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Aim: The development of a polyarginine cell-penetrating peptide (CPP) could enable the treatment of age-related macular degeneration, with drugs like bevacizumab, to be administered using eye drops instead of intravitreal injections. Topical formulations have a vast potential impact on healthcare by increasing patient compliance while reducing the financial burden. However, as the ocular preparations may contain several doses, it is essential to understand the stability of the bevacizumab+CPP conjugate produced.

Materials and Methods: In this work, we examine the stability of a bevacizumab solution with and without cell-penetrating peptide using dynamic light scattering and circular dichroism to assess the physical stability. We use HPLC to assess the chemical stability and ELISA to assess its biological activity. We also examine the potential of the CPP to be used as an antimicrobial agent in place of preservatives in the eye drop.

Results: The structural stability of bevacizumab with and without the CPP was found not to be affected by temperature: samples stored at either 20 °C or 4 °C were identical in behavior. However, physical instability was observed after five weeks, leading to aggregation and precipitation. Further investigation revealed that the addition of the polypeptide led to increased aggregation, as revealed through dynamic light scattering and concentration analysis of the peptide through HPLC. Complexing the bevacizumab with CPP had no effect on biological stability or degradation.

Conclusions: Our findings suggest that the shelf life of CPP+bevacizumab complexes is at least 38 days from its initial formulation. Currently, the mechanism for aggregation is not fully understood but does not appear to occur through chemical degradation.

Keywords: Ocular drug delivery, stability, eye drops, Cell-penetrating peptide (CPP); vascular endothelial growth factor (VEGF)

Introduction

Age-Related Macular Degeneration (AMD) is a leading cause of blindness, affecting around 600,000 people in the U.K. [1] and 10 million people in the US [2]. Neovascular AMD (nAMD) leads to rapid vision loss as new blood vessels invade the macular region of the retina from the choroid [3]. Anti-VEGF monoclonal antibodies have proved effective in treating nAMD [4,5]. However, this treatment is delivered through intravitreal injections, which must be administered by a healthcare professional and can lead to significant patient discomfort and future complications [6,7].

Topical reformulations have the potential to increase patient compliance while also reducing healthcare costs [8]. Cell-penetrating peptides offer a potential mechanism to deliver drugs across biological membranes and are actively being researched as complexing agents for siRNA targeting [9,10]. Topical reformulations involving cell-penetrating peptides have been successful for retinal neovascularisation inhibition by topical delivery of a dodecapeptide-KV11 conjugate, with no tissue toxicity [11].

The development of a novel polyarginine cell-penetrating peptide (CPP) has opened the opportunity to treat nAMD using eye drops rather than intravitreal injections. The potential effectiveness of samples consisting of CPP complexed with bevacizumab has already been explored [12]. Previous studies compared the effect of a topically-instilled bevacizumab+CPP formulation against topical and intravitreally-injected bevacizumab in the treatment of mice that had received photocoagulation treatment of their retinas. The bevacizumab+CPP formulation was shown to suppress choroidal neovascularisation as effectively as intravitreally-injected bevacizumab [12]. However, despite being shown as an effective drug-delivery mechanism, there has been no study

into the pharmaceutical stability of such formulations, which is essential for the progression of this work towards the clinic.

All medicinal products, regardless of formulation, should be manufactured to a high standard to ensure patient safety and efficacy [13,14]. Ophthalmic products are more challenging to formulate than the majority of medicines because they often require multiple doses, therefore increasing the risk to patient safety. Thus, the shelf life of a product of this nature is of increased importance as the drug will be expected to remain stable throughout the specified treatment regimen.

In this work, we address questions surrounding the effects of CPP on bevacizumab by examining the physical, chemical and biological stability of a bevacizumab+CPP complexes using the techniques described within the NHS Quality Assurance Committee Stability protocols [15,16]. This work will provide the basis for the progression of this impactful advance in the delivery of a key treatment for one of the leading causes of blindness.

Materials and Methods

CPP (NH_2- RRRRRR-COOH, CPP) was procured from Genscript, New Jersey. Bevacizumab (Roche Pharmaceuticals, Welwyn Garden City) at 25 mg/mL was used as supplied. 10 mg of CPP was diluted into 2 mL aliquots of bevacizumab to produce solutions containing 5 mg/mL CPP in 25 mg/mL of bevacizumab to produce the bevacizumab+CPP complexes.

Physical Stability

Dynamic Light Scattering (DLS) and visual inspections were used to determine the physical stability of the bevacizumab with and without CPP. Bevacizumab+CPP solutions were stored either at room temperature or at 4 °C for up to 57 days. DLS was carried out using a Nanoseries Zetasizer at 658 nm, set at 20 °C on 0.5 mL solution aliquots. Each sample was recorded five times and in triplicate, with the analysis completed using the Nanoseries software based on a refractive index of 1.33 and viscosity of 0.89 cP. No dilution of the bevacizumab was required before scanning. The CPP titration was performed by titrating 1 μL aliquots of a 200 mg/mL CPP solution.

Chemical and Structural Stability

Chemical stability was examined using HPLC. Six samples were prepared and stored as described above. All samples were run at a flow rate of 1 ml/min, and the column and mobile phases kept at a constant temperature of 25 ºC. Aliquots were run on an Agilent Infinity II series 1290 analytical HPLC with an Aeris Widepore C18-XB RP-HPLC column (250 x 21.2 C18 3.6 μm 200 Å Axia packed) in water. The gradient solvent system used consisted of solvent A (water + 0.05% trifluoroacetic acid) and solvent B (water + 0.1 % trifluoroacetic acid). The gradient was run from 0-100% B over 40 minutes, with the absorbance monitored at 280 nm. Calibration samples were run from

fresh bevacizumab alone, CPP alone, and bevacizumab+CPP stock solutions at concentrations ranging from 0 – 6.25 mg/mL for bevacizumab and 0 – 5 mg/mL for CPP. Uridine was run as an internal standard. The method gave a limit of quantification of 0.0856 mg/mL for bevacizumab and 0.172 mg/ml for CPP, and precisions of 3.48 % and 12.0 % for bevacizumab and CPP respectively. Test samples were prepared from stock by diluting 30 µL of stock solutions into 200 µL of ultrapure water. The concentration of the CPP and of bevacizumab analyzed by the peak area ratio between the analyte and the internal standard and the concentration confirmed by reference to the calibration curve. Samples were tested over the length of the study.

The bevacizumab samples with and without CPP were examined for structural stability using circular dichroism (CD) spectroscopy. This analysis is key when studying biopharmaceuticals as their structure and conformation, and thus biological activity, can be changed by chemical and physical changes. CD spectra of bevacizumab and bevacizumab+CPP were recorded on a Jasco J-715 spectropolarimeter, and scans were recorded across 260-190 nm. The scans were baseline corrected, and readings averaged over five replicates. The absolute ellipticity was recorded in millidegrees and converted to mean residual ellipticity. CD spectra for the stability samples were recorded over eight weeks, and the intensity of the minima at 218 nm plotted as a function of time. To determine differences in degradation with and without the peptide, samples were prepared by dissolving TCEP into both bevacizumab and bevacizumab+CPP to a concentration of 5 mg/mL. These samples were then incubated at room temperature overnight. An additional baseline correction, containing 5 mg/mL TCEP was applied to all denatured spectra.

Biological Stability

The level of bevacizumab in stability samples at different time points was measured using a Protein Detection ELISA kit (KPL, Gaithersburg, MD, USA) with an anti-human antibody (309-001-003; Jackson Immuno Research Laboratory, West Grove, PA, USA). High-affinity 96-well plates (Sigma-Aldrich) were coated with 0.1 mg/mL anti-human antibody for 1 hour, followed by addition of the test samples containing varying concentrations of bevacizumab. The analysis was carried out according to the manufacturer's instructions.

A series of bevacizumab and CPP samples were tested at various time points and their concentration was determined from a calibration curve ranging from 2.5 pg/mL to 25 μg/mL bevacizumab and a new calibration curve is required for each 96-well plate. The stability samples were diluted to 25 μg/mL bevacizumab prior to use. All samples were also recorded with a bevacizumab control.

Antimicrobial Efficacy

The antimicrobial efficacy of CPP solutions was tested using the BP (British Pharmacopeia) Efficacy of Antimicrobial Preservation test [17]. Samples of CPP and bevacizumab+CPP were inoculated with *Staphylococcus aureus* to a final concentration of 10^6 colony-forming organisms per mL. Two strains of *S. aureus* were tested: a BP standard laboratory strain (*S. aureus* NCTC 8532) and a clinical isolate. Samples were kept in the dark and incubated at 20-25 °C. 1 mL samples were taken at intervals throughout the time course and 100 ⌈L aliquots of serial dilutions (ranging from 10^1-10^6) were incubated on LB agar plates at 37°C for a minimum of 24 hours to quantify CFU. Independent samples were taken at 24 hours, 7 days, and 14 days.

Results

Physical Stability

In order to assess the impact of CPP on the physical stability of bevacizumab, the solutions were stored at either 4 °C or room temperature and monitored for changes. The bevacizumab only and CPP only solutions showed no visual changes over 57 days when stored at either 4 °C or room temperature. When formulated with CPP, the bevacizumab solutions showed no changes when stored at either temperature for the first six weeks. However, by day 57, varying amounts of precipitate were observed in all samples. This was then probed using Dynamic Light Scattering (DLS). DLS analysis of the bevacizumab+CPP samples showed no change in particle distribution for up to 38 days (16.8 nm ± 5.0 nm) regardless of storage condition (Table 1). This matches literature data which showed stability up to 30 days[18]. This size is consistent with those found previously [19] for the size distribution of fresh bevacizumab suspensions. However, by 57 days, particle formation was observed by DLS (Table 1). Physical stability does not extend beyond six weeks due to the formation of excipient-induced aggregates caused by the presence of CPP. Titrating increasing amounts of CPP into bevacizumab showed no significant change in particle size up to 5 mg/mL, which is the concentration used in the bevacizumab+CPP complex solution here (Summarized in Supplementary Data Table S1).

Chemical Stability

HPLC chromatograms of the bevacizumab and CPP content within the bevacizumab+CPP samples showed a reduction in the solution concentrations of both over time, which is in agreement with visual inspection of precipitate formation. However, there was a notable sample to sample variation. Thus, the mean change in

bevacizumab and CPP over the study period was not statistically significant (Figure 1). The change in concentration of individual samples can be found in Supplementary Data (Figures S1-S4).

No degradation peaks were observed in any of the HPLC chromatograms, suggesting that the loss in concentration is due to physical instability, not to chemical degradation. Regardless of storage condition, the CD spectra did not change over the length of the study period (Figure 2a). This observation indicates that the structure remains unchanged, and demonstrates the high structural stability of bevacizumab, which is in agreement with the HPLC results. This finding is also in agreement with previous findings that showed bevacizumab samples were stable for at least six months [20]. When degradation was instigated by incubation with TCEP there was a significant change in the spectra compared to the original samples. However, there were no significant differences between the bevacizumab alone or the bevacizumab+CPP in the presence of TCEP (Figure 2b). Additionally, on titrating increasing amounts of CPP into bevacizumab, the CD spectra remained invariant after correcting for the spectrum of the CPP alone. Data can be found in the Supplementary Information (Figures S5-6). The invariance of the ellipticity indicates that the association of CPP and bevacizumab is via physical interaction (such as hydrogen bonding) rather than a chemical one, which would have resulted in a change in protein conformation.

Biological Stability

The concentration of bevacizumab was measured using ELISA to determine biological stability. Over the six week period, we saw no significant differences between bevacizumab alone or bevacizumab complexed with CPP (Figure 2c). Over the 56 day period we did see a slight, but not statistically significant, trend to a higher

bioavailability in both bevacizumab alone and bevacizumab complexed with CPP over time. These data show that when CPP is complexed to bevacizumab there is no effect on the biostability of bevacizumab.

Antimicrobial Efficacy

Previous work has shown that CPP demonstrates some antimicrobial efficacy, therefore we hypothesized that CPP could potentially replace preservatives in the bevacizumab+CPP eye drops. Solutions of the model organism *S. aureus* were incubated with CPP over a time course of 14 days and CFU were quantified at regular intervals. The CPP solutions showed antimicrobial properties against *S. aureus* NCTC 8532, with an average log reduction of 4 after 24 hours compared to t=0 (Figure 3a). This reduction is significantly greater than the required 3 log reduction stated within the British Pharmacopoeia requirements [17]. Furthermore, after seven days, no bacteria were recovered from the samples of CPP. However, the efficacy was significantly reduced upon the addition of bevacizumab (Figure 3a). This trend may have occurred due to preferential binding of the arginine sequence to the MAb over the cell membranes of the bacteria. However, against a strain of *S. aureus* isolated from a clinical sample, CPP showed limited antimicrobial activity (Figure 3b). Only a log reduction of 1 was observed at 14 days, suggesting that the antimicrobial efficacy of CPP will not be able to replace preservatives in a real-world application of the bevacizumab+CPP eye drops.

Conclusion

We have used a range of different methods to determine the stability of CPP+bevacizumab complexes. While the chemical data shows no degradation over the eight-week study period regardless of CPP addition, the physical stability data

demonstrates significant precipitation at 6 weeks. Based on DLS data our findings suggest that the shelf life of CPP+bevacizumab complexes is at least 38 days from its initial formulation. The antimicrobial data suggests that although there is limited efficacy of the CPP against some bacterial strains, this is not robust and preservatives would need to be included in bevacizumab+CPP eye drops.

Acknowledgments

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Declaration of Interest

The authors report no conflicts of interest.

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Tables

Table 1: Particle sizes of samples containing 25 mg/mL bevacizumab and 5mg/mL CPP at different time points when stored at room temperature or 4 °C.

Time (Days)	Mean particle diameter (nm)
0	16.8 ± 5.0
28 (4 °C)	15.8 ± 3.5
38 (4 °C)	15.9 ± 4.0
57 (4 °C)	16.7 ± 4.7 (94 %), 624.2 ± 163.5 (6 %)
57 Room temperature	16.4 ± 4.3 (93 %), 894.3 ± 280.4 (7 %)

Figures

Figure 1

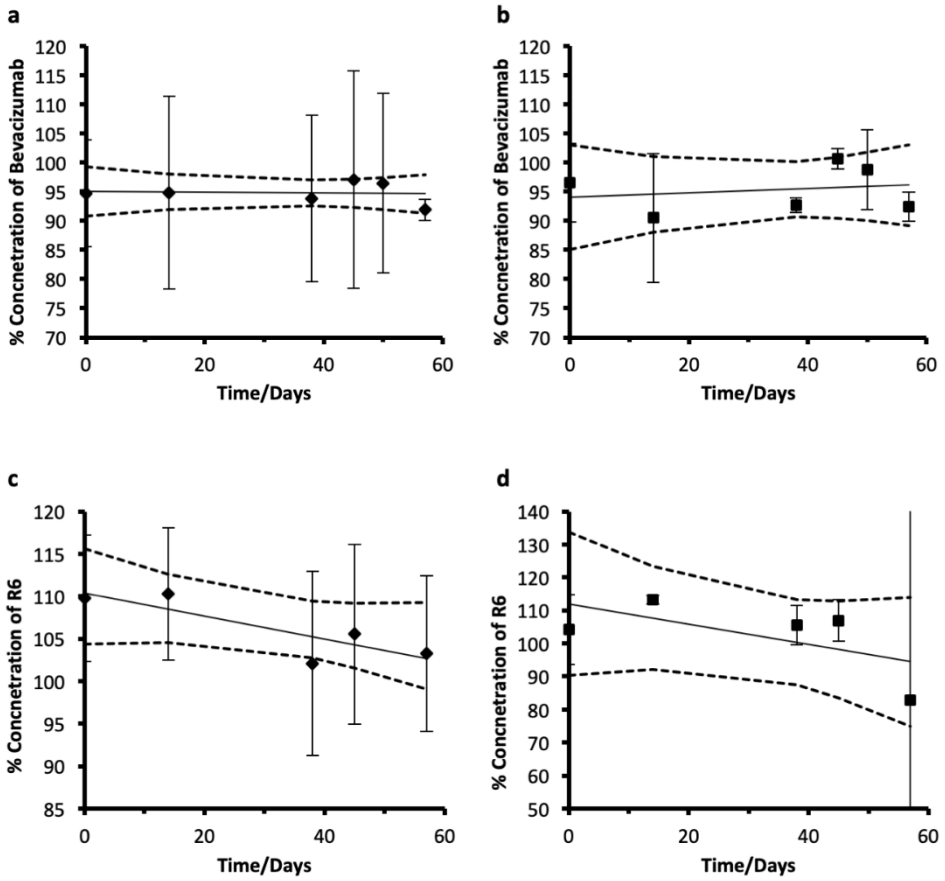

Figure 1: Concentration stability of bevacizumab and CPP, measured by HPLC and recorded as a percentage of 25 mg/mL for bevacizumab and as a percentage of 5 mg/mL for CPP**.** a) Stability of bevacizumab in samples stored at 4 °C**.** b) Stability of bevacizumab in samples stored at room temperature. c) Stability of CPP in samples stored at 4°C. d) Stability of CPP in samples stored at room temperature. The average of six replicates was used for each condition. All data fit to a linear regression (black line) error bars show standard deviation with the 95% confidence interval of the slope shown as a dashed black line.

Figure 2

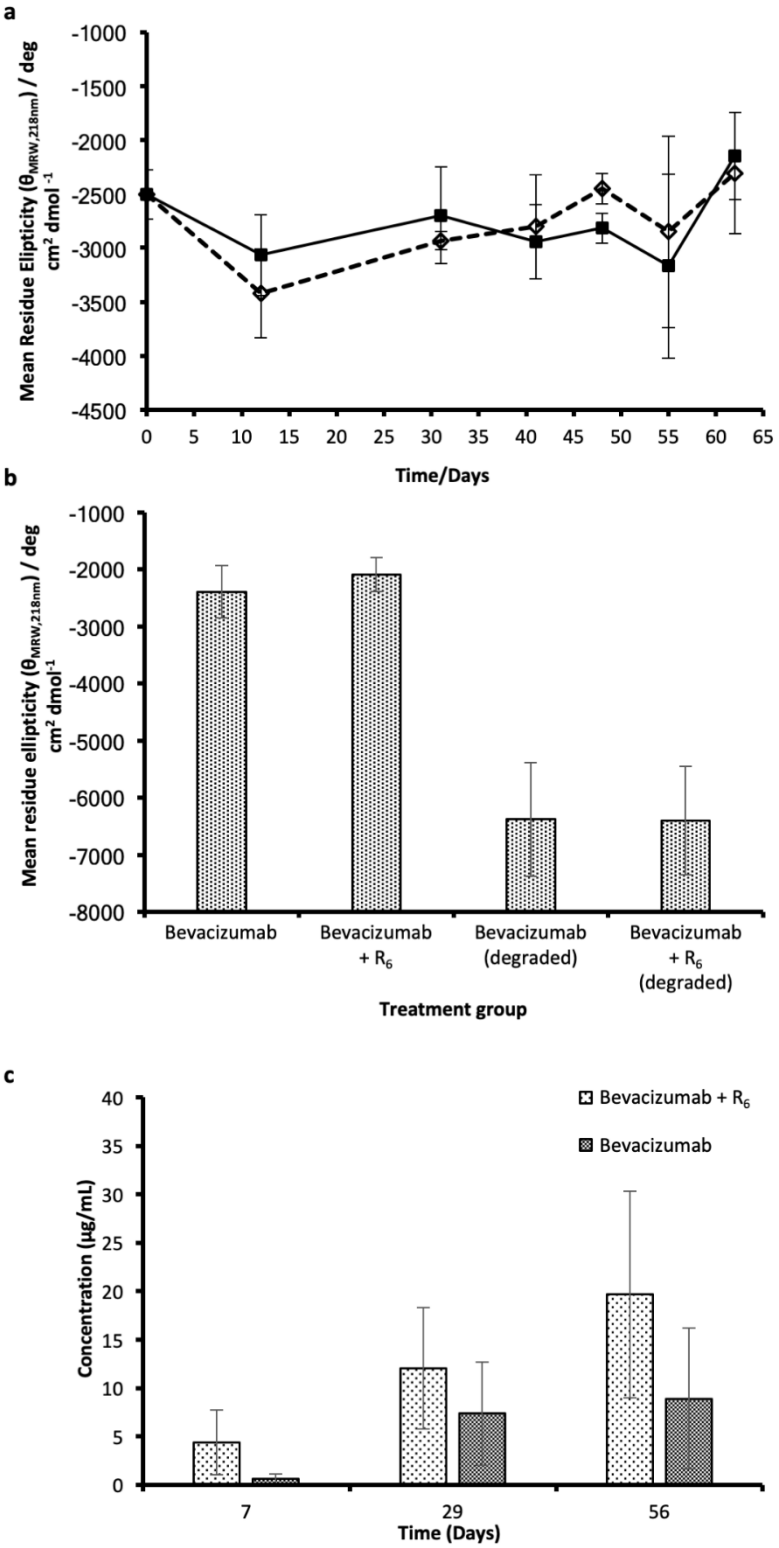

Figure 2: The average mean residue ellipticity a) of bevacizumab+CPP samples measured at 218 nm of samples stored at 4 °C (open diamond markers) or room temperature (solid square markers) over 63 days. All errors are based on 95 % confidence intervals of averaged data for six samples in each condition. b) bevacizumab

and bevacizumab+CPP samples degraded through incubation with TCP, n=3 error bars show standard deviation. All circular dichroism spectra were recorded at 20 °C in a 1 mm path length cuvette. c)The mean concentration of bevacizumab in samples measured using ELISA, n=3 error bars show standard error of the mean.

Figure 3

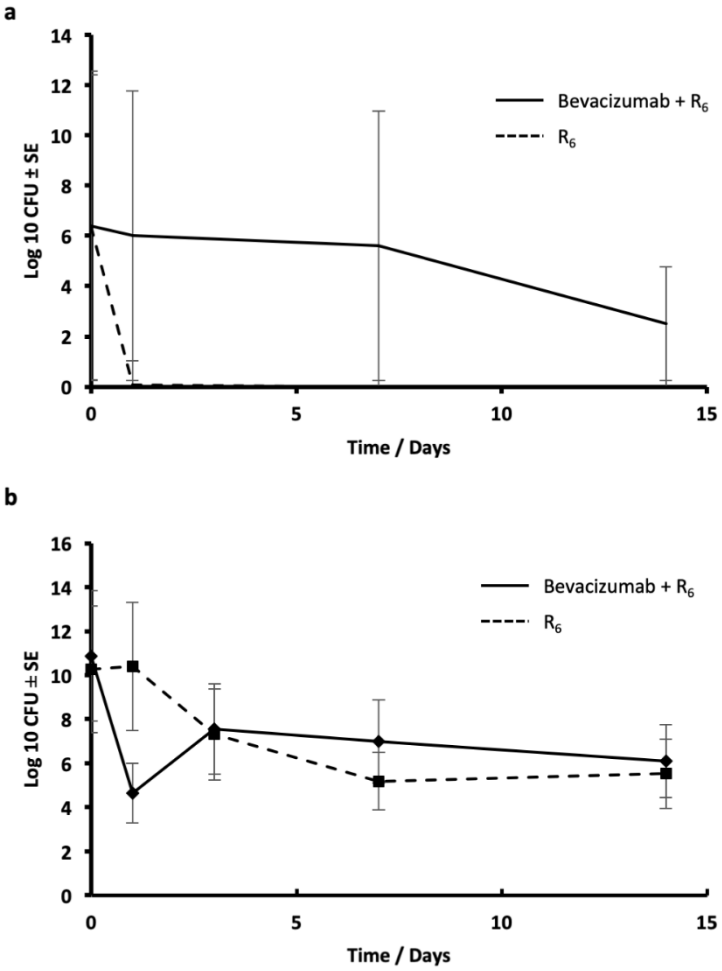

Figure 3: Antibacterial efficacy testing of CPP (CPP only, dashed line) and bevacizumab+CPP samples (solid line) in triplicate with error bars showing the standard deviation of the mean. a) Number of colony-forming units (CFU) of *S.* aureus NCTC 8532 for CPP and bevacizumab+CPP samples over 14 days. b) Antibacterial efficacy testing showing the number of CFU of *S. aureus* isolated from clinical infections for CPP and bevacizumab+CPP samples over 14 days.