Osmol/kg.

2.2.4. Evaluation of physical stability of mAb

Samples were evaluated for the physical stability of mAb by various analytical techniques e.g., microscopically, HIAC liquid particle counter, differential scanning calorimetry (DSC), size exclusion chromatography (SEC) and capillary electrophoresis sodium dodecyl sulfate-non gel sieving (CE-SDS-NGS).

2.2.4.1. Micro-differential scanning calorimetry (DSC). To evaluate the physical stability of the protein in the matrix, samples were evaluated for the melting temperature and melting point onset by differential scanning calorimetry (DSC, VP-DSC MicroCalorimeter (MicroCal™)). Briefly, 300 μL of samples (controls and test samples) were injected into the DSC cell equipped with auto injector (MicroCal, LLC) and the temperature was raised from 10 °C to 110 °C at the rate of 1 °C/min. The data was processed with Origin-7 software and the resulting thermograms were plotted for kCal/mole/°C *vs* temperature (°C).

2.2.4.2. Microscopic evaluation of insoluble particles. Proteins are generally very sensitive towards the physical, chemical and environmental stress which can easily generate soluble and insoluble particles (large aggregates). To evaluate insoluble particles, at each time intervals 2 mL of the sample was transferred aseptically in sterile FTU tubes. The samples were then assessed microscopically (Keyence VHX-600 digital microscope) for the presence of visible particles. Additionally, opalescence was then evaluated.

2.2.4.3. Estimation of sub-visible particles. In order to evaluate sub-visible particles ($\geqslant$2, $\geqslant$5, $\geqslant$10 and $\geqslant$25 μm per mL), samples (negative control, buffer + mAb and VH + mAb) were analyzed by HIAC liquid particle counter (Hiac Royco 3000A, Pacific Scientific, United Kingdom). Briefly, 1.8 mL of each sample was transferred into a sterile, particle free FTU tube. Samples were then analyzed as per the protocol documented in the EP. The number of particles for each different size range were calculated and reported in the graph (particle size *vs* particle count).

2.2.4.4. Size exclusion chromatography (SEC). The level of fragments and soluble aggregates of mAb were assessed using size-exclusion chromatography (SEC) coupled with a UV–visible detector. The SEC method was developed in-house for the estimation of mAb. Briefly, 50 μL of samples (controls and test specimens) were injected into the separation column procured from Tosch Bioscience (TSK gel, G3000SWXL, 7.8–300 mm, 5 μ). The separation of aggregates and fragments were carried out at a flow rate of 0.5 mL/min using 0.2 M phosphate buffer (pH-7.0) as a mobile phase. UV–visible detection was performed at 280 nm with a Waters-2489 detector (Water Corp. MA, USA). Analysis was executed on a Waters 2695 HPLC (Waters Corp. MA, USA) and the data was processed utilizing Empower-2 software.

2.2.4.5. Capillary electrophoresis sodium dodecyl sulfate-non gel sieving (CE-SDS-NGS). Levels of soluble aggregates and fragments in the stability samples were further confirmed by the CE-SDS-NGS. CE-SDS-NGS analysis was performed under both reducing and non-reducing conditions. Before analysis (with the Beckman Coulter Capillary Electrophoresis System Proteome Lab PA800), samples were incubated with SDS (and Dithiothreitol (DTT) in reducing conditions) for 5 min at 70 °C. The capillary was rinsed at 70 psi with 0.1 mM NaOH (5 min), 0.1 mM HCl (1 min) and deionized water (1 min). The SDS MW gel buffer was loaded in the capillary at 50 psi. Reduced and non-reduced samples were then injected electrokinetically at 10 kV and analysis was carried out at 15 kV. The data was processed using 32-Karat software. The area of the main peak, aggregates, fragments, light-chain and heavy-chain were calculated and the results were plotted as peak area (%) *vs* time.

2.2.5. Evaluation of chemical stability of mAb

Protein-L affinity chromatography (GenScript, Cat # L00239) was performed to isolate mAb from the VH matrix. MAb isolation was carried out as per the suppliers protocol. Briefly, sodium phosphate buffer (20 mM Na_2HPO_4, 0.15 M NaCl, pH 8.0) was used as wash buffer whereas 0.1 M glycine (pH 2.5) was utilized as elution buffer. Following elution, the pH of the eluent was immediately neutralized with 1 M Tris-HCl, pH 8.5. The resulting purified mAb was analyzed by SEC to monitor the presence of any contaminants from the VH. The extracted mAb (negative control, buffer + mAb and VH + mAb) was characterized by following methods to observe the chemical modifications.

2.2.5.1. Ion exchange chromatography (IEC). Changes in the mAb charge variants were examined using a weak cation exchange chromatography. Separation of charge variants was carried out on a ProPac WCX-10 analytical cation exchange column (4.0 × 250 mm; Dionex Softron GmbH, #054993) by applying a step gradient of eluent-A (20 mM histidine/histidine HCl buffer, pH 6.0) and eluent-B (20 mM histidine/histidine HCl buffer, 1 M NaCl, pH 6.0) at a flow rate of 1 mL/min. Prior to injection, samples were pre-treated with carboxypeptidase-B (5 mg/mL, Roche Diagnostics GmbH, Cat # 1010323300) for 2 h at 37 °C and 50 μg of mAb was injected for the chromatographic analysis. Analysis was performed on a Waters 2695 HPLC (Waters Corp. MA, USA). Separation of charged species was monitored at 280 nm and the data was processed utilizing Empower software. Charge variants were grouped as pre-peak, acidic species, main peak and basic species. Results were plotted for the main peak (area %)/acidic variants (area %)/basic variants (area %) *vs* time intervals.

2.2.6. Evaluation of binding affinity of mAb by surface Plasmon Resonance (SPR) Biacore

The specific interaction of mAb stability samples with their target antigens was assessed using a SPR-Biacore T100/T200 instru-

ment (GE Healthcare). The bridging SPR affinity assay was performed to evaluate the interactions between mAb and target antigens. Briefly, a Biacore CM5-biosensor chip was equilibrated at room temperature followed by target antigen-1 immobilization via standard amine coupling to achieve a coupling density of >3000 RU. The analysis was performed at room temperature using PBS as the running and dilution buffer. The mAb stability samples (extracted from the VH/PBS) were injected at a flow rate of 5 μL/min, at room temperature with association time of 60 s followed by 60 s of dissociation period. Final step involved the injection of an antigen-2 solution at a flow rate of 5 μL/min, with an association and dissociation phase of 30 s and 120 s, respectively. The chip surface was regenerated by the 30 s (5 μL/min) injection of 10 mM glycine buffer (pH 2.0). The relative binding efficiency of the mAb was estimated by comparing the response with the reference standard. The binding affinity of individual binding domain to their respective antigens was also evaluated and plotted against the time. The activity of the reference material was considered to be 100%.

3. Results

3.1. ExVit-HS model performance

3.1.1. pH stabilization of VH

In-vivo, the VH is always buffered to physiological pH. Therefore, in order to mimic the long-term stability of mAb, it is very important to maintain the pH of the isolated VH to physiological values [20]. We can clearly depict from the data shown in Fig. 2, that the ExVit-HS model developed in our lab has efficiently and constantly maintained the physiological pH of the VH throughout the study period of 3 months. The VH remained unchanged at pH values of 7.4 ± 0.1 for the controls. As expected, even after addition of 50 μL of a pH 6.0 mAb formulation, the pH of VH did not change. These data suggest that the model can be utilized to investigate protein stability in the isolated VH for the duration of 3 months.

3.1.2. Diffusion and loss of mAb from VH in ExVit-HS

The concentration of mAb in the VH-compartment was measured by various analytical techniques mainly UV–visible spectroscopy, Micro-BCA™ and ELISA, which provided consistent and comparable results. As shown in Fig. 3, almost 50% of the protein diffused out of the VH- into the buffer-compartment during the 3 month study period. The rate of diffusion was non-linear with approximate 25% loss in the first 4 weeks and 25% in next 8 weeks.

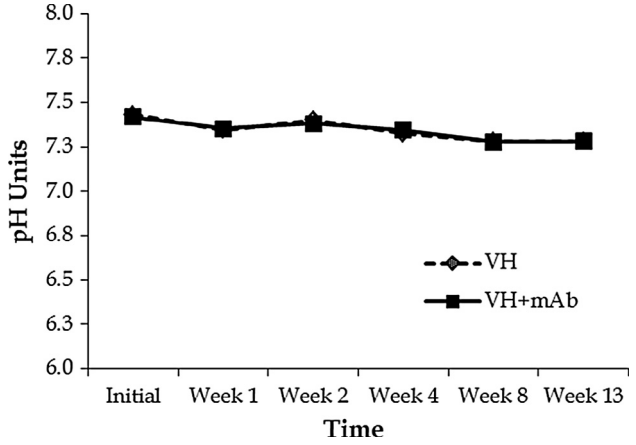

Fig. 2. Change in the pH of –ve control (VH) and test article (VH + mAb) at different time points. Results are expressed in mean ± std. dev.

However, the rate of loss of mAb from the VH-to the buffer-compartment was similar for the control (buffer + mAb) and test samples (VH + mAb). After 3 months of incubation, the concentration of mAb protein retained in the VH-compartment was sufficient to perform all physicochemical analysis with and without purification from the VH.

3.1.3. Osmolality and distribution of mAb formulation in VH

After addition of 50 μL of pH 6.0 isotonic mAb formulation into the VH, the osmolality was measured to confirm the homogenous dispersion of the formulation in the VH. During the entire study, osmolality of the VH and test article were maintained between 310 and 340 mOsmol/kg (Fig. 4). These data suggests that the mAb formulation was always homogeneous with the VH without altering its osmolality.

3.2. Physical stability of mAb in VH

3.2.1. Structural integrity and micro-DSC

The evaluation of the melting on-set and melting point (T_m) of mAb (by micro-DSC) would be an indicator of the structural changes in the mAb. Melting on-set and T_m of the mAb were recorded at 60 °C and 73 °C, respectively. As demonstrated in Fig. 5, even after 3 months incubation in the VH or buffer, melting on-set and T_m of mAb remained unaltered. Also, the data indicate that VH does not have any interfering signals in the melting range (60–85 °C) of the model mAb. Although VH contains multiple proteins, these proteins are deemed unlikely to interfere, at least at their respective (quite low) concentration.

3.2.2. Formation of particles and insoluble aggregates

Controls and test samples were assessed for the presence of visible particles using microscopic analysis. Results exhibited in Fig. 6 (A) demonstrated no visible particles in the controls (VH alone and buffer + mAb) or test samples (VH + mAb) after 3 months of incubation at 37 °C. Also, no immediate precipitation or particle formation was observed when 50 μL of the mAb formulation was injected into the VH or buffer. Sub-visible particles were quantified by light obscuration (HIAC). As reported in Fig. 6(B), the VH with and without mAb did not show significant increase in the sub-visible particles (⩾2, ⩾5, ⩾10 or ⩾25 μm). Particle counts for ⩾2 μm and ⩾5 μm showed significant fluctuation but the fluctuation was consistent for control and test articles. Thus, we refrain to interpret it as a significant change. The results were also confirmed by the turbidimetric analysis (data not shown), where turbidity readings remained below 10 FTU during the study period and were comparable with the controls.

3.2.3. Aggregation and fragmentation by SEC

Typically, mAbs undergo aggregation and/or fragmentation if subjected to a sub-optimal environment. Physiological pH and temperature are not optimal conditions for mAb stability and hence it is important to closely monitor the long-term stability in the VH at 37 °C. Formation of soluble aggregates, although not experimentally proven, may have an impact on the safety and/or efficacy of protein drug. SEC results in Fig. 7(A and B) demonstrate no change in the monomer content of mAb following 3 months of incubation in the VH. This is an interesting finding suggesting that mAb does not generate high molecular weight species (HMWS, soluble aggregates) and low molecular weight species (LMWS, fragments) in the VH. On the other hand, significant loss in the main peak (2.3%) and HMWS area (2.4%), and concurrent increase in the LMWS peak area (4.7%) were observed when mAb was incubated in the PBS at 37 °C for the same amount of time (Fig. 7(C and D), VH-compartment).

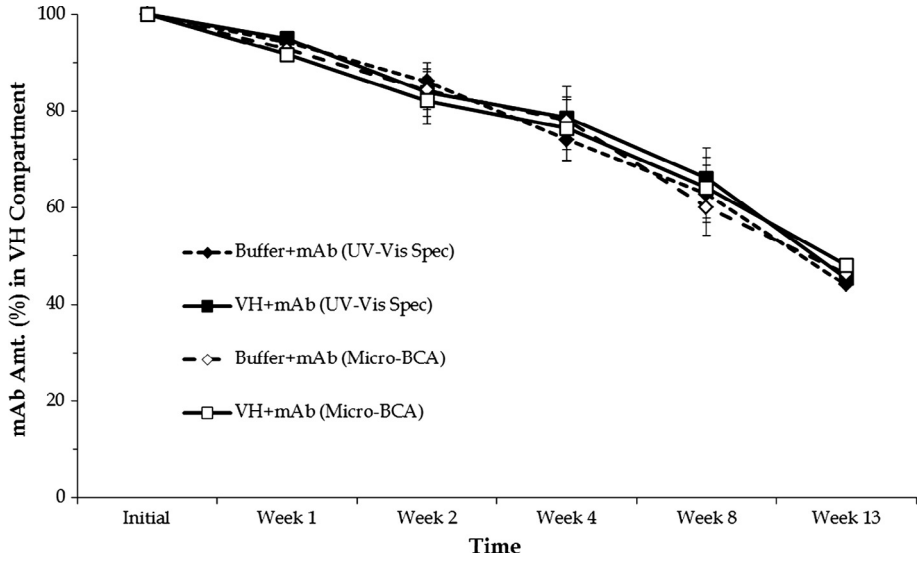

Fig. 3. Concentration of mAb estimated in the VH-compartment at different time intervals by UV–vis spectroscopy and Micro-BCA™ following incubation in the buffer and VH.

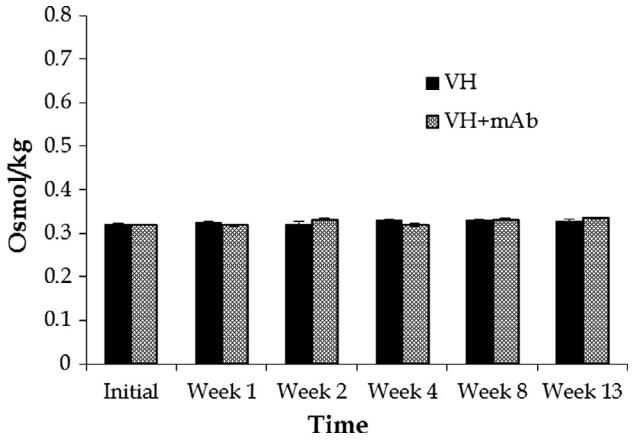

Fig. 4. Change in the osmolality of −ve control (VH) and test article (VH + mAb) at different time points. Results are expressed in mean ± std. dev.

SEC analysis of the mAb in the buffer-compartment for both test (VH + mAb) and control (buffer + mAb) was also performed (results are not shown). SEC chromatograms of the buffer-compartment samples revealed that intact mAb slowly diffused from the VH to the buffer-compartment separated by a diffusion controlling membrane. Small amounts of LMWS and the absence of HMWS were observed in the buffer-compartment when mAb was incubated in the porcine VH. However, in the control (Buffer + mAb) samples, LMWS along with the intact mAb molecules were observed in the buffer-compartment. In an additional control (VH alone), low Mw components (Mw < 50 kDa) of VH (Supplementary information Fig. S1) diffused rapidly from the VH to the buffer-compartment and established equilibrium within week-1. Relatively large VH proteins (MW 50–150 kDa) diffused slowly from the VH to the buffer-compartment and took 2 months to establish the equilibrium between the two compartments. A very large species (Mw > 500) always remained in the VH and never diffused.

3.2.4. Characterization of mAb by CE-SDS-NGS

CE-SDS-NGS analysis was performed to evaluate physical stability and to assess mainly fragmentation of mAb after incubation in the PBS and VH at 37 °C. As illustrated in Fig. 8(A), analysis of non-reduced samples of the VH alone exhibited the presence of VH proteins with smaller molecular weights for both initial and 3 month time points. No peaks of high-molecular weight protein species were observed in the electropherogram of VH alone. Electropherograms of the control (buffer + mAb) and test (VH + mAb) items represented in Fig. 8(A and B), demonstrate the loss of a main peak (peak-7) area and concurrent increase in various smaller molecular weight species (peaks 1, 2, 3, 5 and 6). Loss of the main peak was around 40% in the control sample (buffer + mAb) which was almost twice that of the test article (Fig. 8(C)). Moreover, the area of the LMWS for the control sample was increased more than 3 times compared to the test article.

Reduced CE-SDS-NGS data for the stability samples exhibited a significant drop of heavy-chain peak area and an increase in light-chain peak area. In the test solution (VH + mAb), the heavy chain area reduced by 4% and light-chain increased by 2%, whilst in the control there was a 10% reduction and a 7% increase, respectively. Also, a minor increase in the non-reduced or partially-reduced species was observed.

3.3. Chemical stability of mAb

Protein-L affinity resin-based chromatography was utilized to isolate mAb from the stability samples (control and test specimens) retrieved at different time intervals. The SEC chromatograms of the purified samples of test articles were devoid of any VH contaminants and contained only mAb which confirmed the specificity and selectivity of the purification method. The protein separation and purification efficiency was calculated from the area of the monomer peak in the SEC chromatogram. The yield was approximately 50%. The purified samples were then analyzed for chemical modifications and binding activity of the mAb.

3.3.1. Ion exchange chromatography (IEC)

Weak-cation exchange chromatography was used to separate various charge variants of mAb. As described in Fig. 9, there was approximately a 57% decrease in the main peak area for test samples was observed when compared with the initial values. The acidic variants in the test and control samples were significantly increased by 56% from the initial value. No significant increase in the basic degradants was observed in control or test articles at

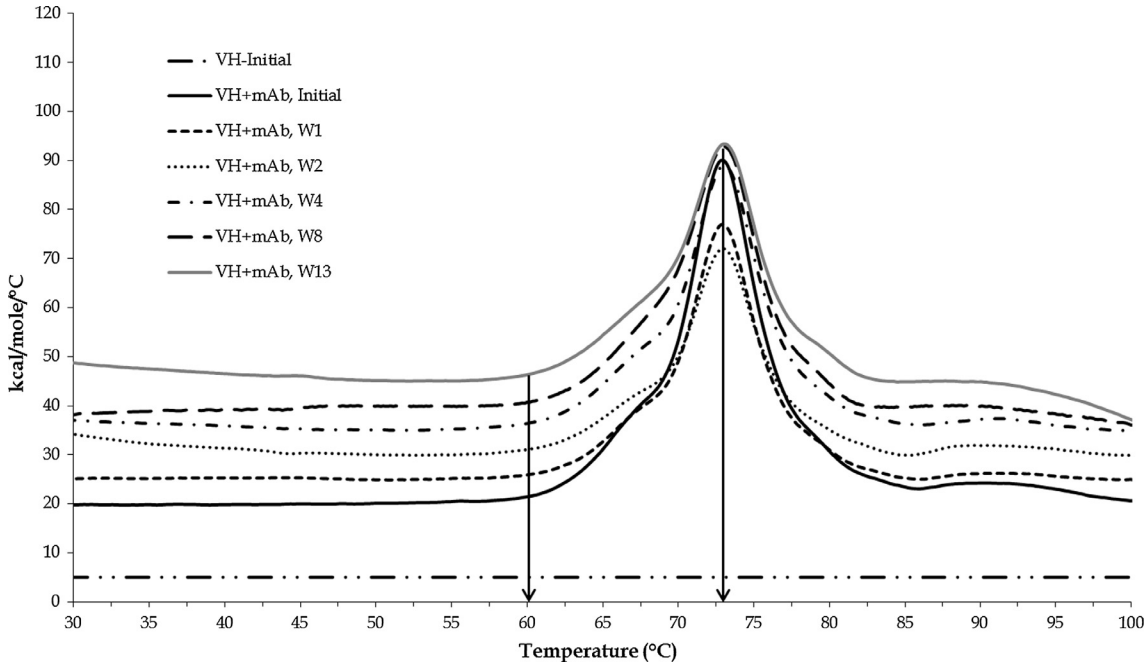

Fig. 5. Melting point evaluation of mAb by DSC at different time points following incubation in VH.

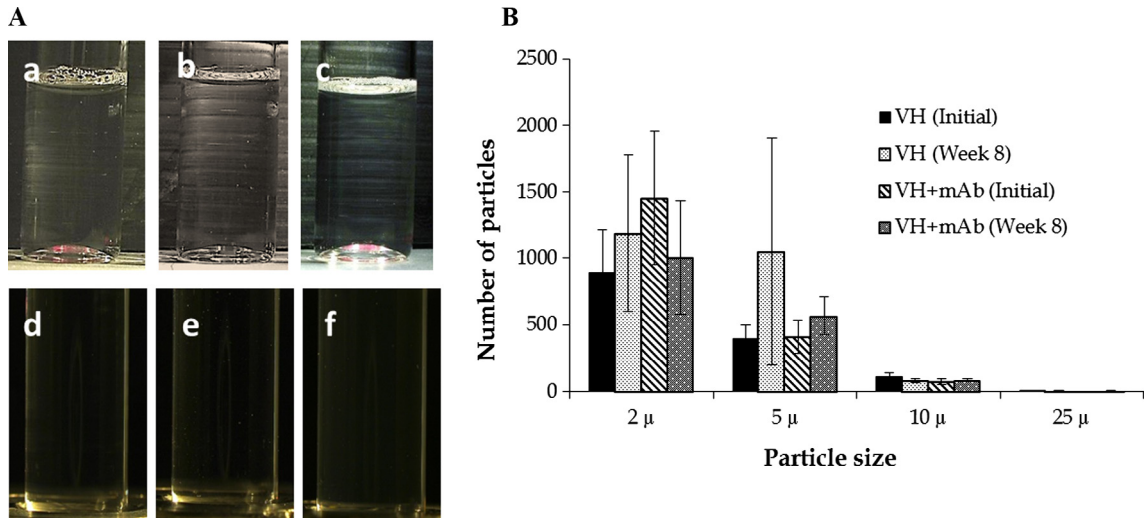

Fig. 6. (A) Microscopic evaluation of visible particles in initial samples ((a) VH, (b) PBS + mAb and (c) VH + mAB) and after 3 months of incubation ((d) VH, (e) PBS + mAb and (f) VH + mAB). Change in the sub-visible particles of different size evaluated at different time points for −ve control (VH) and test article (VH + mAb). Results are expressed in mean ± std. dev.

any given time intervals (Supplementary information Fig. S2). Similar results were observed with the control sample (buffer + mAb) (Supplementary information Fig. S2) suggesting chemical modifications are mainly induced by pH and temperature with minimal or no contribution of VH components.

3.4. Binding affinity of mAb by SPR-Biacore

Binding affinity of mAb in the stability samples were evaluated by SPR-Biacore. As reported in Fig. 10(A), a significant drop in the binding affinity of mAb was observed in both control and test samples after prolonged incubation at 37 °C. mAb in buffer and VH samples exhibited a similar loss of binding affinity until week-8; however, at the 3 month time point, the mAb in buffer sample exhibited around 15% higher loss in potency. Both domains of the bispecific mAb showed a significant loss in the binding affinity compared with initial time point as denoted in Fig. 10(B). Also, the extent of loss was similar in control and test samples until the 2 month time point. Interestingly, thereafter, mAb in buffer solution exhibited a noticeable loss, 12 and 18% in the binding affinity of domain-1 and domain-2, respectively.

4. Discussion

The stability of a protein drug injected into the back of the eye of patients is a great matter of discussion, especially when the injected protein drug is supposed to be retained in the eye for several weeks. Presently, no reliable tool is available to study the stability of protein drugs or drug delivery systems in the isolated VH. As reported in our previously published manuscript, as soon as the

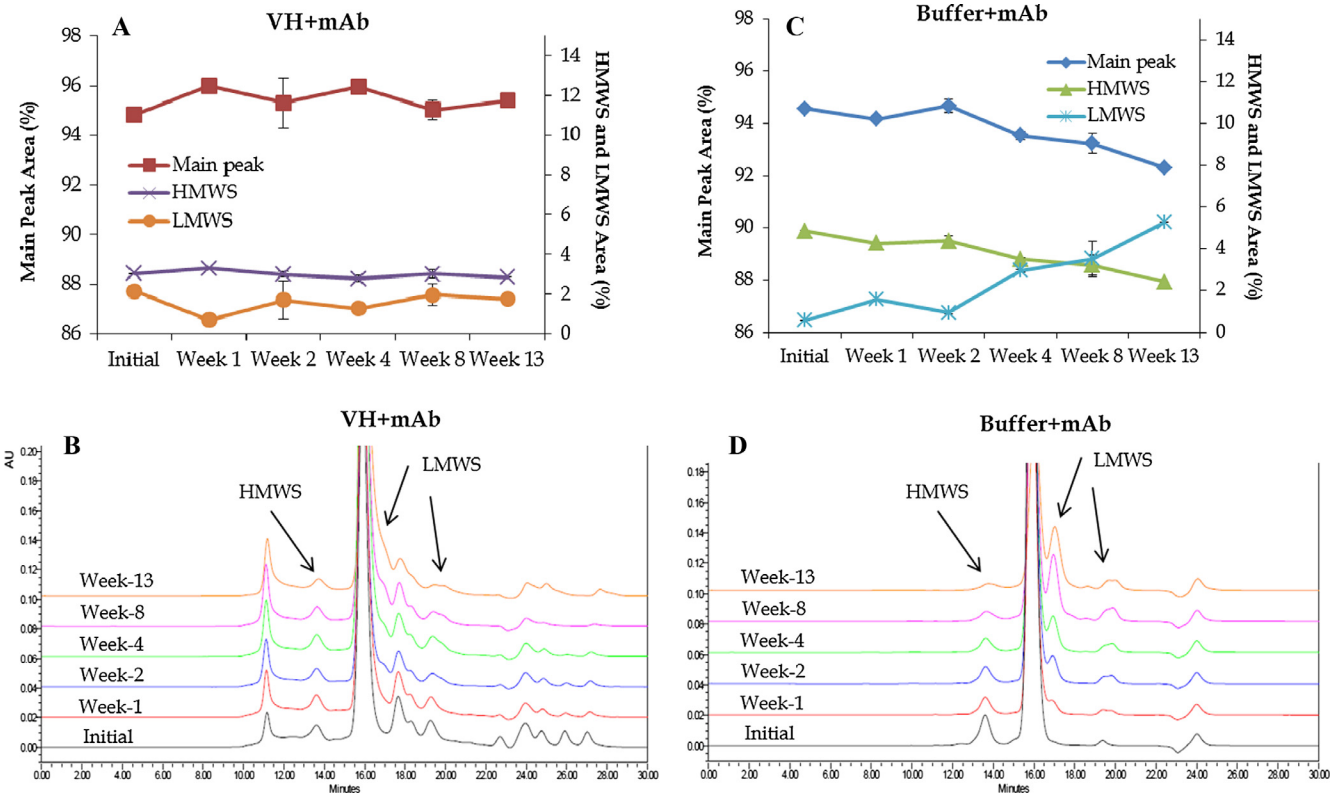

Fig. 7. (A) SEC (VH + mAb, VH-compartment, graphs), (B) SEC (VH + mAb, VH-compartment, chromatograms), (C) SEC (Buffer + mAb, VH-compartment, graphs), and (D) SEC (Buffer + mAb, VH-compartment, Chromatograms).

VH is isolated from the eye, the pH of the VH increases rapidly (ca pH 8.5). The scientific rationale for this alkaline pH shift is not well understood, but our previous findings point towards the speedy alterations in the microenvironment of the VH upon isolation and/or the accumulation of degradation products of the VH [5,8,20–22]. The elevated pH value (ca pH 8.5) and concentrated degradants in the isolated VH can have a detrimental impact on the protein stability and can stimulate degradation of mAb which does not occur in *in vivo* situation and make this study prone to artefacts. Therefore, it is very important to remove the degradation products and to maintain the pH of the VH at physiological values to ensure a better representativeness of the *ex-vivo* stability model for the *in vivo* situation. It is important to note that this model will never be a true representation of human eye but can be a valuable research tool to investigate protein stability and interactions within VH. As described in the results (Fig. 2), the ExVit-HS system effectively maintained the physiological pH of the VH (pH 7.4 ± 0.1) during the entire study period of 3 months. The degradants of VH freely diffused-out from the VH- to the buffer-compartment with the ionic species diffused-in from buffer to VH- compartment. This unique approach of stabilizing the pH of the VH makes ExVit-HS a suitable model to investigate protein drug stability in the presence of the VH.

ExVit-HS, being a dynamic model, maintains the pH of the VH by diffusion but at the same time protein drug also diffuses out from the VH [20]. This is comparable to the *in vivo* situation where protein is eliminated relatively rapidly from the eye depending on the animal species, for example, the rabbit (t1/2 ca 3 days). This makes the physicochemical stability assessment difficult for the long-term study as there is not enough concentration of mAb is available for the various analysis. In this study, we have adapted the design of ExVit to a horizontal side-by-side diffusion in order to achieve a sufficiently high concentration of mAb in direct contact with the VH throughout the study period. From the results described in Fig. 3, it is evident that approximately half of the protein concentration is retained in the VH-compartment even after 3 months of incubation. These results were confirmed by three different analytical techniques, UV–visible assay, Micro-BCA™ and ELISA. ExVit-HS has significantly reduced the rate of diffusion from the VH- to buffer compartment, in comparison with the vertical diffusion model [20]. As expected, at any given time point, the diffusion of mAb from the VH-compartment was comparable between the control (buffer + mAb) and test article (VH + mAb). This also points towards the diffusion of intact mAb from the VH- to the buffer-compartment mainly being controlled by the molecular weight cut-off of the membrane. A few pores, large enough to allow the diffusion of globular protein, might have slowly diffused the intact mAb from the VH- to the buffer-compartment over 3 months. Overall, the protein quantity retained for 3 months in VH-compartment of ExVit-HS was sufficient to perform physicochemical and potency assays.

Since the ExVit-HS model has met two of our key requirements, maintaining physiological pH and confining sufficient protein drug in the VH-compartment, we have added 50 μL mAb formulations in the VH-compartment of the ExVit-HS model. Spiking of the isotonic mAb formulation in the VH did not alter the osmolality of the VH due to the formulation being rapidly diluted in the 4 mL of VH (Fig. 4). After dilution, mAb was fully exposed in the VH for 3 months at physiological temperature and VH components. Various parameters including pH, temperature and interaction with the VH components might introduce physicochemical and functional changes in the mAb. These key changes may induce compromised structural integrity, aggregation and/or fragmentation. The results from the micro-DSC (Fig. 5) indicate that the melting point (~73 °C) and the onset of melting (~60 °C) remained constant at all

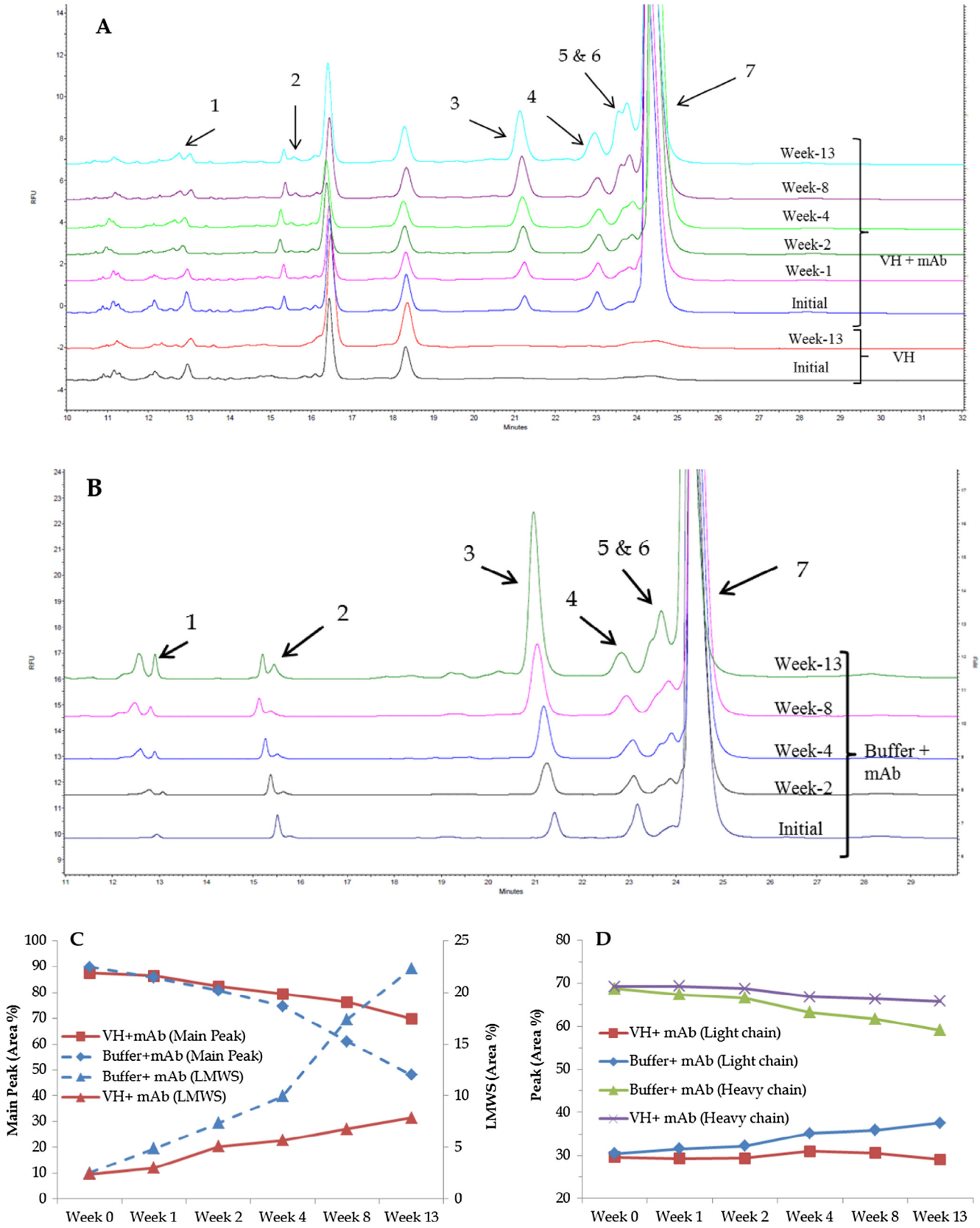

Fig. 8. (A) CE-SDS-NGS electropherogram of VH + VA2 (non-reduced samples), (B) CE-SDS-NGS electropherogram of Buffer + VA2 (non-reduced samples), (C) change in the main peak and LMWS area (%) evaluated by CE-SDS-NGS (non-reduced samples), and (D) changes in the light chain and heavy chain peak area (%) evaluated by CE-SDS-NGS (reduced samples).

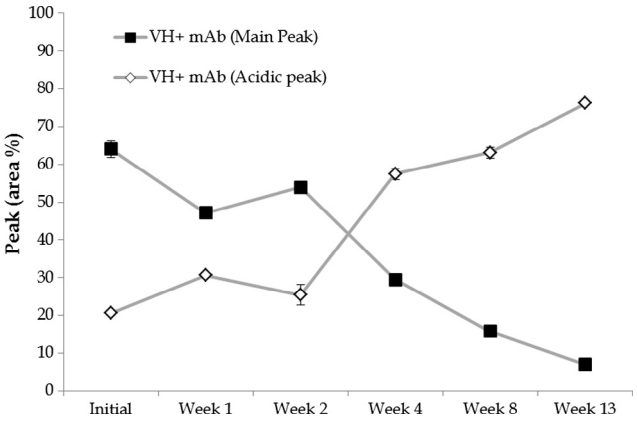

Fig. 9. Change in the main peak area (%) and acidic peak area (%) at different time intervals following incubation in the VH.

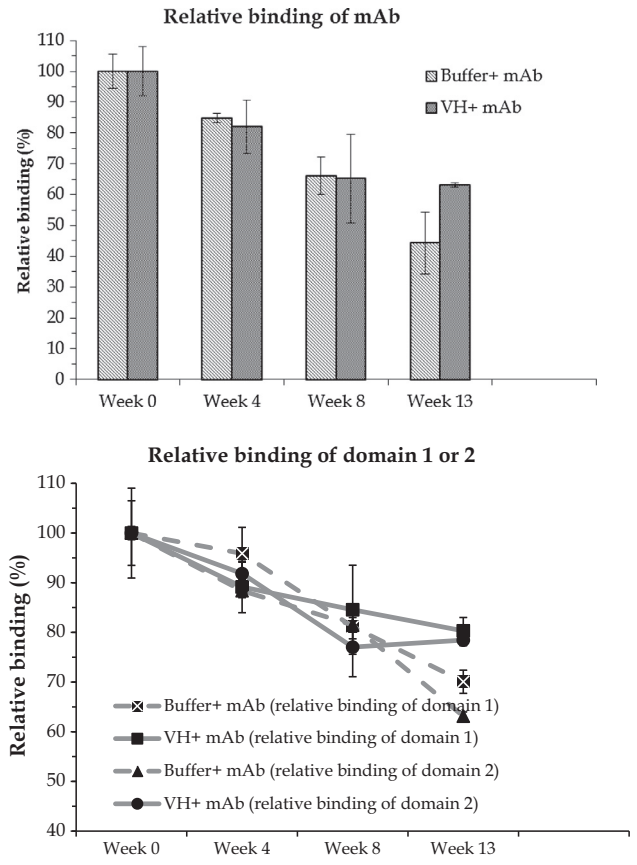

Fig. 10. (A) Relative potency of mAb and (B) relative potency of domain-1 & 2 following incubation at 37 °C in VH up to week-13.

time points, suggesting retention of structural conformational integrity of mAb.

Protein aggregation is always a matter of discussion related to quality and safety aspects of protein drugs, also for the intravitreal route of administration. Results expressed in Fig. 6(A and B) show visible and sub-visible particles. This suggests no significant increase in insoluble aggregates over the 3 months incubation of mAb in the test or control samples. Here, it is worth noting that the VH alone also remained stable for 3 months in the ExVit-HS model and did not generate any insoluble aggregates. These results clearly suggest absence of measurable colloidal, intermolecular interactions between mAb and VH components or intramolecular interactions between mAb molecules which may lead to the formation of insoluble particles or aggregates. This finding is further confirmed by the low level of soluble aggregates observed in SEC data (Fig. 7(A)) which did not increase in the test samples analyzed from the VH-compartment after 3 months.

Very interestingly, the mAb incubated in the PBS showed a significantly higher fragmentation compared to the mAb formulation incubated in the VH. CE-SDS-NGS analysis also confirmed the higher degradation of mAb in the PBS compared to the VH and resulted in two main degradation products, peak 3 and peaks 5 & 6 (Fig. 8(A and B)). These degradation products were identical with the published literature [23]. It is anticipated that peak-3 represents the fragmented mAb with the loss of two light-chains (LC) and peaks-5 & 6 represent the non-glycosylated mAb. Also, looking at the fragmentation pattern in the CE-SDS-NGS electropherogram, it can be hypothesized that the fragmentation products were generated as a result of disulfide bond hydrolysis and the majority of them were LC/LC dimers. Although, it is a topic of further research in order to understand why mAb showed higher fragmentation in PBS than in the VH, one possible explanation could be due to the presence of stabilizing agents in the VH such as glutathione [24]. These agents in the VH might have decreased the stability of LC/LC dimers and permitted reassembly with the LC missing mAb (peak 3). Thus, it is likely that the presence of the reducing agent is probably more efficient in shifting the equilibrium towards the intact mAb via recycling of LC. Additional studies are ongoing in order to understand the fragmentation kinetics in the presence of reducing agents in an artificial system with various low Mw components commonly present in VH (Supplementary information Tables S1 and S2), but this is beyond the scope of the present manuscript. This indicates that mAb may be even better protected and stabilized in the VH environment compared to PBS, although this phenomenon can be mAB specific and requires further assessment before extending to other mAbs or proteins.

The chemical degradation of mAb by IEC in test and control samples, following 3 months of incubation at 37 °C, revealed no noticeable differences. The mAb mainly generated an acidic variants as it is very sensitive to elevated pH (7.4) and temperature (37 °C) which is also in-line with our internal data (data not shown). As the temperature and pH is constant in the control and test samples, mAb exhibited a similar level of acidic species. Samples were also analyzed using LC-MS/MS (data not shown) to locate chemical modifications in complementarity determining regions (CDR) such as succinimide formation, isomerization and methionine oxidation. A minor increase in the non-CDR methionine oxidation (Met258, 268, 434, 444) was observed whilst no tryptophans were impacted. LC-MS/MS data also showed a minor increase of the *iso*-aspartic acid levels in test samples compared to the control samples. Asparagine in CDR was mainly affected during the stability due to the elevated pH (7.4) and temperature (37 °C). Unfortunately, we were not able to estimate the rate of deamidation in this study.

The chemical alteration in the CDR of the antibody in most cases has direct impact on its binding affinity towards the antigen. In fact, the binding affinity of the mAb was reduced significantly following incubation in control PBS or in the VH. Again, mAb in the presence of VH, showed a slightly lower rate and lower extent of loss in binding than mAb in PBS. Also, it was evident from the results that both the binding domain of bispecific mAb exhibited a significant loss in the binding affinity towards their respective antigens. In general, the loss in the binding affinity of mAb was directly correlated to the formation of acidic variants due to deamidation which is in agreement with our internal data for this mAb. The potency loss in PBS was higher probably due to the significant fragmentation and loss in LC compared to that in VH.

5. Conclusions

The novel ExVit-HS model has been successfully designed and evaluated to investigate protein stability following IVT injection. The ExVit-HS model is an alternative and effective tool which can be adopted to research stability of protein or other large molecules following IVT injection. This is the first report, to the best of our knowledge, which demonstrates a real-time 3 months stability assessment of mAb in the VH by mimicking the physiological condition of the eye. The mAb, under investigation, exhibited a higher fragmentation in PBS relative to the VH, suggesting that the VH components even stabilized the mAb. Also, mAb showed a significant chemical degradation and loss in binding activity over three months which is critical information for the prolonged delivery in the eye. The study indicated that buffer is not a realistic representation to VH and it was better protected in the VH environment compared to PBS. However, this finding requires further investigation before extending to other mAbs or proteins.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2016.10.028.

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