Stability of cell-penetrating peptide anti-VEGF formulations for the treatment of age-related macular degeneration

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11 **Stability of Cell-Penetrating Peptide anti-VEGF Formulations for the**

12 **Treatment of Age-Related Macular Degeneration**

13 *Aim:* The development of a polyarginine cell-penetrating peptide (CPP) could

14 enable the treatment of age-related macular degeneration, with drugs like

15 bevacizumab, to be administered using eye drops instead of intravitreal

16 injections. Topical formulations have a vast potential impact on healthcare by

17 increasing patient compliance while reducing the financial burden. However, as

18 the ocular preparations may contain several doses, it is essential to understand the

19 stability of the bevacizumab+CPP conjugate produced.

20 *Materials and Methods:* In this work, we examine the stability of a bevacizumab

21 solution with and without cell-penetrating peptide using dynamic light scattering

22 and circular dichroism to assess the physical stability. We use HPLC to assess the

23 chemical stability and ELISA to assess its biological activity. We also examine

24 the potential of the CPP to be used as an antimicrobial agent in place of

25 preservatives in the eye drop.

26 *Results:* The structural stability of bevacizumab with and without the CPP was

27 found not to be affected by temperature: samples stored at either 20 °C or 4 °C

28 were identical in behavior. However, physical instability was observed after five

29 weeks, leading to aggregation and precipitation. Further investigation revealed

30 that the addition of the polypeptide led to increased aggregation, as revealed

31 through dynamic light scattering and concentration analysis of the peptide

32 through HPLC. Complexing the bevacizumab with CPP had no effect on

33 biological stability or degradation.

34 *Conclusions:* Our findings suggest that the shelf life of CPP+bevacizumab

35 complexes is at least 38 days from its initial formulation. Currently, the

36 mechanism for aggregation is not fully understood but does not appear to occur

37 through chemical degradation.

38 Keywords: Ocular drug delivery, stability, eye drops, Cell-penetrating peptide

39 (CPP); vascular endothelial growth factor (VEGF)

40

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

64   into the pharmaceutical stability of such formulations, which is essential for the

65   progression of this work towards the clinic.

66   All medicinal products, regardless of formulation, should be manufactured to a high

67   standard to ensure patient safety and efficacy [13,14]. Ophthalmic products are more

68   challenging to formulate than the majority of medicines because they often require

69   multiple doses, therefore increasing the risk to patient safety. Thus, the shelf life of a

70   product of this nature is of increased importance as the drug will be expected to remain

71   stable throughout the specified treatment regimen.

72   In this work, we address questions surrounding the effects of CPP on bevacizumab by

73   examining the physical, chemical and biological stability of a bevacizumab+CPP

74   complexes using the techniques described within the NHS Quality Assurance

75   Committee Stability protocols [15,16]. This work will provide the basis for the progression

76   of this impactful advance in the delivery of a key treatment for one of the leading causes

77   of blindness.

78

79   **Materials and Methods**

80   CPP (NH$_2$- RRRRRR-COOH, CPP) was procured from Genscript, New Jersey.

81   Bevacizumab (Roche Pharmaceuticals, Welwyn Garden City) at 25 mg/mL was used as

82   supplied. 10 mg of CPP was diluted into 2 mL aliquots of bevacizumab to produce

83   solutions containing 5 mg/mL CPP in 25 mg/mL of bevacizumab to produce the

84   bevacizumab+CPP complexes.

85   *Physical Stability*

86   Dynamic Light Scattering (DLS) and visual inspections were used to determine the

87   physical stability of the bevacizumab with and without CPP. Bevacizumab+CPP

88   solutions were stored either at room temperature or at 4 °C for up to 57 days. DLS was

89   carried out using a Nanoseries Zetasizer at 658 nm, set at 20 °C on 0.5 mL solution

90   aliquots. Each sample was recorded five times and in triplicate, with the analysis

91   completed using the Nanoseries software based on a refractive index of 1.33 and

92   viscosity of 0.89 cP. No dilution of the bevacizumab was required before scanning. The

93   CPP titration was performed by titrating 1 µL aliquots of a 200 mg/mL CPP solution.

94   *Chemical and Structural Stability*

95   Chemical stability was examined using HPLC. Six samples were prepared and stored as

96   described above. All samples were run at a flow rate of 1 ml/min, and the column and

97   mobile phases kept at a constant temperature of 25 ºC. Aliquots were run on an Agilent

98   Infinity II series 1290 analytical HPLC with an Aeris Widepore C18-XB RP-HPLC

99   column (250 x 21.2 C18 3.6 µm 200 Å Axia packed) in water. The gradient solvent

100   system used consisted of solvent A (water + 0.05% trifluoroacetic acid) and solvent B

101   (water + 0.1 % trifluoroacetic acid). The gradient was run from 0-100% B over 40

102   minutes, with the absorbance monitored at 280 nm. Calibration samples were run from

103   fresh bevacizumab alone, CPP alone, and bevacizumab+CPP stock solutions at

104   concentrations ranging from 0 – 6.25 mg/mL for bevacizumab and 0 – 5 mg/mL for

105   CPP. Uridine was run as an internal standard. The method gave a limit of quantification

106   of 0.0856 mg/mL for bevacizumab and 0.172 mg/ml for CPP, and precisions of 3.48 %

107   and 12.0 % for bevacizumab and CPP respectively. Test samples were prepared from

108   stock by diluting 30 µL of stock solutions into 200 µL of ultrapure water. The

109   concentration of the CPP and of bevacizumab analyzed by the peak area ratio between

110   the analyte and the internal standard and the concentration confirmed by reference to the

111   calibration curve. Samples were tested over the length of the study.

112   The bevacizumab samples with and without CPP were examined for structural stability

113   using circular dichroism (CD) spectroscopy. This analysis is key when studying

114   biopharmaceuticals as their structure and conformation, and thus biological activity, can

115   be changed by chemical and physical changes. CD spectra of bevacizumab and

116   bevacizumab+CPP were recorded on a Jasco J-715 spectropolarimeter, and scans were

117   recorded across 260-190 nm. The scans were baseline corrected, and readings averaged

118   over five replicates. The absolute ellipticity was recorded in millidegrees and converted

119   to mean residual ellipticity. CD spectra for the stability samples were recorded over

120   eight weeks, and the intensity of the minima at 218 nm plotted as a function of time. To

121   determine differences in degradation with and without the peptide, samples were

122   prepared by dissolving TCEP into both bevacizumab and bevacizumab+CPP to a

123   concentration of 5 mg/mL. These samples were then incubated at room temperature

124   overnight. An additional baseline correction, containing 5 mg/mL TCEP was applied to

125   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.

149 **Results**

150 *Physical Stability*

151 In order to assess the impact of CPP on the physical stability of bevacizumab, the

152 solutions were stored at either 4 °C or room temperature and monitored for changes.

153 The bevacizumab only and CPP only solutions showed no visual changes over 57 days

154 when stored at either 4 °C or room temperature. When formulated with CPP, the

155 bevacizumab solutions showed no changes when stored at either temperature for the

156 first six weeks. However, by day 57, varying amounts of precipitate were observed in

157 all samples. This was then probed using Dynamic Light Scattering (DLS). DLS analysis

158 of the bevacizumab+CPP samples showed no change in particle distribution for up to 38

159 days (16.8 nm ± 5.0 nm) regardless of storage condition (Table 1). This matches

160 literature data which showed stability up to 30 days[18]. This size is consistent with those

161 found previously [19] for the size distribution of fresh bevacizumab suspensions.

162 However, by 57 days, particle formation was observed by DLS (Table 1). Physical

163 stability does not extend beyond six weeks due to the formation of excipient-induced

164 aggregates caused by the presence of CPP. Titrating increasing amounts of CPP into

165 bevacizumab showed no significant change in particle size up to 5 mg/mL, which is the

166 concentration used in the bevacizumab+CPP complex solution here (Summarized in

167 Supplementary Data Table S1).

168 *Chemical Stability*

169 HPLC chromatograms of the bevacizumab and CPP content within the

170 bevacizumab+CPP samples showed a reduction in the solution concentrations of both

171 over time, which is in agreement with visual inspection of precipitate formation.

172 However, there was a notable sample to sample variation. Thus, the mean change in

173   bevacizumab and CPP over the study period was not statistically significant (Figure 1).

174   The change in concentration of individual samples can be found in Supplementary Data

175   (Figures S1-S4).

176   No degradation peaks were observed in any of the HPLC chromatograms, suggesting

177   that the loss in concentration is due to physical instability, not to chemical degradation.

178   Regardless of storage condition, the CD spectra did not change over the length of the

179   study period (Figure 2a). This observation indicates that the structure remains

180   unchanged, and demonstrates the high structural stability of bevacizumab, which is in

181   agreement with the HPLC results. This finding is also in agreement with previous

182   findings that showed bevacizumab samples were stable for at least six months [20]. When

183   degradation was instigated by incubation with TCEP there was a significant change in

184   the spectra compared to the original samples. However, there were no significant

185   differences between the bevacizumab alone or the bevacizumab+CPP in the presence of

186   TCEP (Figure 2b). Additionally, on titrating increasing amounts of CPP into

187   bevacizumab, the CD spectra remained invariant after correcting for the spectrum of the

188   CPP alone. Data can be found in the Supplementary Information (Figures S5-6). The

189   invariance of the ellipticity indicates that the association of CPP and bevacizumab is via

190   physical interaction (such as hydrogen bonding) rather than a chemical one, which

191   would have resulted in a change in protein conformation.

192   ***Biological Stability***

193   The concentration of bevacizumab was measured using ELISA to determine biological

194   stability. Over the six week period, we saw no significant differences between

195   bevacizumab alone or bevacizumab complexed with CPP (Figure 2c). Over the 56 day

196   period we did see a slight, but not statistically significant, trend to a higher

197   bioavailability in both bevacizumab alone and bevacizumab complexed with CPP over

198   time. These data show that when CPP is complexed to bevacizumab there is no effect

199   on the biostability of bevacizumab.

200   *Antimicrobial Efficacy*

201   Previous work has shown that CPP demonstrates some antimicrobial efficacy, therefore

202   we hypothesized that CPP could potentially replace preservatives in the

203   bevacizumab+CPP eye drops. Solutions of the model organism *S. aureus* were

204   incubated with CPP over a time course of 14 days and CFU were quantified at regular

205   intervals. The CPP solutions showed antimicrobial properties against *S. aureus* NCTC

206   8532, with an average log reduction of 4 after 24 hours compared to t=0 (Figure 3a).

207   This reduction is significantly greater than the required 3 log reduction stated within the

208   British Pharmacopoeia requirements [17]. Furthermore, after seven days, no bacteria were

209   recovered from the samples of CPP. However, the efficacy was significantly reduced

210   upon the addition of bevacizumab (Figure 3a). This trend may have occurred due to

211   preferential binding of the arginine sequence to the MAb over the cell membranes of the

212   bacteria. However, against a strain of *S. aureus* isolated from a clinical sample, CPP

213   showed limited antimicrobial activity (Figure 3b). Only a log reduction of 1 was

214   observed at 14 days, suggesting that the antimicrobial efficacy of CPP will not be able

215   to replace preservatives in a real-world application of the bevacizumab+CPP eye drops.

216

217   **Conclusion**

218   We have used a range of different methods to determine the stability of

219   CPP+bevacizumab complexes. While the chemical data shows no degradation over the

220   eight-week study period regardless of CPP addition, the physical stability data

221   demonstrates significant precipitation at 6 weeks. Based on DLS data our findings

222   suggest that the shelf life of CPP+bevacizumab complexes is at least 38 days from its

223   initial formulation. The antimicrobial data suggests that although there is limited

224   efficacy of the CPP against some bacterial strains, this is not robust and preservatives

225   would need to be included in bevacizumab+CPP eye drops.

231   **Declaration of Interest**

232   The authors report no conflicts of interest.
233

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301

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.

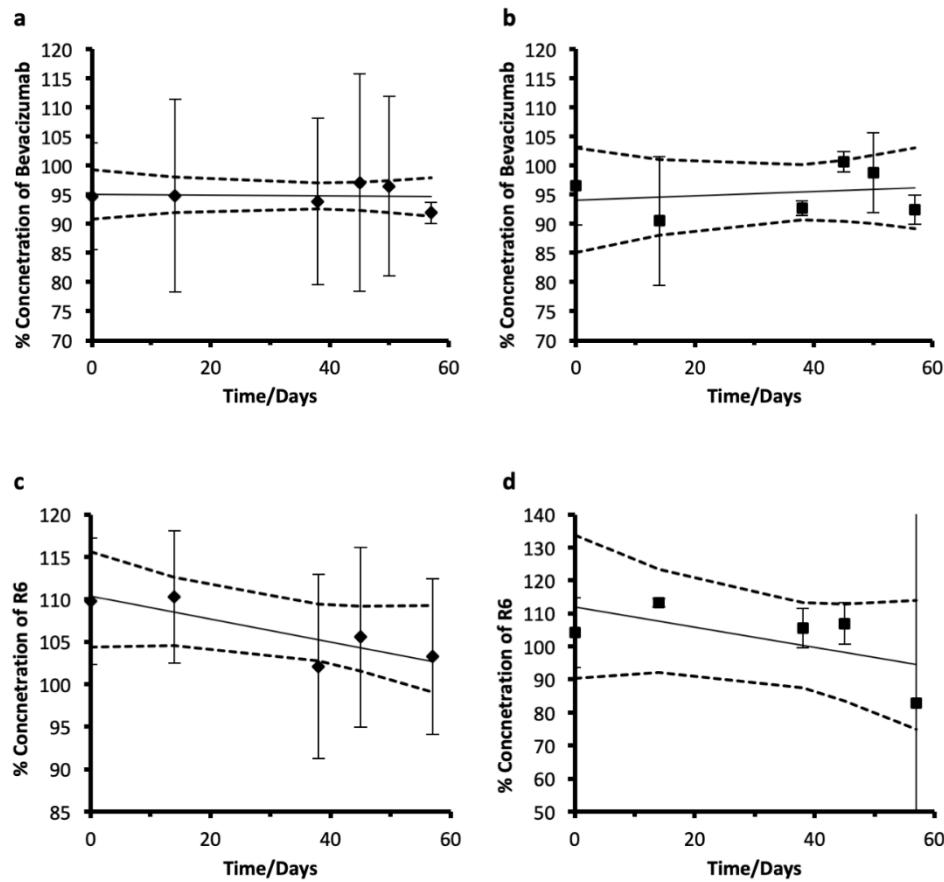| Time (Days)	Mean particle diameter (nm)
0	16.8 ± 5.0
28 (4 °C)	15.8 ± 3.5
38 (4 °C)	15.9 ± 4.0
57 (4 °C)	16.7 ± 4.7 (94 %), 624.2 ± 163.5 (6 %)
57 Room temperature	16.4 ± 4.3 (93 %), 894.3 ± 280.4 (7 %)

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
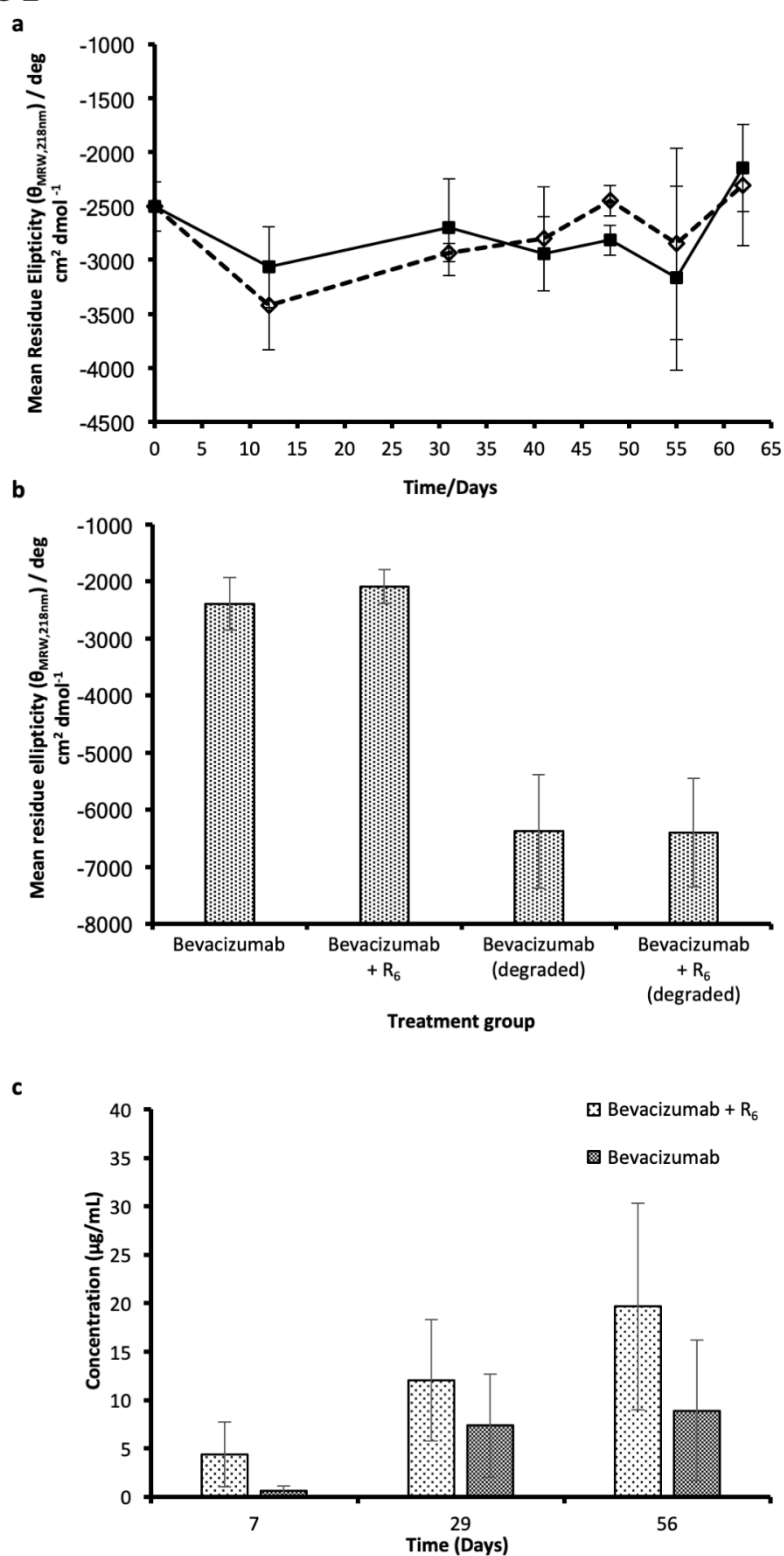
319
320 Figure 2: The average mean residue ellipticity a) of bevacizumab+CPP samples

321 measured at 218 nm of samples stored at 4 °C (open diamond markers) or room

322 temperature (solid square markers) over 63 days. All errors are based on 95 %

323 confidence intervals of averaged data for six samples in each condition. b) bevacizumab

324 and bevacizumab+CPP samples degraded through incubation with TCP, n=3 error bars

325 show standard deviation. All circular dichroism spectra were recorded at 20 °C in a 1

326 mm path length cuvette. c)The mean concentration of bevacizumab in samples

327 measured using ELISA, n=3 error bars show standard error of the mean.
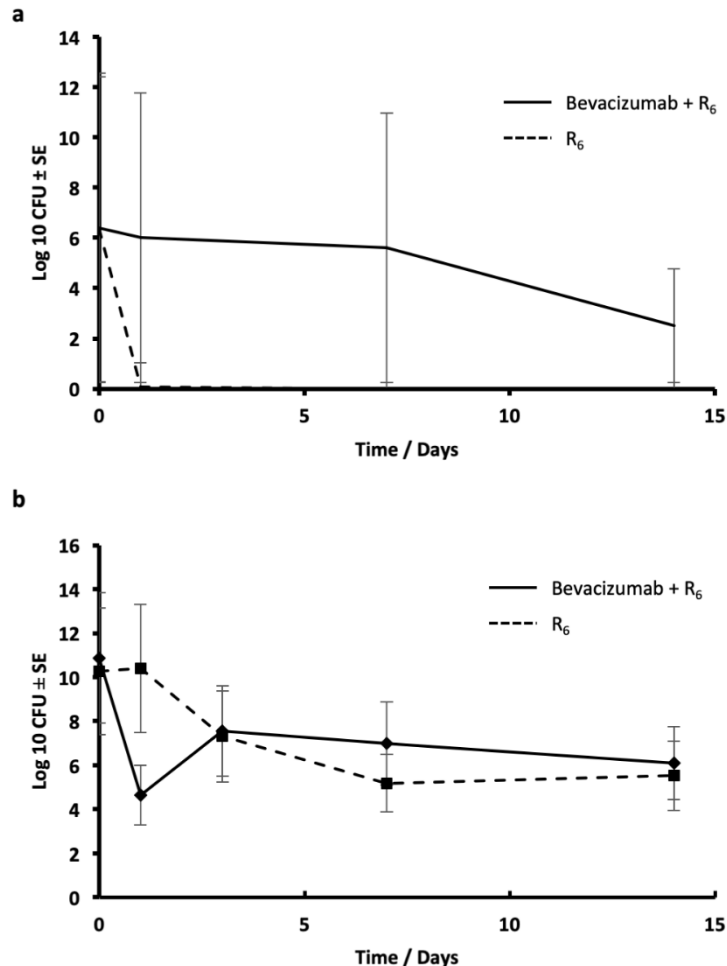
Figure 3

328

329 Figure 3: Antibacterial efficacy testing of CPP (CPP only, dashed line) and

330 bevacizumab+CPP samples (solid line) in triplicate with error bars showing the standard

331 deviation of the mean. a) Number of colony-forming units (CFU) of *S*. aureus NCTC

332 8532 for CPP and bevacizumab+CPP samples over 14 days. b) Antibacterial efficacy

333 testing showing the number of CFU of *S. aureus* isolated from clinical infections for

334 CPP and bevacizumab+CPP samples over 14 days.

335