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Research article

**CIPROFLOXACIN LOADED VASCULAR PROSTHESES FUNCTIONALIZED
WITH POLY-METHYLBETA- CYCLODEXTRIN: THE IMPORTANCE OF IN
VITRO RELEASE CONDITIONS**

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27 **Abstract**

28 Synthetic Vascular Graft Infection (SVGI) can be very serious for patients with dramatic
29 consequences (up to 6 %). Polyester vascular grafts (PET) were modified with polymerized
30 cyclodextrin (Poly-Me β CD) and loaded with ciprofloxacin (CFX) for the prevention of
31 postoperative infections. The aim of this study was to investigate the CFX/ Poly-Me β CD
32 interactions and the importance of the type of the dissolution technique. The solubility of CFX
33 was significantly improved upon Poly-Me β CD, and the interaction between CFX and Poly-
34 Me β CD were observed by NMR (Nuclear Magnetic Resonance). Drug release was measured
35 in phosphate buffer saline pH 7.4 at 37 °C using: (i) agitated flasks, (ii) the paddle apparatus,
36 (iii) the conventional flow-through cells, (iv) the modified flow-through cells with agarose gel
37 at different flow rates. Importantly, CFX release depends on the flow rate as well as the
38 experimental set-up *in vitro*. CFX release from virgin prostheses (PET) was faster than from
39 functionalized prostheses (PET-Me β CD), irrespective of the flow rate, which indicates the
40 superiority of Poly-Me β CD in the control of CFX release. The CFX diffusion from PET-
41 Me β CD into agarose gel showed a continuously progressive diffusion during 7 days. Thus, this
42 test can be highly appropriate for *in vitro* characterization of such drug delivery systems.

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47 *Keywords:* Vascular prostheses, modified flow-through cell, ciprofloxacin, Poly-Me β CD, PET-

48 Me β CD, controlled drug delivery, diffusion test

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52 **1. Introduction**

53 Synthetic Vascular Graft Infection (SVGI) occurs for up to 6 % of the surgical acts but can be
54 very serious for patients [1,2,3] as the mortality rate reaches 25-88 % and the risk of amputation
55 is very high (21 %) [4]. The applied treatment consists in removing the infected prosthesis and
56 debridement of the surrounding tissues. Thus, the replacement of the prosthesis is ensured by
57 drug coated prostheses, biosynthetic prostheses [5], homografts or xeno-pericardial aortic tube
58 grafts [6,7]. In addition to drug coated prostheses, antibiotics and silver loaded grafts are widely
59 used as bioactive agents in order to reduce the risk of postoperative infections.

60 Vascular graft impregnated with rifampicin have shown interesting results in the
61 treatment of SVGI [8]. The antibacterial efficacy of rifampicin or rifampicin/tobramycin loaded
62 prostheses was demonstrated by infected dog model [9,10]. However, the spectrum of activity
63 of this combination of antibiotics is limited to gram positive bacteria, and thus, clinical results
64 in terms of efficacy and integration have shown controversial effects [11,12]. Compared to
65 antibiotic loaded grafts, the efficacy of silver, however, is much lower as expected
66 [13,14,15,16]. Nevertheless, the silver acetate coating seems to promote endothelialisation of
67 the graft without inducing host tissue inflammation [17,18]. Interestingly, the combination of
68 silver with triclosan have shown a significantly improved activity against gram negative
69 bacteria, gram positive bacteria as well as fungi species [2,19,20].

70 Unfortunately, drug is rapidly washed by the bloodstream in the case of conventional
71 antibiotic loaded grafts (< 24 hours), which can lead to less efficacy of the treatment [21,22,23].
72 To overcome this restriction, modified polyester-based vascular grafts have been developed
73 [24,25]. The concept is to functionalize the prostheses with polymer of cyclodextrins (Poly-
74 CD) in order to increase the antibiotic-loading capacity and subsequently provide locally
75 sustained drug delivery during the critical postoperative period. The functionalized prostheses
76 with Poly-CD should deliver large amount of loaded antibiotics such as rifampicin, vancomycin
77 and particularly ciprofloxacin [26,27]. Polyester vascular grafts loaded with the appropriate

78 antibiotic can be efficient against a large variety bacterial species, which are currently involved
79 in human SVGI. These bacteria are especially gram-positive organisms (75-80 % of SVGI)
80 from the endogenous flora (e.g. *S. aureus* and *S. epidermidis*) and gram negative bacteria (e.g.
81 *E. coli*, *P. aeruginosa* and *E. cloacae*, 20-25 % of SVGI) [28]. Ciprofloxacin is an antibiotic,
82 which is already used in our previous studies to treat large spectre of bacteria (gram + and gram
83 -) with vascular grafts polymerized with cyclodextrins [24,25] and also in order to be
84 homogenously and consistently with these studies. Furthermore, it has been shown using
85 fluorescence spectroscopy, ¹H-NMR, and other physical analysis that ciprofloxacin interacts
86 with βCD or HPβCD, leading to the formation of a stable 1:1 stoichiometric complex in solution
87 and also the physical state of the drug into the cavity of the cyclodextrins [29,30].

88 However, one of the major hurdles for the development of this type of advanced drug
89 delivery systems is the lack of an established *in vitro* drug release test. In the field of synthetic
90 vascular graft, different techniques were used to study the *in vitro* drug release behaviours.
91 Unfortunately, there is no established dissolution test, which can be found in the
92 pharmacopoeias for the characterisation *in vitro* of grafted vascular prosthesis or drug eluting
93 stents. Nowadays, closed flasks containing the release medium will be used in order to study
94 the drug release profile *in vitro*. Different techniques have been described in the literature using
95 various drugs into vascular prosthesis or drug eluting stents. Grafted vascular prosthesis
96 “Dacron prosthesis: a polyester fiber” containing antibiotics such as vancomycin, rifampicin,
97 gentamicin as well as ciprofloxacin have been already evaluated *in vitro* as well as *in vivo*, and
98 compared with commercially marketed products. For instance, either vascular grafts release the
99 total drug (100 %) within 48h or the drug remains into the prosthesis for a long period, which
100 can retard the treatment efficacy or even the failure of the treatment [31,32]. Importantly, these
101 systems should release drug during approximately one week and surely evaluated with an
102 appropriate *in vitro* dissolution test. Other antibiotics have been used and recommended for the
103 treatment of prosthetic vascular graft infections but administered either orally or intravenously.

104 However, these routes of administration could be very long and could affect the vitality of
105 patients [33].

106 The measurement of the antibiotic relative release rate from vascular graft was
107 determined using UV-spectrophotometry after a predetermined time in-human plasma (37 °C,
108 pH 7.4) but using static conditions [34] or in different simple release media (water, phosphate
109 buffer) [24]. Furthermore, the antibacterial activity of the antibiotic loaded graft has been also
110 assessed using agar gel diffusion test at predetermined time interval upon exposure to phosphate
111 buffer [21] or human plasma [25]. Recently, homemade dynamic systems were developed to
112 mimic blood flow with systolic-diastolic flow [35], continuous flow (35 ml/min) [36] or shear
113 and friction forces [37]. The development of vessel-simulating flow-through cell were deeply
114 studied for the investigation of bio-relevant drug-eluting stent and also to simulate the local
115 drug delivery for such site-specific drug delivery in vitro [38,39,40,41,42].

116 The aims of this study were to (1) evaluate ciprofloxacin delivery from poly-methyl beta
117 cyclodextrin (Poly-Me β CD) functionalized vascular prostheses in vitro, and (2) identify a
118 clinically relevant dissolution apparatus/test for such medical devices. In vitro drug release
119 profile was measured according to different systems and conditions in table 1:

120 (i) Agitated flasks semi-dynamic conditions, (ii) the paddle apparatus (USP apparatus 2) semi-
121 dynamic conditions, (iii) conventional flow-through cells (USP apparatus 4) at 5 to 35 mL/min
122 flow rate, and (iv) modified flow-through cells, better mimicking *in vivo* conditions at
123 35 mL/min, according to [38]. Drug diffusion into the tissue has been simulated in this study
124 by agarose gel- diffusion test.

125

126 **Materials and methods**

127 **2.1. Materials**

128 Woven poly(ethylene terephthalate) vascular prostheses (Polythese[®], PET; Perouse
129 Médical, Ivry-Le-Temple, France). Methyl beta- cyclodextrin (Me β CD, Crysmeb[®], substitution
130 degree = 0.5; Roquette, Lestrem, France). Citric acid (CTR) and sodium dihydrogen
131 hypophosphite (NaH₂PO₄, 2H₂O) (Aldrich chemicals, Saint Quentin Fallavier, France).
132 Ciprofloxacin base powder (CFX base; INRESA, Bartenheim, France) and ciprofloxacin
133 hydrochloride solution (CFX: 200 mg/100mL; Kabi, Paris, France). Gen Agaroseose LE
134 (agarose powder; Genaxxon BioScience, Ulm, Germany). Acetic acid glacial (Fisher Scientific,
135 Meadow Road, Loughborough, UK). Demineralized water ultrapure (18.2 M Ω , Milli-Q system,
136 Millipore, France).

137 Anionic water-soluble Poly-Me β CD was synthesized according to a method that is already
138 patented [43]. 10 g Citric acid as crosslinking agent, 3 g sodium hypophosphite as catalyst
139 and 10 g Me β CD were dissolved in 100 mL demineralized water. The powder mixture was
140 obtained after removing water by rotative evaporator and drying at 140 °C during 30 min under
141 vacuum. A suspension of the obtained dried powder mixture and water was prepared and
142 afterward dialyzed during 72 h in water using 6-8 kDa membranes (SPECTRAPOR 1,
143 Spectrumlabs; Fisher Scientific, Meadow Road, Loughborough, UK). Finally, the water-
144 soluble anionic cyclodextrin polymer (Poly-Me β CD) was freeze- dried. The obtained Poly-
145 Me β CD contained 74 % (w/w) of the pure Me β CD, which was determined by ¹H NMR.
146 The calculated carboxylate group's content was 2.7 \pm 0.3 mmol/g. The molecular weight of
147 Poly Me β CD was 8000 g/mol, that was measured by size exclusion chromatography (SEC) in
148 water equipped with a light scattering detector [44].

149

150

151

152 **2.2. Preparation of grafted prostheses containing ciprofloxacin**

153 The cyclodextrin (CD) grafting process is based on the pad-dry-cure textile finishing method
154 previously published and patented [45]. The PET prostheses were impregnated and roll-
155 squeezed in an aqueous solution containing Me β CD, catalysts (NaH₂PO₄·2H₂O) and citric acid
156 (CTR). Functionalization occurred in a thermo-fixation oven (Minithermo[®], Roaches, UK) at
157 160 °C during 30 minutes. Afterwards, prostheses are thoroughly washed with water by soxhlet
158 extractor for 3 hours in order to remove the unreacted products. The cyclodextrin modified
159 prostheses (PET-Me β CD) used in the present study show a functionalization rate of 18 ± 0.5 %
160 (w/w), which are determined from the weight gain of samples upon functionalization according
161 to our previous paper [24].

162 Functionalised PET prostheses samples were loaded by dipping into CFX (200 mg/100
163 mL) during 4 hours at room temperature under shaking (240 rpm). Samples were rinsed one
164 minute with demineralized water in order to remove the excess of antibiotic solution
165 (nonspecific interactions between PET-Me β CD coating and CFX). The prostheses were dipped
166 in 0.05 M NaOH (4h, 240 rpm, 37 °C) in order to hydrolyse the Poly-Me β CD coating and
167 thereby release CFX that interacted with this coating. According to our previous article, the
168 amount of CFX on virgin PET and PET-Me β CD was 6.3 ± 0.4 mg/g, and 45.7 ± 4.6 mg/g,
169 respectively [25].

170

171 **2.2. Solubility study**

172 Various concentrations of Me β CD and Poly-Me β CD solutions were prepared in demineralized
173 water covering a wide range of concentrations from 2 to 20 mg/mL. The pH was adjusted to
174 5.5 to avoid the influence of pH on drug solubility. CFX base (INRESA) powder was added in
175 excess (4 mg/mL) to the solutions under stirring (240 rpm) for 24 h at 37 °C (horizontal shaker,
176 Innova 40[®], Montesson, France). Solutions were filtered with 0.45 μ m cellulose membrane
177 (VWR syringe filters of cellulose, Fontenay sous Bois, France). Aliquots of the solutions were

178 diluted with demineralized water and analysed UV-spectrophotometrically for their drug
179 content.

180

181 ***2.3. Complexation study by NMR***

182 Interactions between Me β CD and CFX were observed by proton NMR in the case of
183 the “Poly- Me β CD” as well as in the oligomer “Me β CD” . All experiments were recorded using
184 a spectrometer AV NEO 900 21.1 T (proton Larmor frequency of 900.23 MHz). A 5 mm
185 CPTCI 1H-13C/15N/D Z-GRD cryoprobe was used in this study. Two-dimensional ROESY
186 experiments [46] was used to determine the orientation of the guest molecule into the Me β CD
187 cavity and were acquired in the phase-sensitive mode. The probe temperature was regulated to
188 300 K. Each spectrum consisted of a matrix of 32 K (F2) by 32 K (F1) covering a sweep width
189 of 9090 Hz. Spin-lock mixing periods of 200 ms has been used in this work. The Qsine
190 apodization functions were applied in both dimensions prior to Fourier transformation. 304
191 increments were collected with 32 transients in each.

192 ***2.3. Adsorption kinetics experiments***

193 The vascular prostheses samples (\varnothing 10 mm, PET) were introduced in 10 mL of CFX
194 solution (2000 mg/L) during 5 min up to 240 min at room temperature with an agitation of
195 240 rpm (horizontal shaker, Innova 40[®], Montesson, France). Samples were removed, rinsed
196 with demineralized water (1 min) and dipped in 0.05M NaOH (4h, 240 rpm, 37°C). The total
197 amount of CFX loading onto the PET prostheses was measured by UV-Vis spectroscopy (UV-
198 1800 spectrophotometer, Shimadzu, Noisiel, France) at 271 nm.

199 For the isotherm experiments, PET prostheses were incubated during 240 min at room
200 temperature in 10 mL of CFX solution with concentrations varying from 67.5 to 2000 mg/L.
201 Samples were then treated as mentioned above in order to determine the quantity of loaded
202 CFX (q_e , mg/g). The concentration of the solution at the equilibrium (C_e , mg/L) was calculated
203 from q_e . The adsorption isotherm was directly reported under its linearized forms according to

204 the Langmuir isotherm on the one hand

$$205 \quad \frac{Ce}{qe} = \frac{1}{K_L} + \frac{\alpha L Ce}{K_L} \quad \text{Eq. (1)}$$

206

207 where K_L (L/g) and αL are the Langmuir constants that allow the calculation of the theoretical
208 monolayer capacity, and the Freundlich isotherm on the other hand

$$209 \quad \ln(qe) = \ln(K_f) + \frac{1}{n} \ln(Ce) \quad \text{Eq. (2)}$$

210

211 where K_f (L/g) is the Freundlich constant and $1/n$ is the relative adsorption capacity
212 characteristic [47].

213

214 **2.4. *In vitro* drug release studies**

215 PET-Me β CD were placed in 10 mL of ciprofloxacin solution for 4 h and then dried
216 overnight at room temperature.

217 To determine the drug sorption, the PET-Me β CD were then exposed to 20 mL of
218 aqueous NaOH 0.1 M overnight, in order to remove the cyclodextrin polymers via hydrolysis,
219 thus releasing the remaining antibiotics and determining the practical drug loading. The amount
220 of antibiotic was measured by UV-spectrophotometry in the aqueous NaOH solution at $\lambda = 278$
221 nm

222 CFX release from PET-Me β CD was measured upon exposure to phosphate buffer saline
223 pH 7.4 at 37 °C, using a UV-spectrophotometer at 270 nm. Different drug release methods were
224 used (Table 1, Figure 1):

- 225 (i) Agitated flasks of 40 mL volume in a horizontal shaker 80 rpm, 37 °C (Innova 40,
226 Eppendorf, Hamburg, Germany). Briefly, tubular prostheses (\varnothing 10 mm, length 3
227 cm) loaded with CFX (200 mg/100mL, 4 hours) were introduced in the container
228 containing 20 mL of phosphate buffer saline pH 7.4. At predetermined time, 20 mL
229 (complete medium change) of the release medium were collected and replaced by

230 fresh medium respecting sink conditions. The media were measured for the drug
231 content with a UV-Vis spectrophotometer (UV-1800, Shimadzu, Noisiel, France) at
232 270 nm (Figure 1A).

233 (ii) The paddle apparatus (Sotax smart AT7 dissolution tester, Aesch Switzerland).
234 Briefly, tubular prostheses (\varnothing 10 mm, length 10 cm) loaded with CFX (200
235 mg/100mL, 4 hours) were introduced in the apparatus containing 900 mL of
236 phosphate buffer saline pH 7.4 under 80 rpm agitation. At predetermined time,
237 10 mL of the release medium were collected and replaced by fresh medium
238 respecting sink conditions. The media were measured for the drug content with a
239 UV-Vis spectrophotometer (UV-1800, Shimadzu, Noisiel, France) at 270 nm
240 (Figure 1B).

241 (iii) Conventional flow-through cells (CE 7 Smart dissolution apparatus, Sotax, Aesch,
242 Switzerland) corresponding to USP 4. Briefly, tubular prostheses (\varnothing 10 mm, length
243 3 cm) loaded with CFX (200 mg/ml, were introduced in the apparatus (specific cell
244 22.6 mL). Different flow rates have been tested in closed system mode (5, 10, 20
245 and 35 mL/min) as well as in open system mode (5 mL/min). In the first case, the
246 media was analysed online at 270 nm by UV-Vis spectroscopy (Lambda 25
247 spectrophotometer, Perkin Elmer, Waltham, USA) connected to a reservoir release
248 medium (PBS, 900 mL) as well as to the flow-through cells “closed modus”. In the
249 second case, all the media was collected every 20 minutes and measured for the drug
250 content with a UV-Vis spectrophotometer (Lambda 25 spectrophotometer, Perkin
251 Elmer, Waltham, USA) at 270 nm (open modus) (Figure 1C).

252 (iv) Modified flow-through cells mimicking *in vivo* conditions. According to [38], a
253 modification of compendia flow-through cell was adapted as illustrated in Figure
254 1D). The tubular prosthesis (\varnothing 10 mm, length 3 cm) was fixed into the cell on a
255 plastic disc, which serves as a sealing ring to prevent leakage at both ends of the

256 graft samples. Agar powder was dissolved in boiling water to prepare 0.6 % (w/w)
257 agar solution, which was cooled (40 °C) and poured in the free volume outside of
258 the prosthesis so that sol-gel transition occurred rapidly. The graft lumen remained
259 free for the medium (PBS pH 7.4) circulation. The release medium flowed through
260 the prosthesis lumen in order to mimic the blood flow arterial vessel while the agar
261 gel simulated the surrounding tissues that are exposed to the outer surface of the
262 artificial graft. The bloodstream is represented by PBS solution, which is pumped
263 with a peristaltic pump through the prosthesis lumen at 35 mL/min by closed
264 systems, mimicking the physiological conditions. The media was analysed online
265 by the UV-Vis spectroscopy (Lambda 25 spectrophotometer, Perkin Elmer,
266 Waltham, USA) which was connected to a reservoir release medium (PBS, 900 mL)
267 as well as to the flow-through cells “USP IV apparatus”.

268 (v) Drug diffusion into agarose gels was carried out as follows:

269 Functionalized or virgin PET prostheses were placed in 10 mL of ciprofloxacin
270 solution for 4 h and then dried overnight at room temperature. Please note that the
271 implants were then exposed to 20 mL of aqueous NaOH 0.1 M overnight, in order
272 to remove the cyclodextrin polymers via hydrolysis, thus releasing the remaining
273 antibiotics into the prostheses for the measurement of the practical drug loading.
274 The amount of antibiotic was measured by UV-spectrophotometry in the aqueous
275 NaOH solution at $\lambda = 278$ nm.

276 Prostheses were placed in 10 mL of ciprofloxacin solution for 4 h and then dried
277 overnight at room temperature.

278 Agarose powder was dissolved in boiling water to prepare 0.6 % (w/w) agarose solution. The
279 pH of the agarose solution was 7.0 ± 0.5 at 37 °C. According to Hoang et al. [48] the prostheses
280 “Ø 10 mm” were placed into the agarose gel (0.6 % w/w) as follows: a half of the required
281 agarose solution was cast into a Petri dish (9 cm in diameter) allowing for gel formation. After

282 solidification of the gel, the implant “functionalized or not “was placed at the center of the gel
283 and the remaining solution (40 – 45 °C) was carefully cast onto the first layer allowing for gel
284 formation. After solidification of the gel, the Petri dish was then placed into a water-filled
285 desiccator in an incubator (non-agitated, n = 3; GFL 3033, Gesellschaft fuer Labortechnik,
286 Burgwedel, Germany) at 37 °C to prevent water evaporation during the experiments. At
287 predetermined time points, cylindrical gel samples were removed at various distances from the
288 implant using a glass tube of 5 mm in diameter (Figure 3E). The samples were weighed and
289 analyzed for their drug content by HPLC as follows: the gel was dissolved in 2 mL of water at
290 100 °C and dispersed using Vortex mixer at 3000 rpm for 5 min (FB15012 Topmix Shaker,
291 Fisher Scientific GmbH, Schwert, Germany). The dispersions were filtered using a PVDF
292 syringe filter (0.45 µm, Millex-HU, Merck Millipore, Tullagreen, Ireland) prior injection into
293 the HPLC system. A Thermo Fisher Scientific Ultimate 3000 Series HPLC, equipped with a
294 LPG 3400 SD/RS pump, an auto sampler (WPS-3000 SL) and a UV-Vis detector (VWD-
295 3400RS) (Thermo Fisher Scientific, Waltham, USA). The separation was performed on a C18
296 column (Kintex EVO 100 A°, 250 x 4.6 mm; 5 µm; Phenomenex, Le Pecq, France); at 30 °C.
297 The mobile phase was 87:13 (v/v) mixture of an aqueous acetic acid solution (50 mL/L) and
298 acetonitrile, and a flow rate of 1.5 mL/min was used. The effluent peaks were monitored at $\lambda =$
299 278 nm.

300

301 **Results and Discussion**

302 ***3.1. Solubility study***

303 Cyclodextrins are well known to be a good solubility enhancer for poorly water soluble drugs.
304 This can be very beneficial for the drug bioavailability and thereby the efficacy of the treatment.
305 As it can be seen in figure 1, ciprofloxacin solubility in water increased with the increase of
306 Poly-Me β CD concentration. It is evident that the polymerized cyclodextrin shows higher CFX-
307 solubility than the monomeric form (Me β CD). This can be attributed to the fact that drug
308 interacts better and in high amount with the polymer, which provides high surface available for
309 complexation. Moreover, the apparent binding constants were calculated as described
310 previously [49] for the Me β CD (monomeric form) as well as Poly-Me β CD (polymeric form) at
311 a pH of 5.5. Interestingly, the K-value of the polymer was much higher ($K_{1:1} = 793.76 \text{ M}^{-1}$) than
312 the one calculated for the monomeric form ($K_{1:1} = 55.92 \text{ M}^{-1}$). It has to be pointed out that this
313 increase of the binding constant can be probably explained by the ionic interactions between
314 the polymer and the drug. This behavior is already confirmed with a previous study [49].
315 Importantly, in addition to the hydrophobic interactions (cyclodextrin cavity) the polymeric
316 network shows hydrophilic interactions, which are highly electrostatic especially when the form
317 is under its ionic state.

318

319 ***3.2. Me β CD or Poly-Me β CD /Ciprofloxacin interaction***

320 The formation of an inclusion complex between α , β , γ and Me β CD with ciprofloxacin
321 has been previously studied using an Ultrashield 400 MHz Bruker spectrometer [24,25]. In all
322 cases, Job plots experiments showed that 1:1 complexes were formed by inclusion of the
323 piperazinyll group inside this cyclodextrin cavity. Though, using a 400 MHz spectrometer
324 superimposition of the signals of H₂ and H₆ protons of piperazinyll group and that of the H₂
325 proton of CD (situated around 3.6 ppm) could provoke a certain doubt on the dipolar
326 interactions between H₂ and H₆ protons of ciprofloxacin with the H₃ protons of the CD at 3.9

327 ppm. Therefore, it could be a handsome profit in this study to use the improved resolution of
328 the AV NEO 900 MHz spectrometer. In fact, the resolution of the 2D NOESY NMR spectrum
329 is clearly improved (Figure 2A and B) when comparing to 400 MHz [24], since protons H₂ and
330 H₆ of CFX are well separated from the H₂ signal of the Me β CD. As a consequence, 2D map
331 precisely displays a cross peak issued from the dipolar interaction between the H₂ and H₆
332 protons of piperaziny group (3.57 – 3.587 ppm) and the proton H₃ of Me β CD situated inside
333 the cavity (3.87 – 3.89 ppm) (Figure 2A and B).

334

335

336 **3.3. Ciprofloxacin sorption kinetic**

337 Figure 4 displays the sorption kinetic of ciprofloxacin onto PET-Me β CD modified samples
338 after impregnation in a 2 g/L CFX solution. A rapid sorption was observed within the first 30
339 minutes, followed by a plateau after 60 minutes of incubation, which indicates the typical
340 equilibrium of CFX sorption. Therefore, an equilibrium sorption time of 240 min was then
341 applied in the following isotherm experiments. Importantly, these medical devices are able to
342 adsorb sufficient amount of CFX that can be mandatory used for the antibacterial effect (45.7
343 ± 4.6 mg/g) as we previously demonstrated by a microbiological evaluation [25].

344 Figure 5a and Figure 5b show the amount of loaded CFX (Q_e) onto PET-Me β CD as a function
345 of the CFX concentration in the impregnation bath before equilibrium (CFX, mg/L) (Figure 5a)
346 and at the equilibrium (C_e , mg/L) (Figure 5b). As it can be seen on these figures, the maximal
347 amount of CFX loaded onto the prosthesis was 48.5 ± 3.4 mg/g, which was obtained after
348 incubation of the PET-Me β CD into the highest concentration of CFX solution (2g/L).

349 Figure 5b illustrates the amount of loaded CFX onto PET-Me β CD “ Q_e ” but in this case as a
350 function of the isotherm plot representing “ C_e ” that indicates the concentration (mg/L) of the
351 CFX in the solution at the equilibrium. The maximal amount of CFX loaded onto PET-Me β CD
352 was 48.5 ± 3.4 mg/g versus 6.3 ± 0.4 mg/g for the virgin prostheses. This outcome can confirm

353 the 7-fold improvement of CFX sorption due to the functionalization of the prostheses.
354 Importantly, the Langmuir model reported in Figure 5c shows a linear response ($r^2 = 0.9971$)
355 and the Langmuir constants were $q_o = 48.54 \text{ mg/g}$; $K_L = 1.05$; $\alpha_L = 0.02163 \text{ g/L}$. However, the
356 release profile with the Freundlich model is not well fitted as the correlation coefficient r^2
357 obtained was equal to 0.9091 (Figure 5d). Obviously, the Langmuir model is an appropriate
358 model for the sorption of ciprofloxacin onto PET-Me β CD (Figure 5c).

359

360 **3.4. *In vitro* ciprofloxacin release**

361 *In vitro*- drug release and setup are one of the most important standard operating systems
362 used in the quality control of pharmaceutical dosage forms prior testing in the animal or human
363 trials. For that reason, the way and manner of the drug release measurements will play a
364 major/distinct role anticipating the release kinetic and drug release behavior in simulated bulk
365 medium. Several equipment for *in vitro* drug release are described in united states
366 pharmacopeia, European pharmacopeia or Japanese pharmacopeia such as paddle apparatus
367 (USP 2) and flow-through cell method (USP 4). However, care should be taken when using
368 dissolution tests described in pharmacopeia for solid oral dosage forms or implanted medical
369 devices due to the non-realistic simulation of the pathophysiological conditions. The better we
370 will simulate the *in vivo* conditions, the more successful the accuracy of the drug release
371 prediction will be. Thus, there is a need of establishing clinically relevant *in vitro* dissolution
372 test for such drug delivery systems “Medical devices”. Figure 3 gives representative schema of
373 the different methods served for the *in vitro* characterization of this drug eluting vascular
374 prosthesis used in this study.

375 **3.4.1. Impact of the flow rate on ciprofloxacin release**

376 Figure 6a and 6b illustrate the relative and absolute ciprofloxacin release rate from
377 functionalized vascular prosthesis with Me β CD upon contact to phosphate buffer pH 7.4
378 according to USP 41 (USP 4). *In vitro* drug release measurements was conducted at various

379 flow rates (5, 10, 20, 35 mL/min) using the conventional flow-through cell apparatus. Clearly,
380 Poly-Me β CD network are able to control the drug release rate in phosphate buffer as it has been
381 shown in previous study [24]. Figure 6a show that the relative drug release rate increased
382 remarkably with increasing the flow rate of the release medium. For instance, 78 %
383 ciprofloxacin (40 mg/g vascular prosthesis) at a flow rate of 35 mL/min versus 71, 61, 57 %
384 (35, 32, 29 mg/g vascular prosthesis) at flow rates of 20, 10, 5 mL/min, respectively, was
385 released during 2 h upon exposure to phosphate buffer pH 7.4. This indicates that CFX loaded
386 vascular grafts were washed out rapidly when increasing the speed of the circulated medium in
387 the case of the conventional flow through cell apparatus, which was also confirmed by coated
388 drug-eluting stents with a mixture of Eudragit RL:Eudragit RS (3:7) at a flow rate of 35 mL/min
389 [38]. Clearly, the absolute drug release rate increased with increasing the flow rate due to the
390 increased shear forces caused by the high passage of the release medium on the prostheses
391 surface (Figure 6b).

392

393 **3.4.2. Impact of the open/closed system on ciprofloxacin release**

394 Figure 7 shows the relative and absolute release rate from functionalised vascular prostheses at
395 a flow rate of 5 mL/min using both modus (closed vs. open). The objective was to evaluate the
396 impact of the type modus on *in vitro* drug release kinetics. Figure 7a and 7b show that the flow-
397 through cells equipment open and closed modus at a flow rate of 5 mL/min did not affect the
398 relative release as well as the absolute release rates from the ciprofloxacin grafted vascular
399 prosthesis with respect to the nature of the coating materials. This can be attributed to the fact
400 that ciprofloxacin hydrochloride is highly soluble in the release medium. Importantly, this drug
401 release data confirmed the previous release data obtained in figure 6, which indicates the good
402 reproducibility of the studied grafted vascular prosthesis. For instance, 57 % ciprofloxacin was
403 released with the closed modus versus 56.5 % with the open modus after 2h, which corresponds
404 approximately to 29 mg/g vascular prosthesis.

405

406 **3.4.3. Impact of the method on ciprofloxacin release**

407 Figure 8 shows the relative (in percent) and absolute (in mg/g) release rate of
408 ciprofloxacin from functionalised vascular prostheses using conventional as well as modified
409 flow-through cell apparatus with closed modus upon exposure to phosphate buffer pH 7.4. For
410 reasons of comparison, the release profile from coated vascular prosthesis was tested during 48
411 hours by USP 2 and in agitated flasks, in order to evaluate the impact of the method for
412 dissolution test on drug release profile in such controlled drug delivery systems.

413 It is worth to mention that the flow rate of the flow-through cell equipment was
414 35 mL/min and the monographs of the USP, Ph. Eur. and JP. recommend flow rates of 30-35
415 mL/min. However, it is unknown if drug washout will be sustained during a long period. The
416 desired drug release profile should occur approximately 5-7 days after implantation in order to
417 overcome the risk of infection period after graft implantation (postoperative risk). In order to
418 measure the remaining drug on the implant, and diffused into the agarose gel that mimics the
419 tissues in contact with the outer face of the graft, ciprofloxacin quantification was assessed in
420 parallel on the prostheses itself and in the gel. Table 2 illustrates the CFX quantities remaining
421 into the gel as well as in the prosthesis (after 4h), that correlates well with the quantity released
422 into the bulk fluid, as 100 % of the initially loaded drug was recovered in the three media (PBS,
423 prostheses, gel). Interestingly, the CFX quantity present into the gel was negligible “1.38 %”
424 and also that are extracted from uncoated vascular prosthesis “0.94 %”. Again here, the effect
425 of cyclodextrin polymers as a carrier for controlled drug delivery from coated vascular
426 prosthesis was a determining factor in the release kinetic. It is to emphasise that the relative *in*
427 *vitro* drug release profile from coated vascular prosthesis with Poly-Me β CD upon modified
428 flow-through cell apparatus was remarkably slower than the conventional flow-through cell and
429 the paddle apparatus. For instance, only 66 % CFX (31 mg/g vascular prosthesis) was released
430 from functionalized vascular prosthesis when using the modified flow-through cell apparatus.

431 However, approximately 100 % CFX (47-51 mg/g vascular prosthesis) was released with the
432 conventional flow-through cell apparatus as well as with the USP 2 apparatus (paddle method),
433 whereas only 74 % CFX was released but if the grafted prostheses were incubated into the
434 agitated flasks. Importantly, the obtained results were confirmed when using drug-eluting stents
435 (DES) containing more potent drugs (e.g. immunosuppressant or cytostatics) [38]. As shown
436 previously, DES containing triamterene or fluorescein sodium show an accelerated release with
437 paddle apparatus as well as compendial flow-through cell [50].

438 The relative release rate of CFX was faster in the case of the virgin prostheses
439 (poly(ethylene terephthalate), PET) than the PET-prostheses functionalized with Me β CD,
440 irrespective of the flow rate (15 minutes vs 120 minutes at a flow rate of 5 mL/min). The PET-
441 Me β CD showed a controlled release of CFX with prolonged profile when using the modified
442 flow-through cell. For instance, 50.9 % and 85.9 % ciprofloxacin was released after 2h from
443 the PET-Me β CD coated prosthesis using the modified flow through cells and the conventional
444 USP method at flow rate of 35 mL/min, respectively.

445 Importantly, the reloading of the polymerized cyclodextrin on the grafted vascular prosthesis is
446 not occurred due to the presence of ions in phosphate buffer (release medium in vitro) and
447 proteins in blood stream (in vivo). The cavity of the cyclodextrin can be rapidly filled with ions
448 (Na⁺, Ca²⁺, Mg²⁺, phosphate etc.) as well as proteins (in blood) and replace the drug position
449 after drug release from the cyclodextrin- complex. Thus, ions and proteins can be considered
450 as a concurrent substance, which can clog the free cavity into cyclodextrins as soon as the drug
451 is released. It has to be pointed out that this phenomenon has been also confirmed in vitro
452 because of the increased drug release during the incubation period. Importantly, we did not
453 observe any reloading of the polymerized cyclodextrins with CFX. In the case of reloading, we
454 have had observed a level-off or a decrease in the absolute release profile.

455 Interestingly, the method of the agitated flasks shows slower release behaviour due to the lower
456 mechanical shear forces into the flasks caused by the paddles in the case of USP 2, for example.

457 Thus, the conditions as well as the method of the *in vitro* drug release measurements influence
458 the kinetic profile of the *in vitro* release data significantly. It is mandatory to check *in vitro* drug
459 release measurements in order to anticipate the release profile behaviour. Moreover, it has to
460 be pointed out that the USP apparatus 2 is not the appropriate method for the quality control of
461 functionalized vascular prosthesis under these conditions. From literature point of view, this
462 type of dissolution method will not be appropriate for the drug release prediction *in vitro* of
463 drug eluting stents [38,50]. In order to be closer to the patho-physiological conditions, flow-
464 through cell apparatus (conventional and modified) could be interesting to this issue in order to
465 investigate correctly and upon realistic conditions *in vitro* drug release from functionalized
466 vascular prostheses. All these information are very important data prior to test such controlled
467 drug delivery systems *in vivo*. However, in some cases the *in vitro*- *in vivo* correlation shows
468 discrepancy behaviour. Therefore, the choice of the dissolution test apparatus is of utmost
469 importance in order to obtain very accuracy prediction of drug release.

470 **3.5. CFX release test in agarose gel**

471 Since the *in vitro* dissolution test only serve as a quality control, other analysis such as
472 drug diffusion in agarose gel [48,51] could be also useful in the evaluation of the drug release,
473 and thereby reinforcing the accuracy of the quality control test. According to Huoang Thi,
474 samples were cut in small circles and embedded in agarose gel (0.6 % w/w). Gel samples were
475 withdrawn at predetermined time points and for their antibiotic content analysed using HPLC
476 system. Figure 9 shows ciprofloxacin concentration analysed after a predetermined time at
477 different distances away from the centre “prosthesis sample” of the Petri dish. It is evident that
478 the relative diffusion rate of CFX in the agarose gel from coated prosthesis with Poly-Me β CD
479 (Figure 9a) is significantly slower than from uncoated vascular prosthesis (Figure 9b). In the
480 case of virgin prosthesis, 30 μ g CFX per g vascular prosthesis was detectable after 6 h
481 incubation into the agarose gel at a distance of 1 cm from the prosthesis versus 22 μ g/g after
482 24 h incubation. This results in unmaintained drug concentration at different distances over a

483 period of time (24, 120, 168h). However, very low and undetectable CFX was analysed at
484 different distances from the uncoated vascular prosthesis (Figure 9a). Interestingly,
485 approximately 15 μg CFX per g vascular prosthesis coated with polymerized Methyl beta
486 cyclodextrin have been detected and maintained during 7 days at different distances from drug
487 source (Figure 9b). This can be attributed to the fact that drug release is controlled by the
488 polymeric network adsorbed onto the vascular prostheses. Importantly, drug diffusion into
489 agarose gel was maintained in a time-controlled manner during 7 days (the critical postoperative
490 period). However, drug concentration diffused from the virgin without Poly-Me β CD was in
491 some cases not detectable and after 24 h the diffused drug into the whole gel amount was
492 unfortunately very low (under limit of detection). Please note that virgin prostheses show a very
493 low adsorbed drug (6 % of the prosthesis). It is to emphasise that diffusion test into agarose gel
494 intends to simulate the pathophysiological conditions *in vivo* better than the classical
495 dissolution test apparatus described in Pharmacopeia. The obtained results confirm the above
496 obtained data *in vitro*, and emphasis the homogeneity of *in vitro* data carried out in this study.
497 However, the behaviour of such system is unknown *in vivo* and all these data will be just a
498 quality control of these medical devices, which need to be proofed *in vivo*.
499

500 **Conclusions**

501 The choice of the dissolution test is of utmost importance in the quality control of
502 vascular prostheses, approaching the establishment of clinically relevant dissolution
503 specifications. Thus, great care has to be taken when defining the experimental conditions for
504 drug release measurements from such drug eluting prostheses: The observed release kinetics
505 fundamentally depend on the type of apparatus and specific conditions. It is to emphasise that
506 in this study modified flow through cell apparatus as well as the diffusion test into the hydrogel
507 have shown to be the most appropriate method for the evaluation of vascular prostheses *in vitro*,
508 approaching the pathophysiological conditions. In the future, it will be important to correlate
509 this type of *in vitro* results with appropriate *in vivo* data in order to overcome the usual
510 occurred *in vitro/in vivo* discrepancy.

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520

521 **Conflicts of interest**

522 All authors declare no conflict of interest. The Editor-in-Chief of the journal is a member
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Table 1: Different methods used for experimentally drug release measurements of ciprofloxacin functionalized or virgin vascular prostheses.

Method	Appartus/container	Media volume	In vitro conditions	Sampling procedure	Sampling time points
USP 4 Conventional (Fig. 5)	Poly(propylene) flow-through cell (1L)	900 mL	Media circulation (<i>closed modus</i>), flow rat 5, 10, 20, 35 mL/min), magnetic stirrer 300 rpm at 37°C	No media change Automated withdrawal Online drug measurement	0, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.33, 2.66, 3, 3.33, 3.66, 4, 4.33, 4.66, 5, 5.33, 5.66, 6 hrs
USP 4 Conventional (Fig. 6)	Poly(propylene) flow-through cell (1L)	900 mL	Media circulation (<i>open or closed</i> _____), flow rate 5 mL/min, magnetic stirrer 300 rpm at 37°C	Manuall withdrawal Replenishment of fresh media	0, 0.33, 0.66, 1, 1.33, 1.66, 2, 2.33, 2.66, 3, 3.33, 3.66, 4, 4.33, 4.66, 5, 5.33, 5.66, 6 hrs
Agitated flask (Fig. 7)	Poly(propylene) vial (40 ml)	20 mL	Horizontal shaker 80 rpm, 37°C	Complete media change	0, 0.08, 0.16, 0.25, 0.5, 0.75, 1, 2, 3, 4, 8, 24, 48 hrs
USP 2 (Fig. 7)	Glass beaker (1L)	900 mL	Media circulation (<i>closed modus</i>), paddle 80 rpm, 37°C	No media change Online drug measurment	0, 0.08, 0.16, 0.25, 0.5, 0.75, 1, 2, 3, 4, 8, 24, 48 hrs
USP 4 Conventional (Fig. 7)	Poly(propylene) flow-through cell (1L)	900 mL	Media circulation (<i>closed modus</i>), flow rat 35 mL/min), magnetic stirrer 300 rpm at 37°C	No media change Automated withdrawal Online drug measurement	0, 0.08, 0.16, 0.25, 0.5, 0.75, 1, 2, 3, 4, 8, 24, 48 hrs
USP 4 Modified (Fig. 7)	Poly(propylene) flow-through cell, agarose gel surrounding the prosthesis (1L)	900 mL	Media circulation (<i>closed modus</i>) flow rate 35 mL/min, magnetic stirrer 300 rpm at 37°C	No media change, online measurment (UV spectrophomter)	0, 0.08, 0.16, 0.25, 0.5, 0.75, 1, 2, 3, 4, 8, 24, 48 hrs

Table 2: The absolute and relative amounts of ciprofloxacin released in the phosphate buffer, agar gel and remained in the functionalized Me β CD prosthesis after 4h exposure to the release medium (agitated flasks).

Ciprofloxacin	Released				Prosthesis				Gel			
	mg/g		%		mg/g		%		mg/g		%	
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
PET-Me β CD	46.26	3.81	97.70	0.51	0.44	0.11	0.94	0.23	0.64	0.18	1.38	0.48

Figure captions

- Fig. 1: In vitro solubility test of ciprofloxacin upon exposure to Me β CD as well as Poly-Me β CD. Please note that ultrapure water was used in this study.
- Fig. 2: 2D-NOESY NMR spectrum in D₂O focused on the Me β CD protons (vertical) and the piperazinyll protons of CFX (horizontal) of the: (a) 1:1 Me β CD/CFX complex (10 mM) and (b) Poly-Me β CD/CFX complex.
- Fig. 3: Schematic presentation of the different in vitro release set-ups used to monitor drug release from the investigated vascular prostheses: a) Agitated flasks, b) USP paddle apparatus, c) conventional flow-through cell (closed or open modus), and c) modified flow-through cell (analogous to Neubert et al., 2008) (closed modus). Details are described in materials and methods section (Table 1).
- Fig 4: The impact of the impregnation time on the ciprofloxacin loading (2 mg/mL) onto the PET-Me β CD Prosthesis (cylindrical prostheses, 10 cm in length, 10 cm in diameter, 80 rpm, 37°C).
- Fig. 5: The impact of the CFX quantity after 4h of impregnation on the sorption capacity of onto PET-Me β CD (a), the Langmuir isotherm of adsorption (c), and Freundlich isotherm of adsorption (d).
- Fig. 6: Conventional flow-through cell (USP 4)– Impact of the flow rate in the **closed modus** (indicated in the diagrams: 5, 10, 20, 35 mL/min) on ciprofloxacin release: a) relative release rates, b) absolute release rates (per g prosthesis) from the investigated vascular prosthesis in phosphate buffer saline pH 7.4 “PBS”.
- Fig. 7: Conventional flow-through cell (USP 4)– Impact of the **modus-type** (indicated in the diagrams: **open modus** vs. **closed modus**) on ciprofloxacin release: a) relative release rates, b) absolute release rates (per g prosthesis) from the investigated vascular prosthesis in phosphate buffer saline pH 7.4 “PBS”. The flow rate was 5 mL/min.

Fig. 8: Impact of in vitro release set-ups on ciprofloxacin release: a) relative release rates, b) absolute release rates (per g prosthesis) from the investigated prosthesis in phosphate buffer saline pH 7.4 "PBS". Agitated flasks at 80 rpm, USP 2 at 80 rpm, conventional flow-through cell, and Modified flow-through cell (USP 4, flow rate 35 mL/min, closed modus).

Fig. 9: In vitro ciprofloxacin diffusion profiles upon agar gel from vascular prosthesis: (a) non- functionalized PET and (b) PET-Me β CD functionalized PET at different exposure times [according to T.H. Hoang Thi].

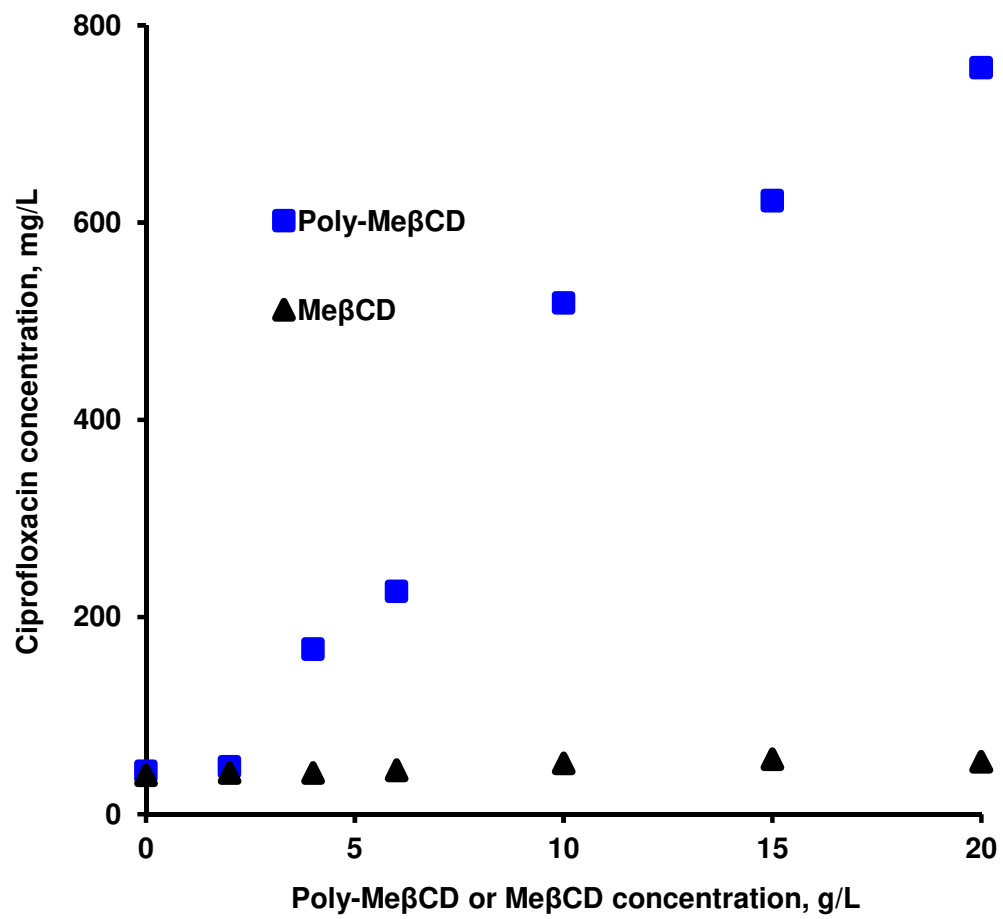


Figure 1

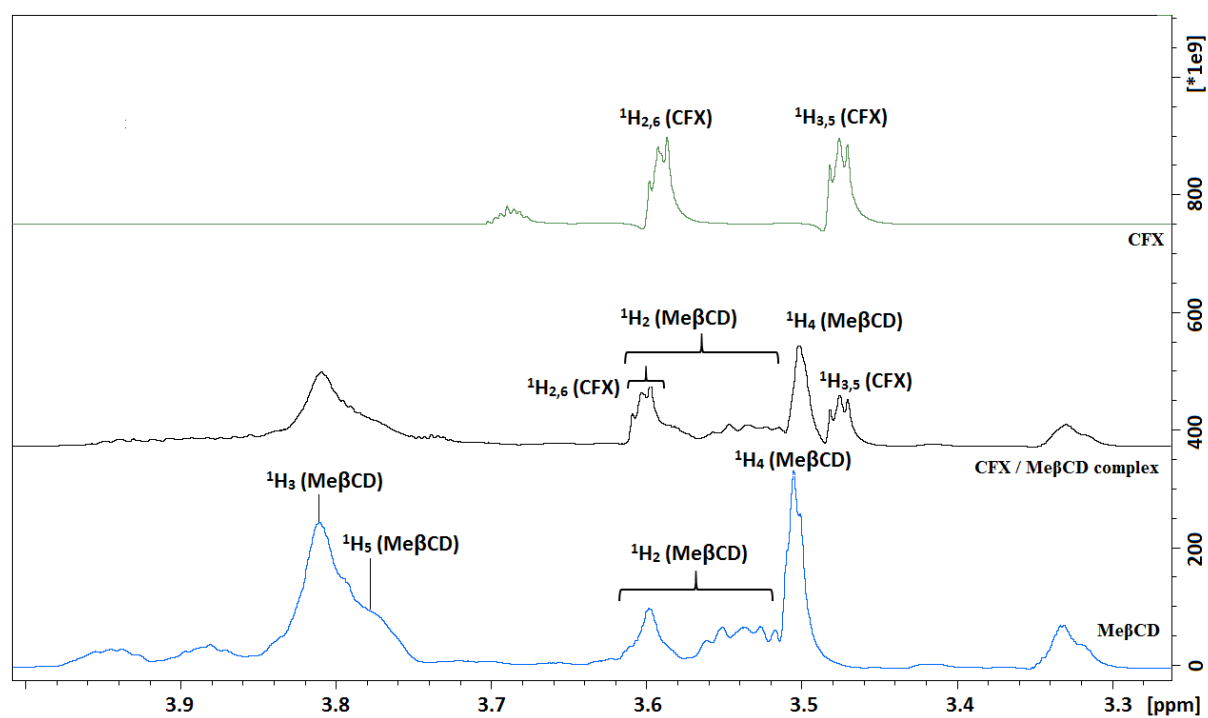
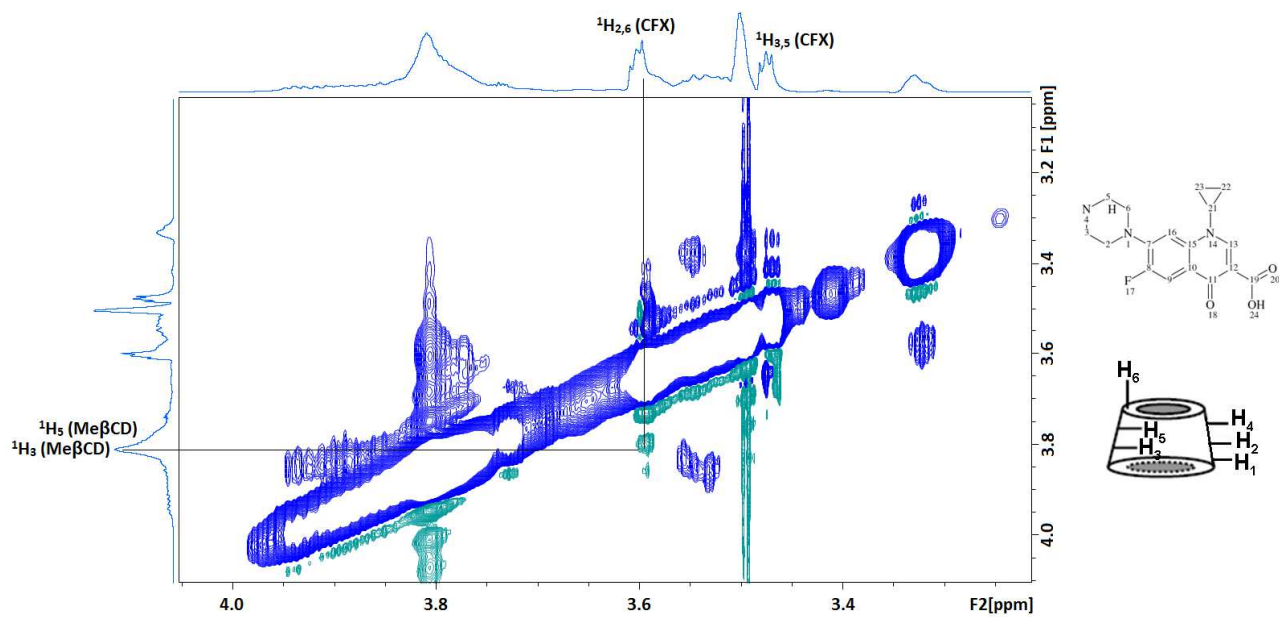


Figure 2A

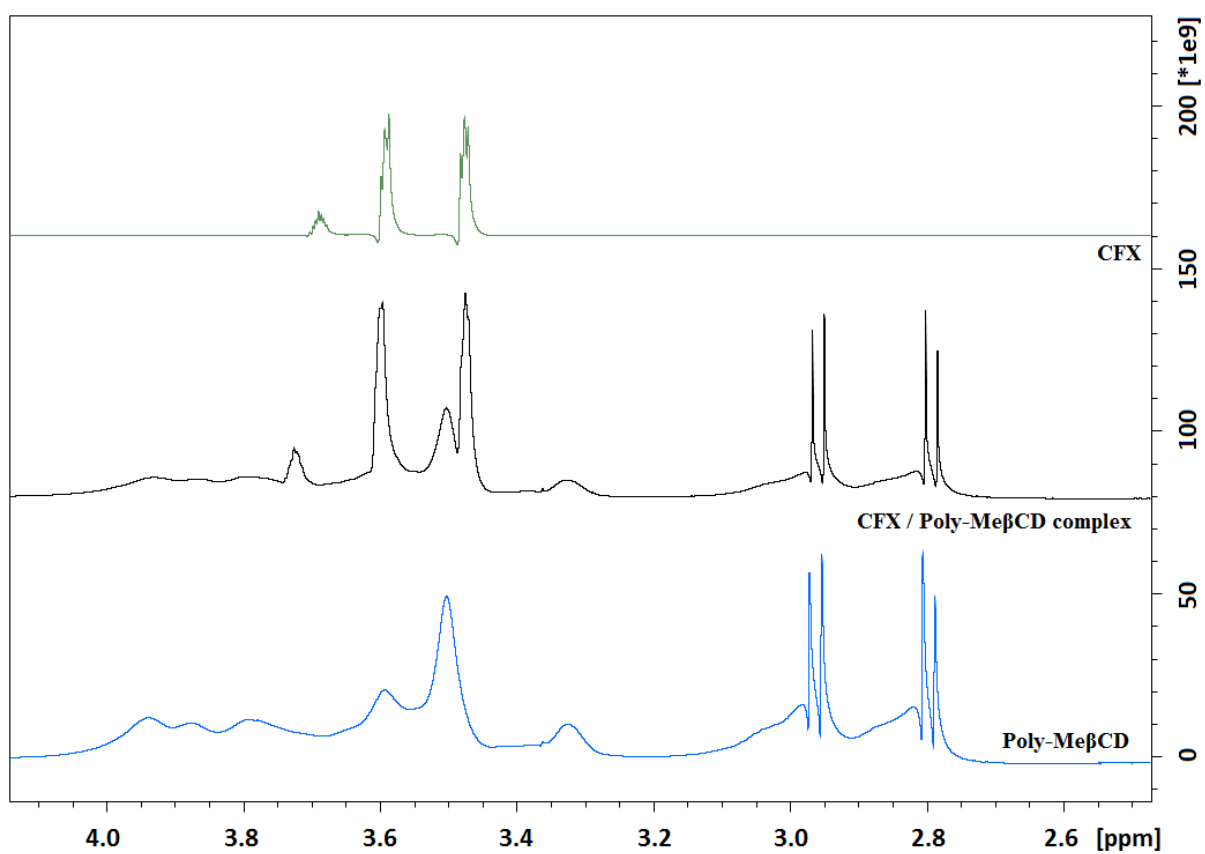
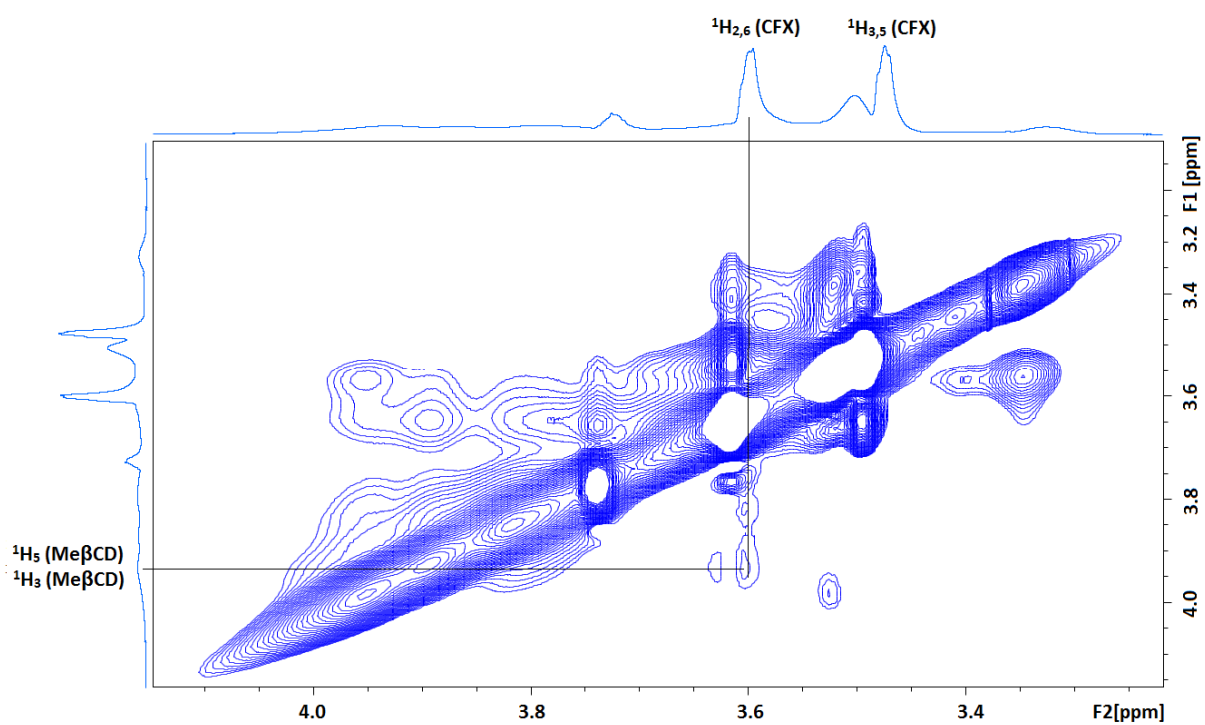


Figure 2B

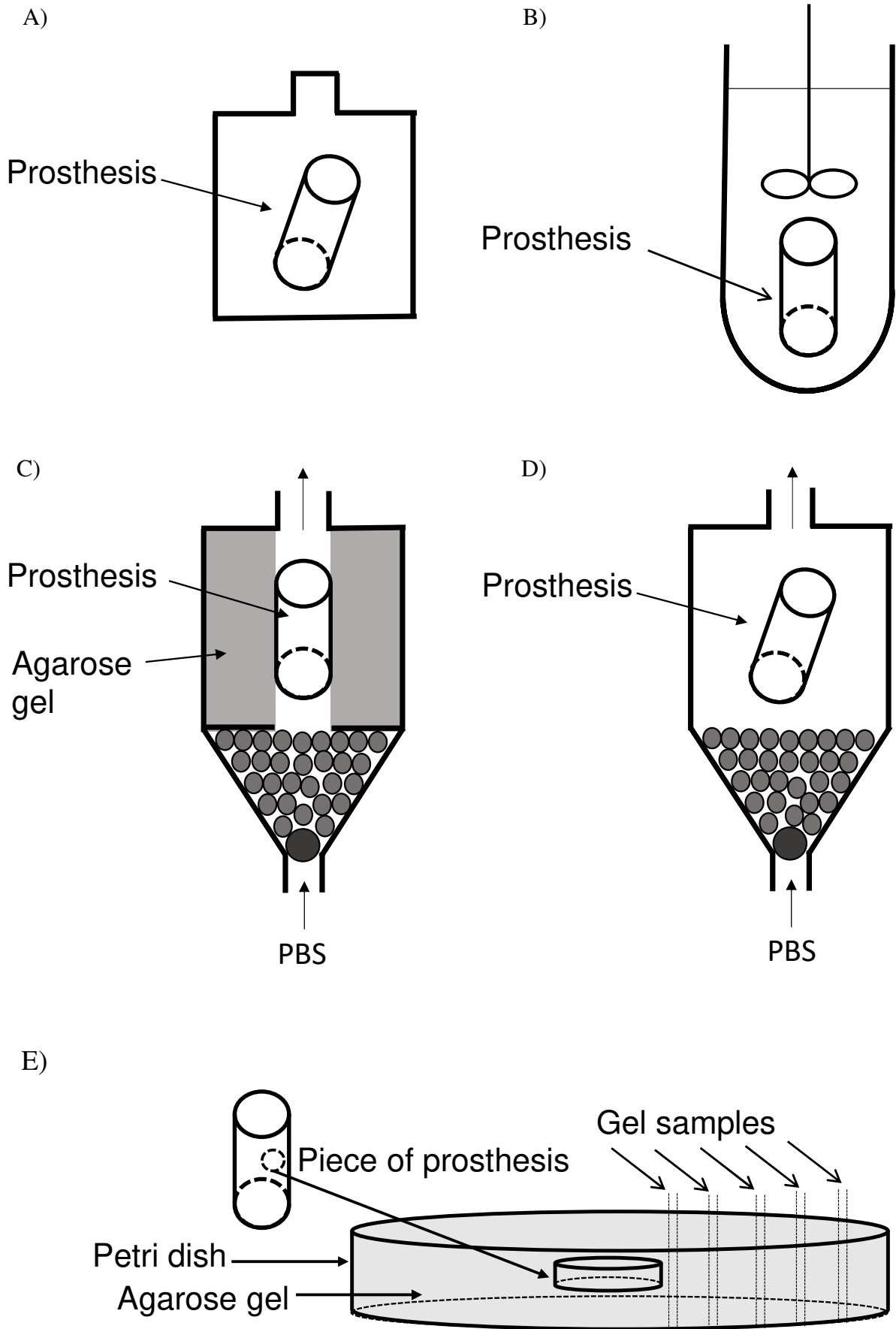
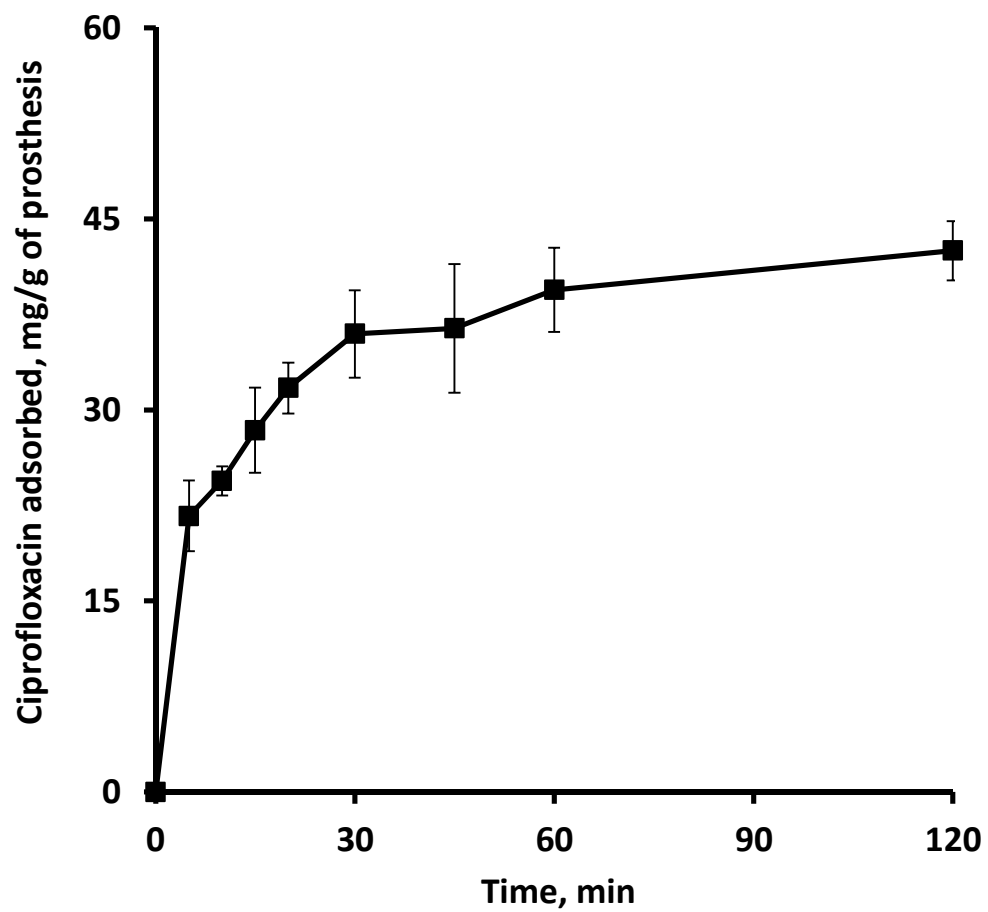


Figure 3

**Figure 4**

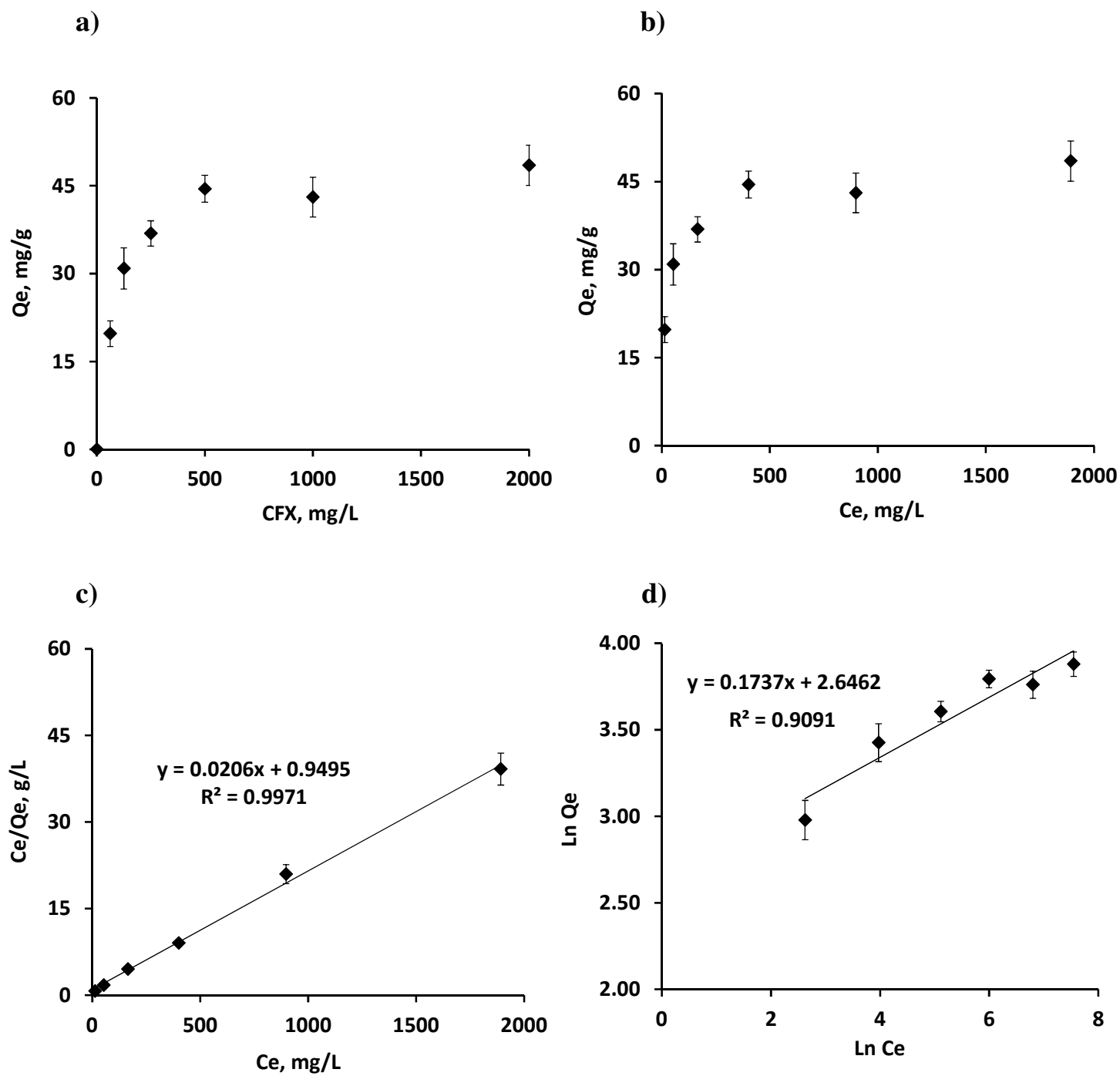


Figure 5

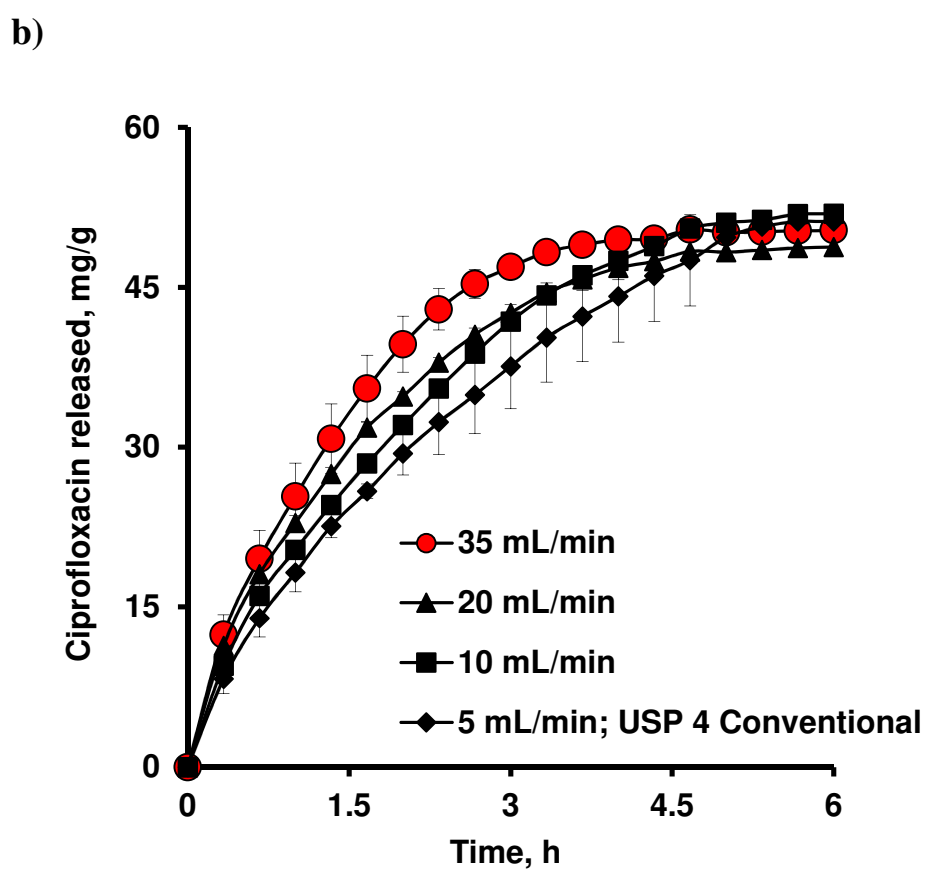
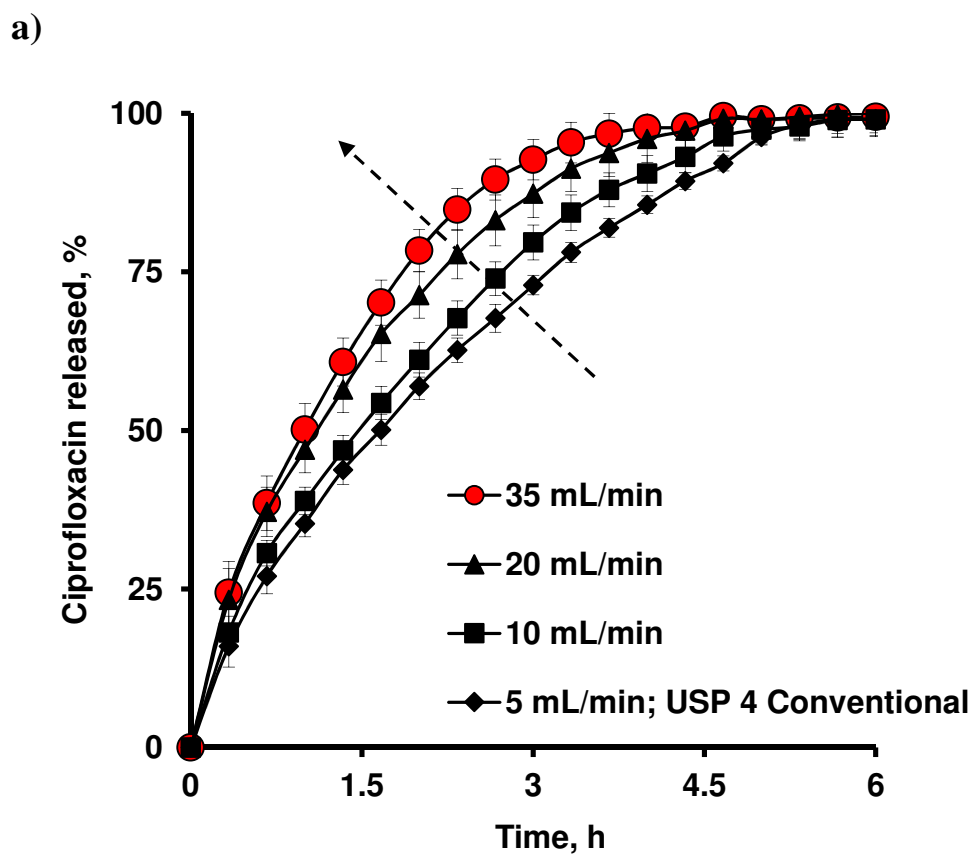
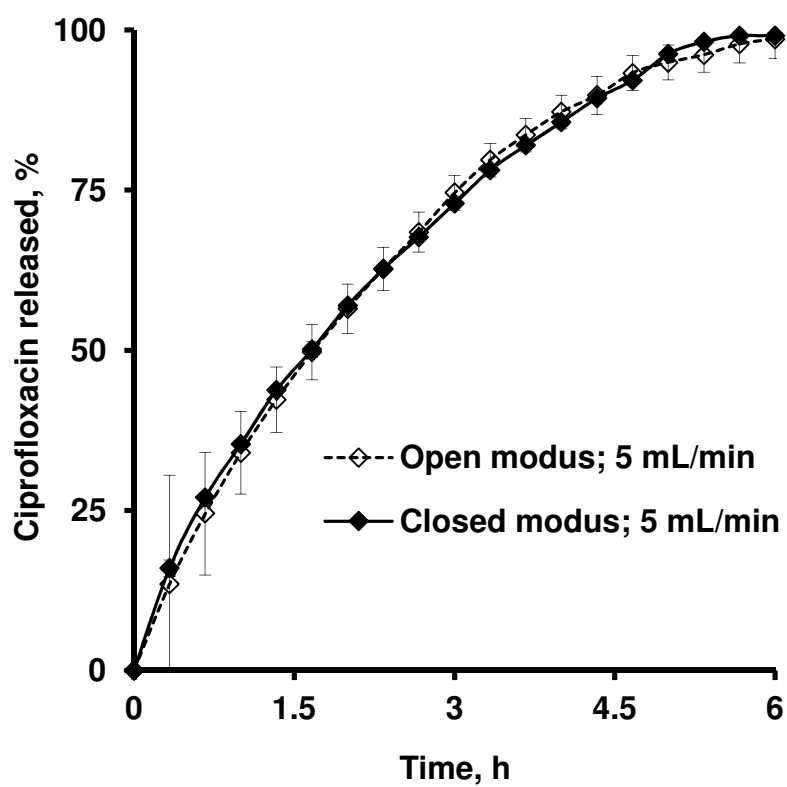


Figure 6

a)



b)

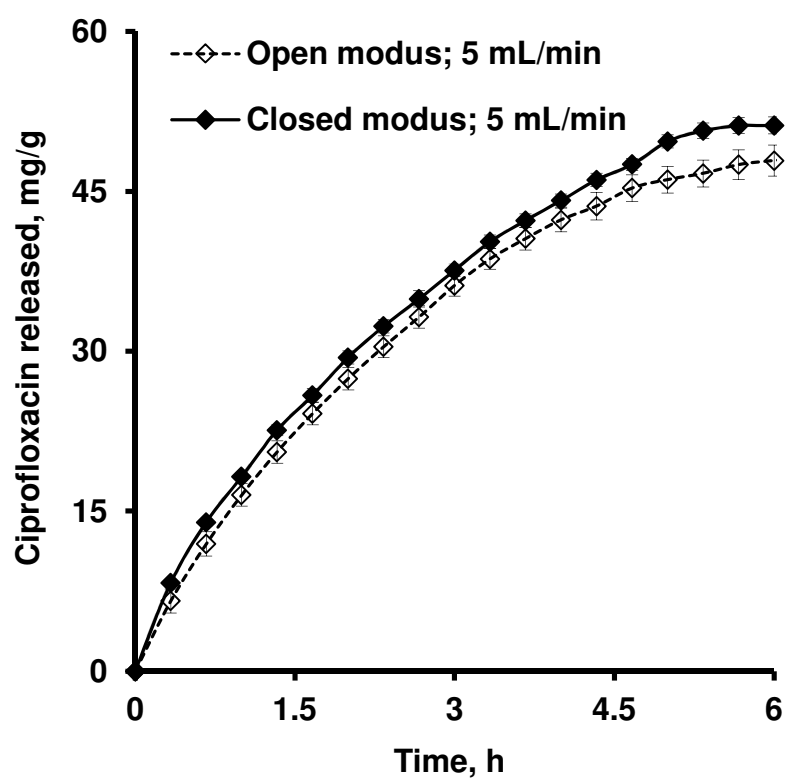


Figure 7

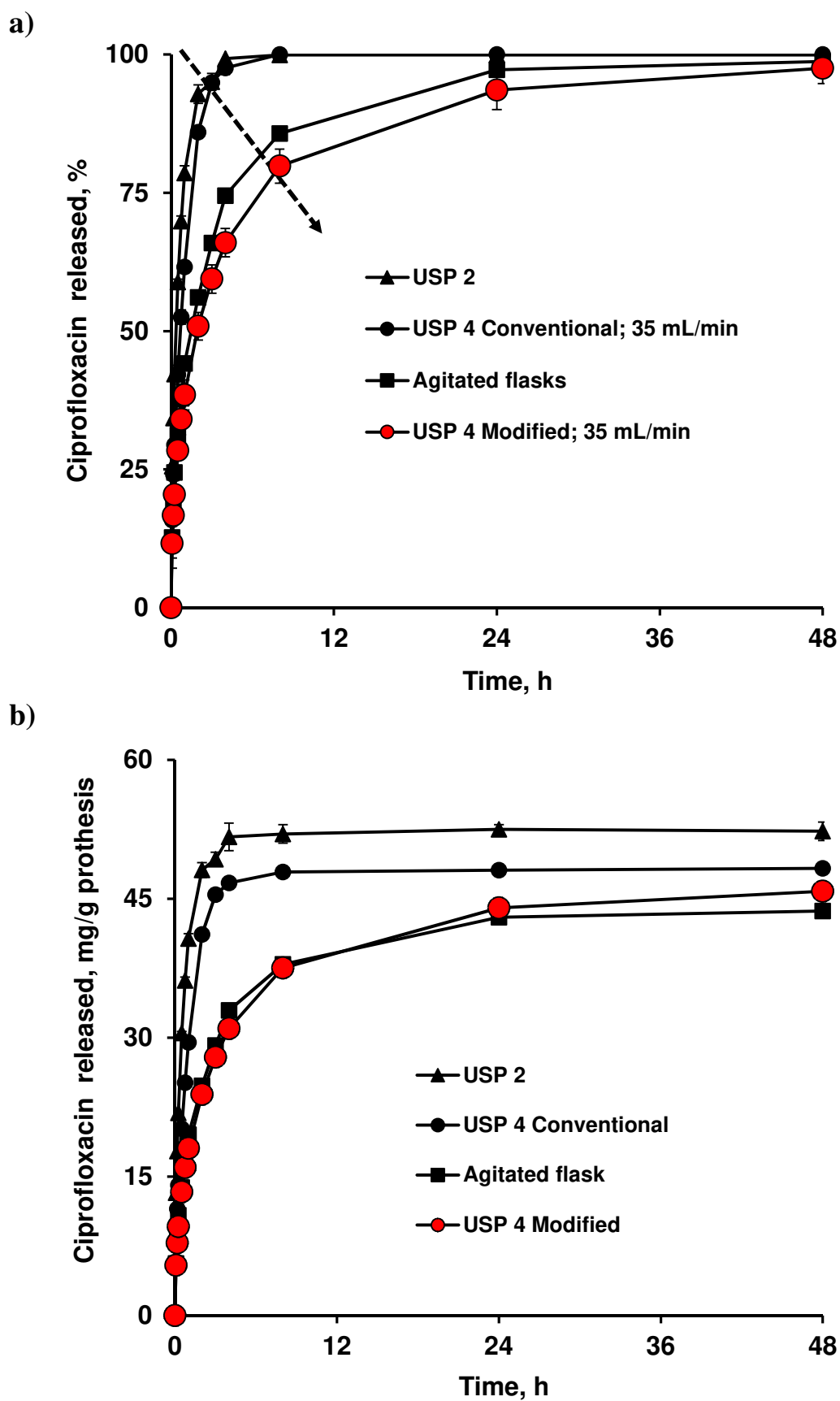


Figure 8

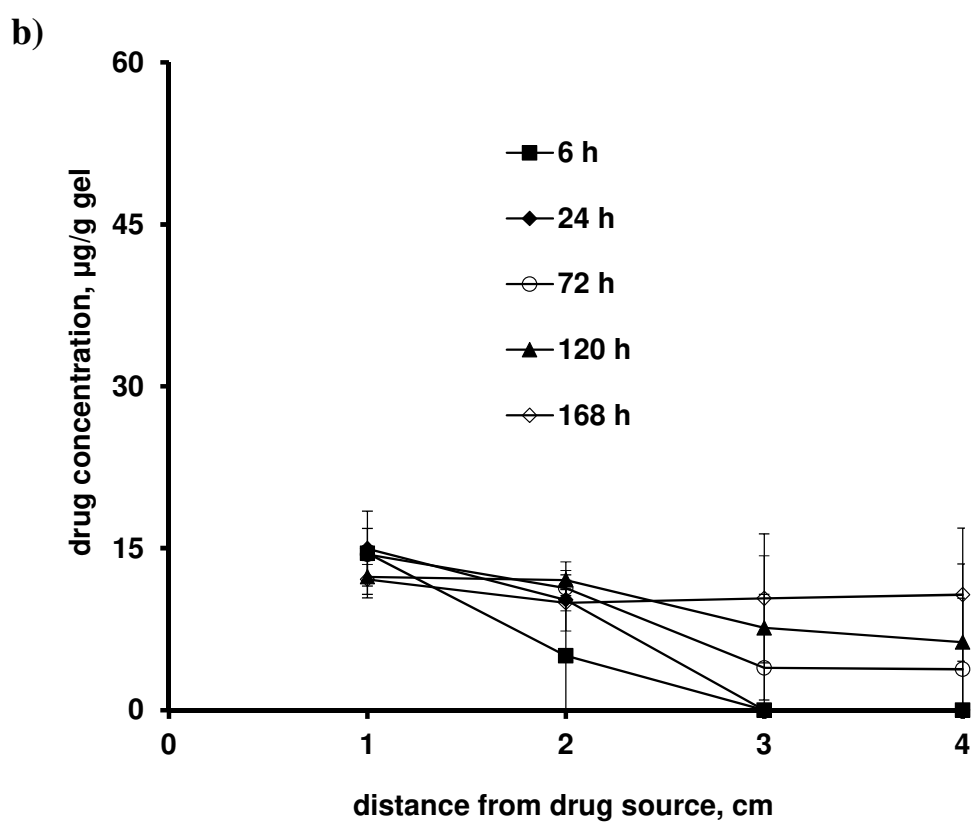
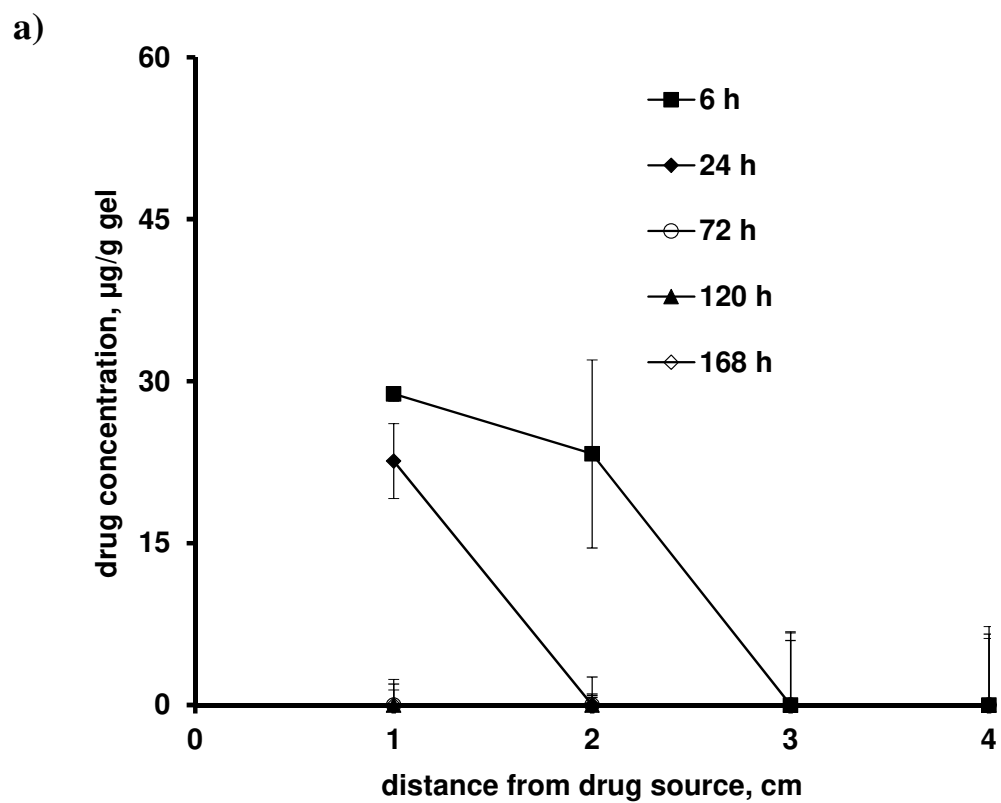


Figure 9

