Ciprofloxacin loaded vascular prostheses functionalized with poly-methylbeta- cyclodextrin: The importance of in vitro release conditions

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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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Abstract

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

Keywords: Vascular prostheses, modified flow-through cell, ciprofloxacin, Poly-MeβCD, PET-MeβCD, controlled drug delivery, diffusion test

1. Introduction

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

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

Unfortunately, drug is rapidly washed by the bloodstream in the case of conventional antibiotic loaded grafts (< 24 hours), which can lead to less efficacy of the treatment [21,22,23]. To overcome this restriction, modified polyester-based vascular grafts have been developed [24,25]. The concept is to functionalize the prostheses with polymer of cyclodextrins (Poly-CD) in order to increase the antibiotic-loading capacity and subsequently provide locally sustained drug delivery during the critical postoperative period. The functionalized prostheses with Poly-CD should deliver large amount of loaded antibiotics such as rifampicin, vancomycin 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, ^{1}H-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-MeβCD) 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

Materials and methods

2.1. Materials

Woven poly(ethylene terephthalate) vascular prostheses (Polythese®, PET; Perouse Médical, Ivry-Le-Temple, France). Methyl beta- cyclodextrin (MeβCD, Crysmeb®, substitution degree = 0.5; Roquette, Lestrem, France). Citric acid (CTR) and sodium dihydrogen hypophosphite (NaH_2PO_4, $2H_2O$) (Aldrich chemicals, Saint Quentin Fallavier, France). Ciprofloxacin base powder (CFX base; INRESA, Bartenheim, France) and ciprofloxacin hydrochloride solution (CFX: 200 mg/100mL; Kabi, Paris, France). Gen Agaroseose LE (agarose powder; Genaxxon BioScience, Ulm, Germany). Acetic acid glacial (Fisher Scientific, Meadow Road, Loughborough, UK). Demineralized water ultrapure (18.2 MΩ, Milli-Q system, Millipore, France).

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

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 MeβCD, catalysts ($NaH_2PO_4.2H_2O$) 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-MeβCD) 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-MeβCD coating and CFX). The prostheses were dipped
166 in 0.05 M NaOH (4h, 240 rpm, 37 °C) in order to hydrolyse the Poly-MeβCD 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-MeβCD 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 MeβCD and Poly-MeβCD 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 µm 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 MeβCD and CFX were observed by proton NMR in the case of
183 the "Poly- MeβCD" as well as in the oligomer "MeβCD" . 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 MeβCD
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 (∅ 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

the Langmuir isotherm on the one hand

$$\frac{Ce}{qe} = \frac{1}{K_L} + \frac{\alpha_L Ce}{K_L} \qquad \text{Eq. (1)}$$

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

$$ln(qe) = ln(Kf) + \frac{1}{n} ln(Ce) \qquad \text{Eq. (2)}$$

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

2.4. In vitro drug release studies

PET-MeβCD were placed in 10 mL of ciprofloxacin solution for 4 h and then dried overnight at room temperature.

To determine the drug sorption, the PET-MeβCD were then exposed to 20 mL of aqueous NaOH 0.1 M overnight, in order to remove the cyclodextrin polymers via hydrolysis, thus releasing the remaining antibiotics and determining the practical drug loading. The amount of antibiotic was measured by UV-spectrophotometry in the aqueous NaOH solution at λ = 278 nm

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

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

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

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

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

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

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

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

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

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

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

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

Results and Discussion

3.1. Solubility study

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

3.2. MeβCD or Poly-MeβCD /Ciprofloxacin interaction

The formation of an inclusion complex between α, β, γ and MeβCD with ciprofloxacin has been previously studied using an Ultrashield 400 MHz Bruker spectrometer [24,25]. In all cases, Job plots experiments showed that 1:1 complexes were formed by inclusion of the piperazinyl group inside this cyclodextrin cavity. Though, using a 400 MHz spectrometer superimposition of the signals of H_2 and H_6 protons of piperazinyl group and that of the H_2 proton of CD (situated around 3.6 ppm) could provoke a certain doubt on the dipolar interactions between H_2 and H_6 protons of ciprofloxacin with the H_3 protons of the CD at 3.9

ppm. Therefore, it could be a handsome profit in this study to use the improved resolution of the AV NEO 900 MHz spectrometer. In fact, the resolution of the 2D NOESY NMR spectrum is clearly improved (Figure 2A and B) when comparing to 400 MHz [24], since protons H_2 and H_6 of CFX are well separated from the H_2 signal of the MeβCD. As a consequence, 2D map precisely displays a cross peak issued from the dipolar interaction between the H_2 and H_6 protons of piperazinyl group (3.57 – 3.587 ppm) and the proton H_3 of MeβCD situated inside the cavity (3.87 – 3.89 ppm) (Figure 2A and B).

3.3. Ciprofloxacin sorption kinetic

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

Figure 5a and Figure 5b show the amount of loaded CFX (*Qe*) onto PET-MeβCD as a function of the CFX concentration in the impregnation bath before equilibrium (CFX, mg/L) (Figure 5a) and at the equilibrium (Ce, mg/L) (Figure 5b). As it can be seen on these figures, the maximal amount of CFX loaded onto the prosthesis was 48.5 ± 3.4 mg/g, which was obtained after incubation of the PET-MeβCD into the highest concentration of CFX solution (2g/L).

Figure 5b illustrates the amount of loaded CFX onto PET-MeβCD "*Qe"* but in this case as a function of the isotherm plot representing "*Ce"* that indicates the concentration (mg/L) of the CFX in the solution at the equilibrium. The maximal amount of CFX loaded onto PET-MeβCD 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 mg/g;* K_L = *1.05*; α_L = 0.02163 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-MeβCD (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 MeβCD 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-MeβCD 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.

3.4.3. Impact of the method on ciprofloxacin release

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

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

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

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

Importantly, the reloading of the polymerized cyclodextrin on the grafted vascular prosthesis is not occurred due to the presence of ions in phosphate buffer (release medium in vitro) and proteins in blood stream (in vivo). The cavity of the cyclodextrin can be rapidly filled with ions (Na^+, Ca^{2+}, Mg^{2+}, phosphate etc.) as well as proteins (in blood) and replace the drug position after drug release from the cyclodextrin- complex. Thus, ions and proteins can be considered as a concurrent substance, which can clog the free cavity into cyclodextrins as soon as the drug is released. It has to be pointed out that this phenomenon has been also confirmed in vitro because of the increased drug release during the incubation period. Importantly, we did not observe any reloading of the polymerized cyclodextrins with CFX. In the case of reloading, we have had observed a level-off or a decrease in the absolute release profile.

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

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

3.5. CFX release test in agarose gel

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

Conclusions

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

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Conflicts of interest

All authors declare no conflict of interest. The Editor-in-Chief of the journal is a member in the same research group "INSERM U1008" but is not co-author of this article. The manuscript has been subject to all of the journal's usual procedures, including peer review, which has been handled independently of the Editor-in-Chief.

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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 (*closed modus*), 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 (*open or closed*), flow rate 5 mL/min, magnetic stirrer 300 rpm at 37°C	Manuall withdrawal Replenishement 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 (*closed modus*), 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 (*closed modus*), 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 surounding the prosthesis (1L)	900 mL	Media circulation (*closed modus*) 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 MeβCD 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-MeβCD	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 MeβCD as well as Poly-MeβCD. Please note that ultrapure water was used in this study.

Fig. 2: 2D-NOESY NMR spectrum in D_2O focused on the MeβCD protons (vertical) and the piperazinyl protons of CFX (horizontal) of the: (a) 1:1 MeβCD/CFX complex (10 mM) and (b) Poly-MeβCD/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-MeβCD 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-MeβCD (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-MeβCD functionalized PET at different exposure times [according to T.H. Hoang Thi].

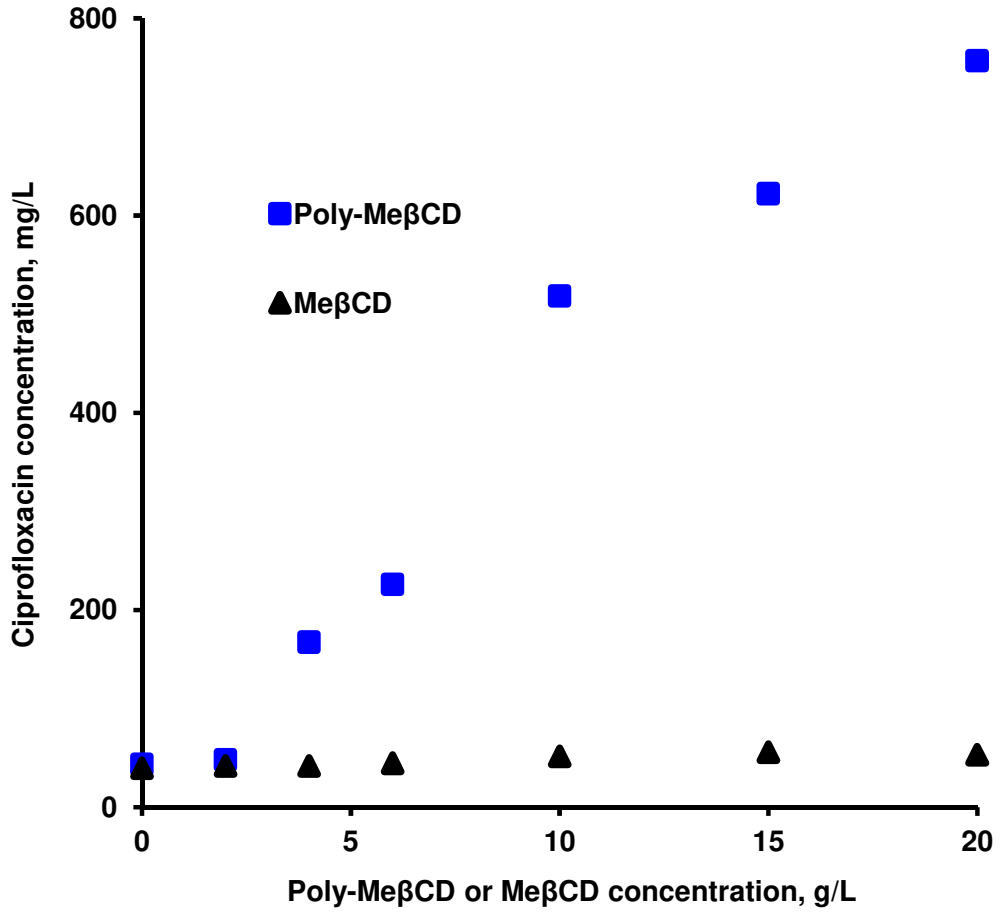

800
600
400
200
0
Ciprofloxacin concentration, mg/L
Poly-MeβCD
MeβCD
0
5
10
15
20
Poly-MeβCD or MeβCD concentration, g/L

Figure 1

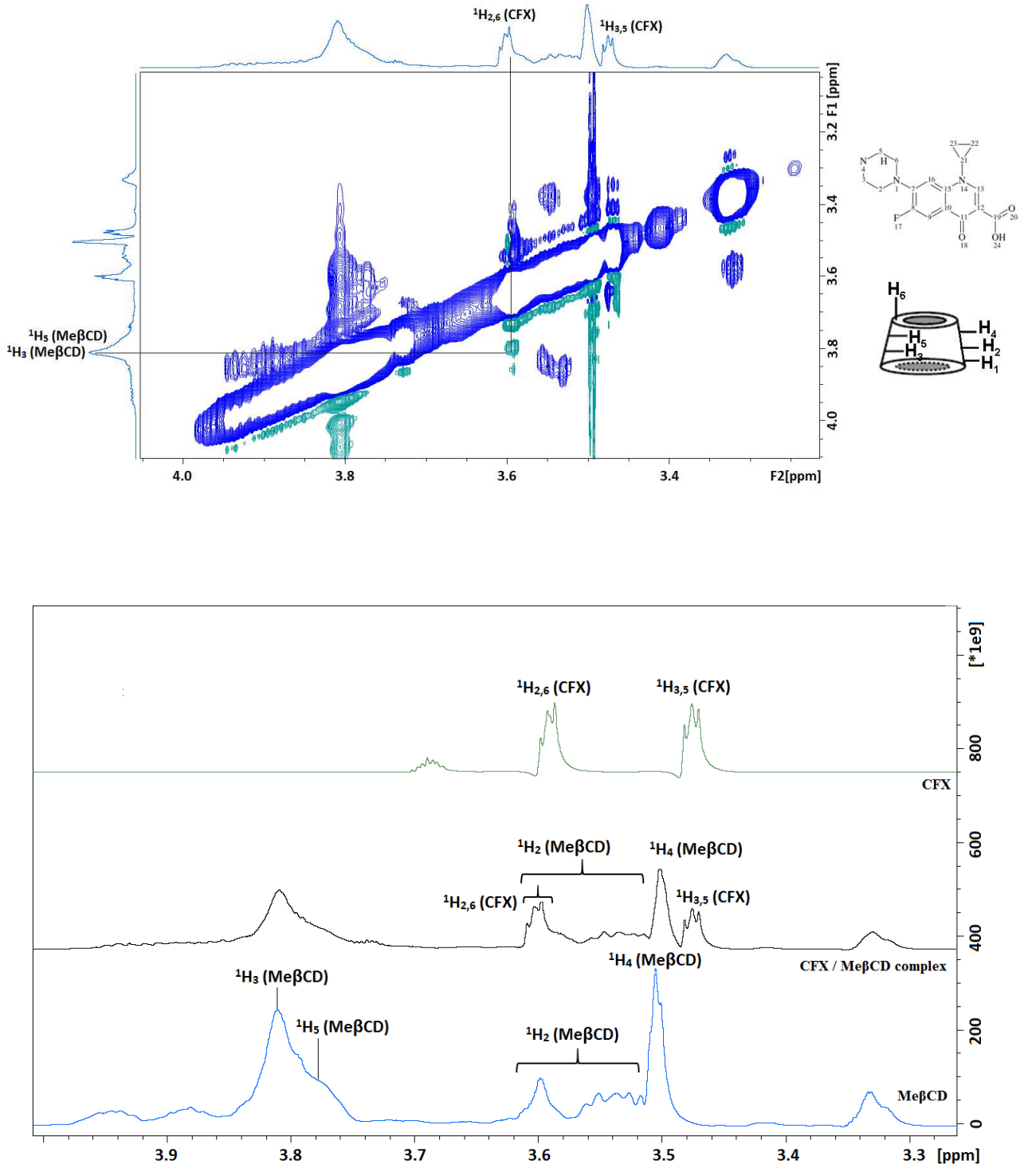

$^1H_{2,6}$ (CFX)
$^1H_{3,5}$ (CFX)
$^1H_5$ (MeβCD)
$^1H_3$ (MeβCD)
F2[ppm]
H6
H4
H5
H2
H3
H1
CFX
CFX / MeβCD complex
MeβCD
$^1H_2$ (MeβCD)
$^1H_4$ (MeβCD)
[ppm]

Figure 2A

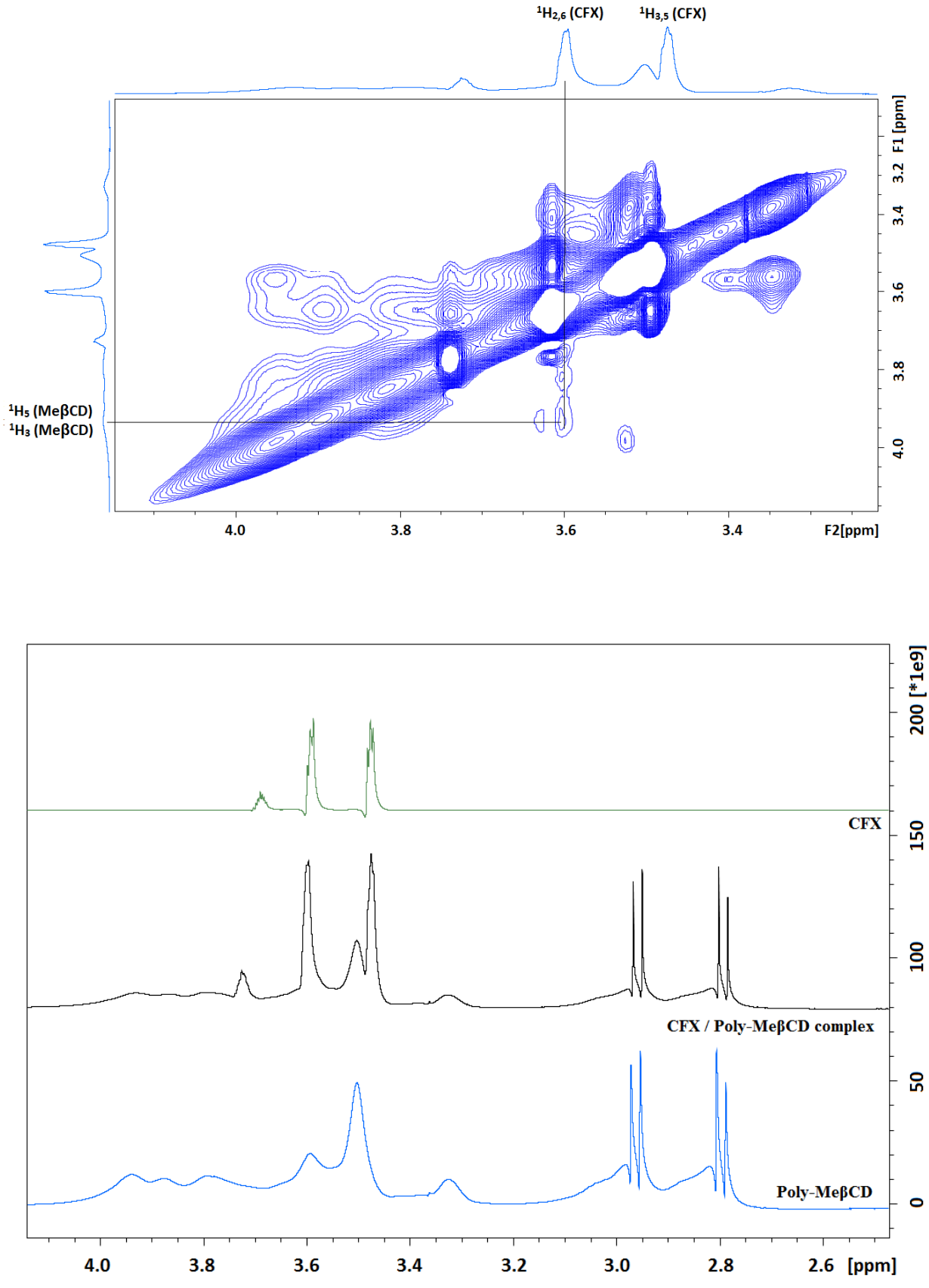

$^1H_{2,6}$ (CFX)
$^1H_{3,5}$ (CFX)
$^1H_5$ (MeβCD)
$^1H_3$ (MeβCD)
F1 [ppm]
F2[ppm]
CFX
CFX / Poly-MeβCD complex
Poly-MeβCD
200 [*1e9]
150
100
50
0
[ppm]

Figure 2B

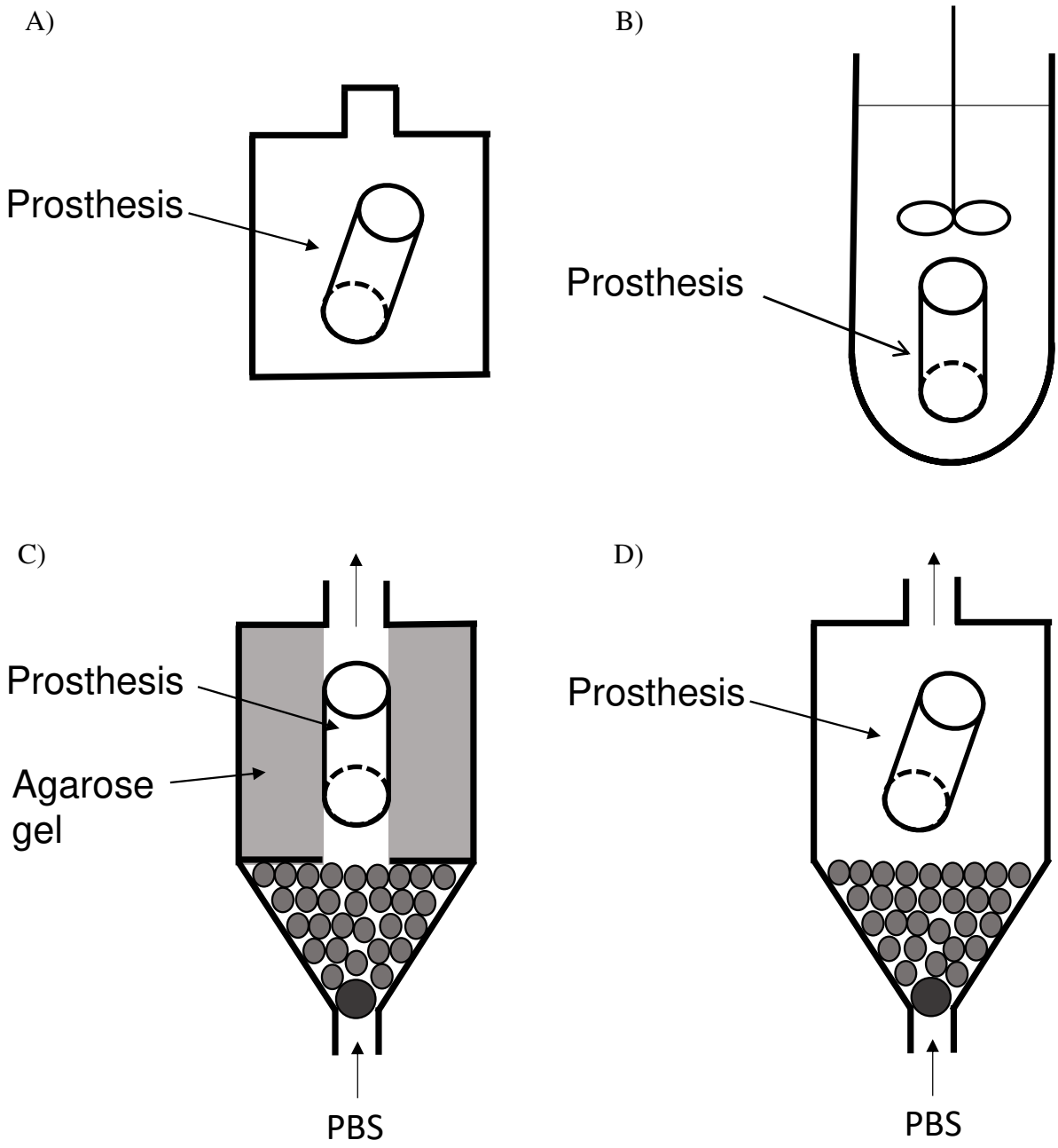

A)
Prosthesis
B)
Prosthesis
C)
Prosthesis
Agarose gel
PBS
D)
Prosthesis
PBS
E)
Piece of prosthesis
Gel samples
Petri dish
Agarose gel

Figure 3

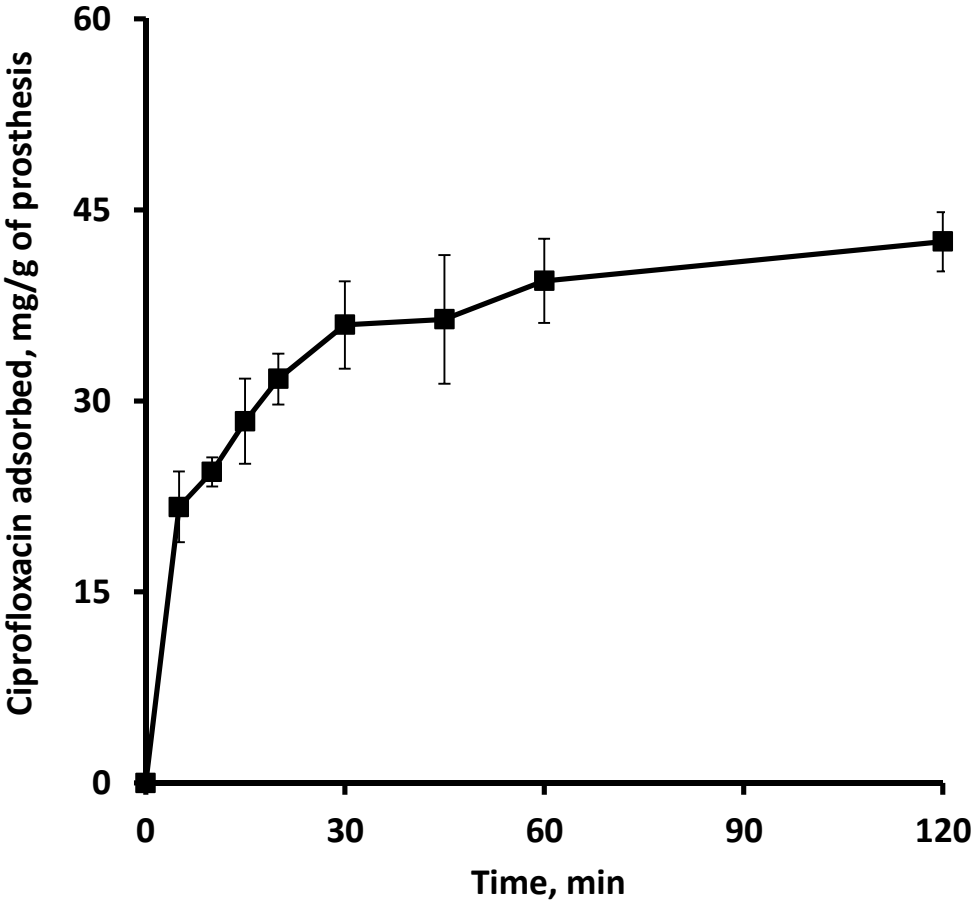

60
45
30
15
0
Ciprofloxacin adsorbed, mg/g of prosthesis
0
30
60
90
120
Time, min

Figure 4

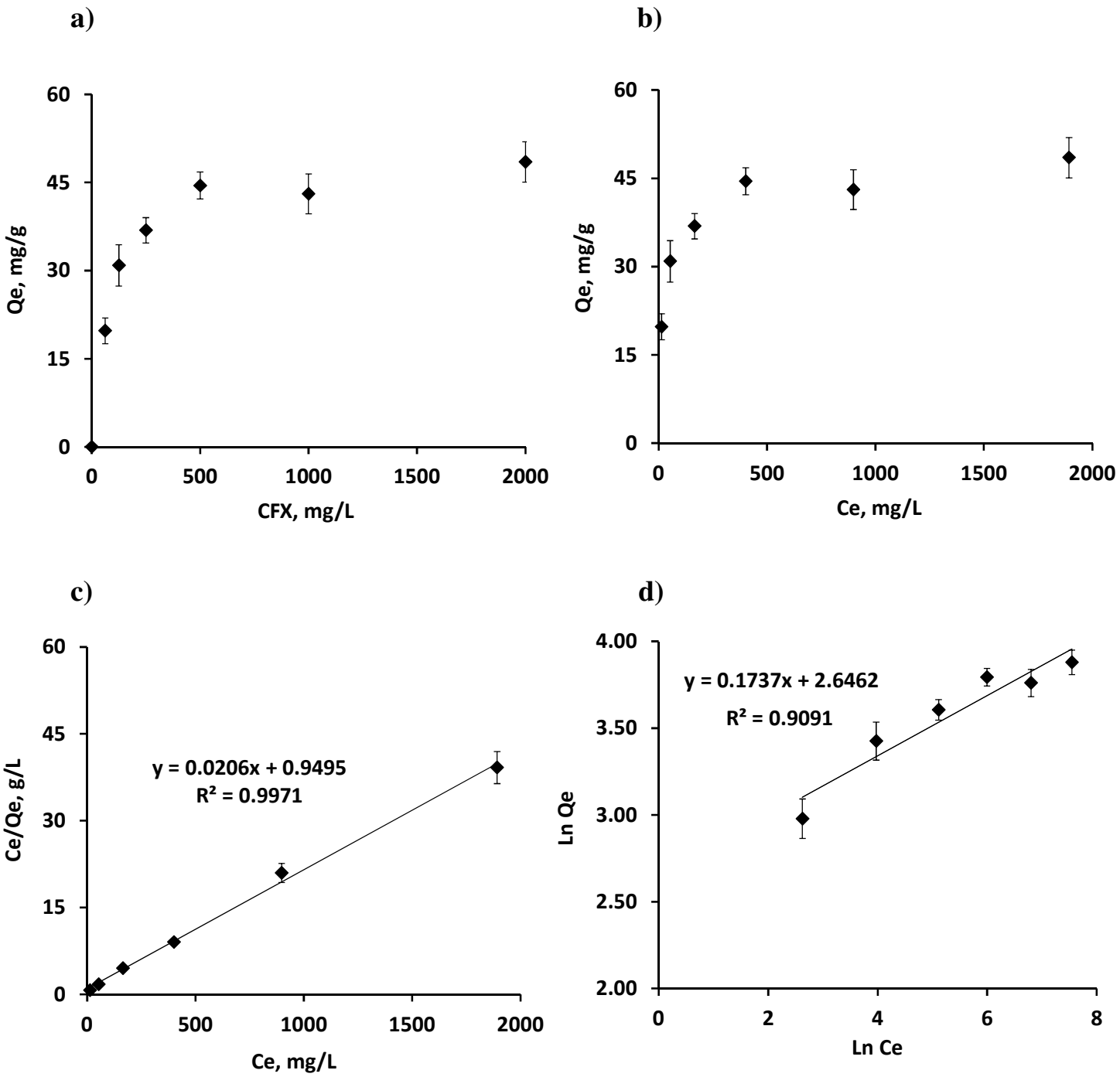
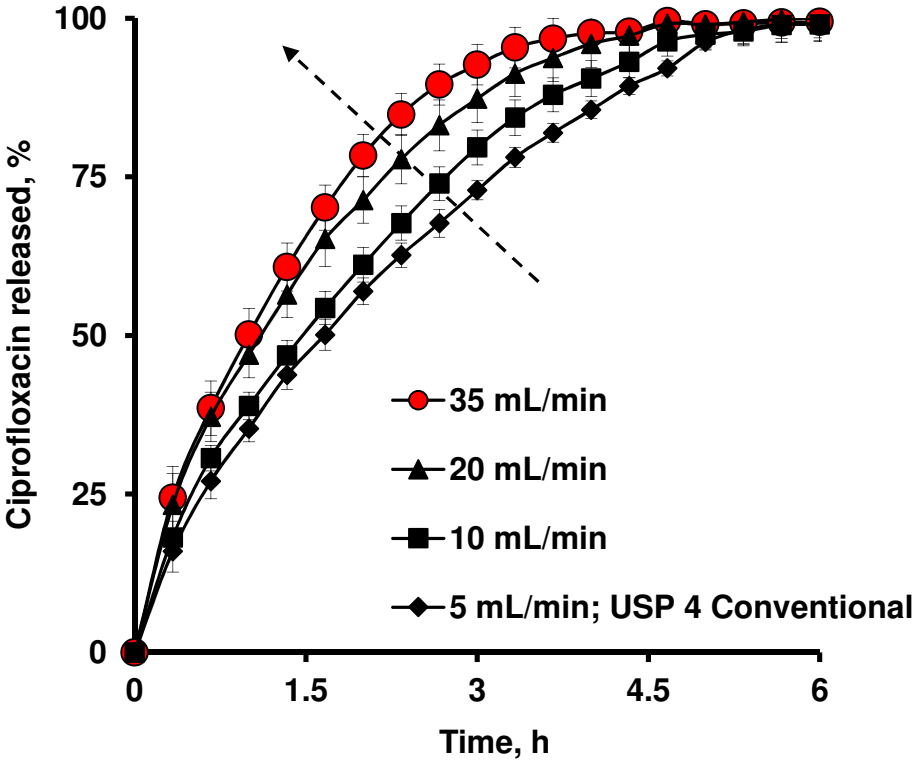

a)
Qe, mg/g
CFX, mg/L
b)
Qe, mg/g
Ce, mg/L
c)
Ce/Qe, g/L
y = 0.0206x + 0.9495
R² = 0.9971
Ce, mg/L
d)
Ln Qe
y = 0.1737x + 2.6462
R² = 0.9091
Ln Ce

Figure 5

a)


Ciprofloxacin released, %
100
75
50
25
0
0
1.5
3
4.5
6
Time, h
35 mL/min
20 mL/min
10 mL/min
5 mL/min; USP 4 Conventional

b)

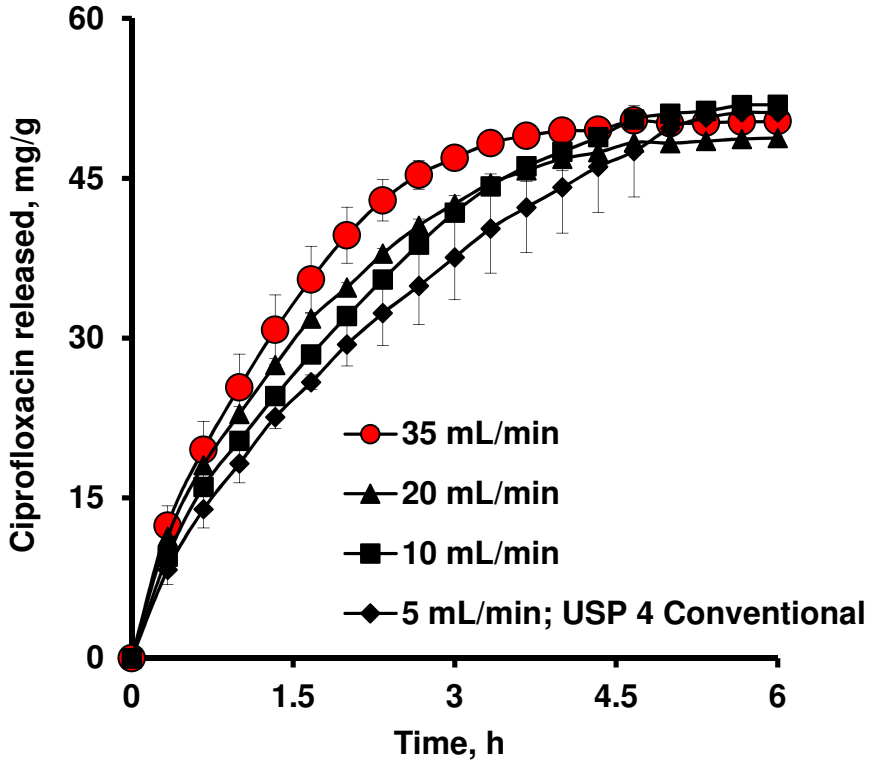
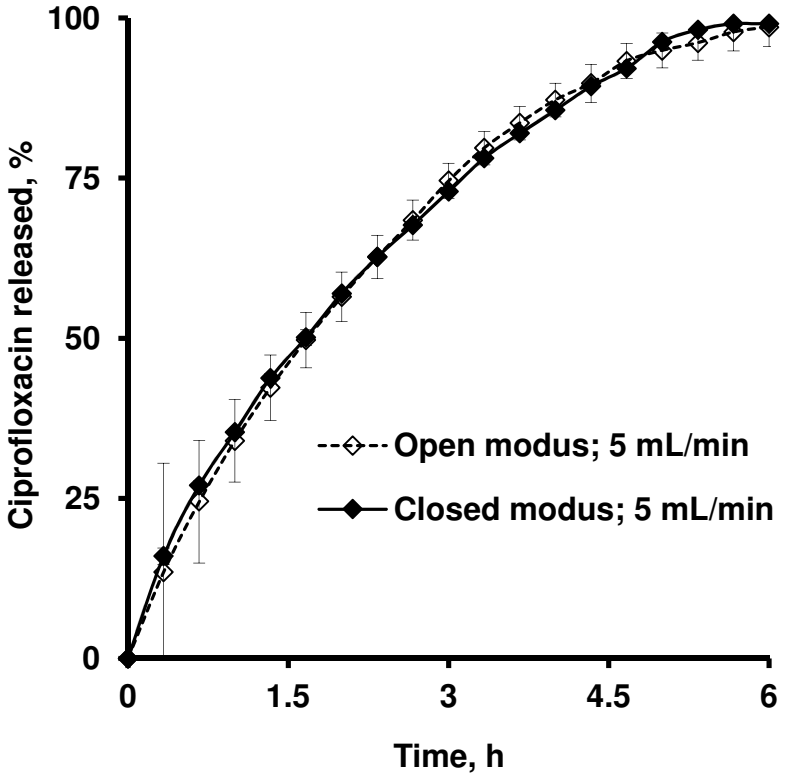

Ciprofloxacin released, mg/g
60
45
30
15
0
0
1.5
3
4.5
6
Time, h
35 mL/min
20 mL/min
10 mL/min
5 mL/min; USP 4 Conventional

Figure 6

a)


100
75
50
25
0
Ciprofloxacin released, %
Open modus; 5 mL/min
Closed modus; 5 mL/min
0
1.5
3
4.5
6
Time, h

b)

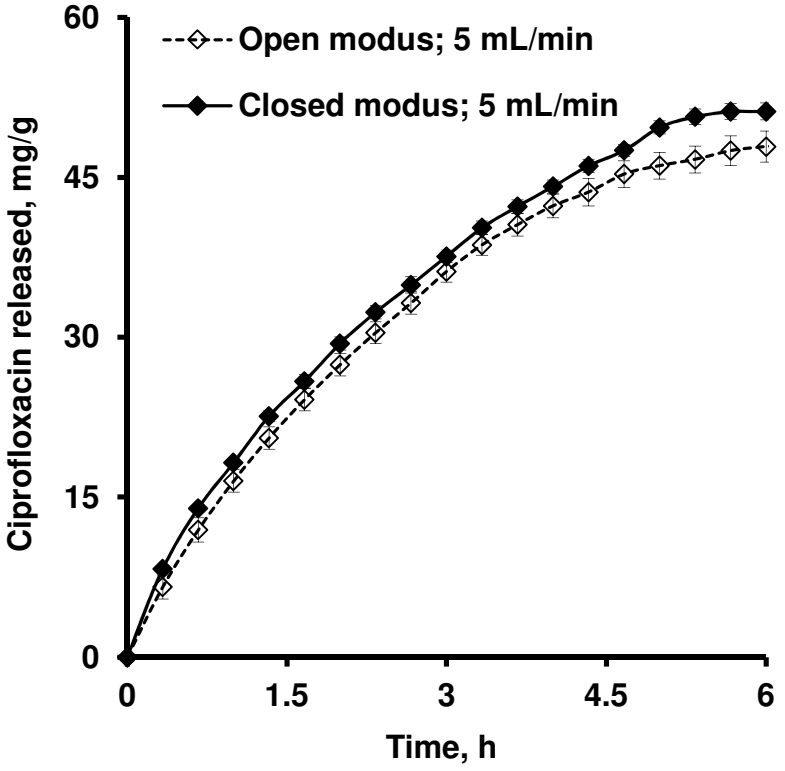
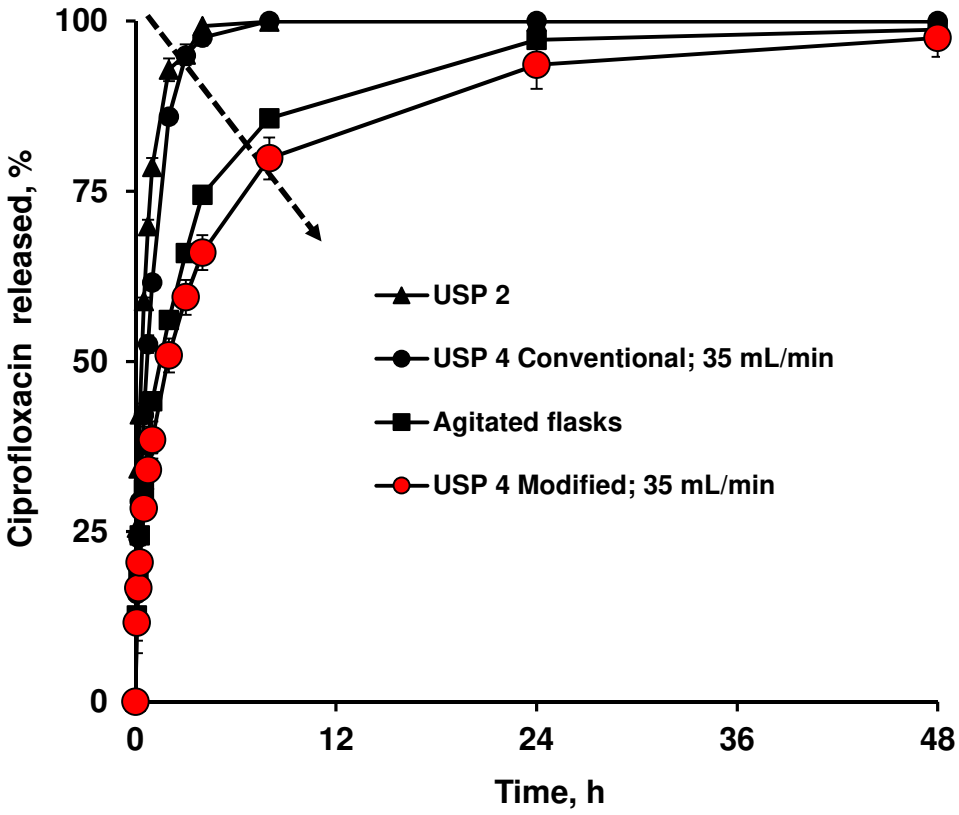

60
45
30
15
0
Ciprofloxacin released, mg/g
Open modus; 5 mL/min
Closed modus; 5 mL/min
0
1.5
3
4.5
6
Time, h

Figure 7

a)


Ciprofloxacin released, %
100
75
50
25
0
0
12
24
36
48
Time, h
USP 2
USP 4 Conventional; 35 mL/min
Agitated flasks
USP 4 Modified; 35 mL/min

b)

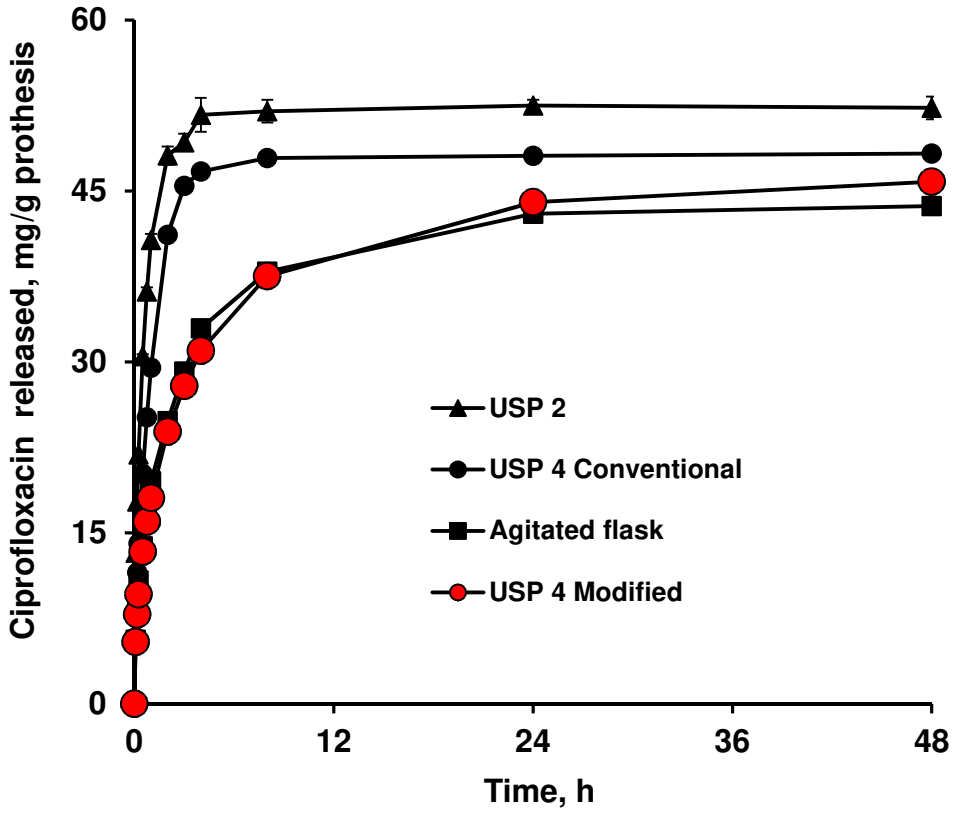
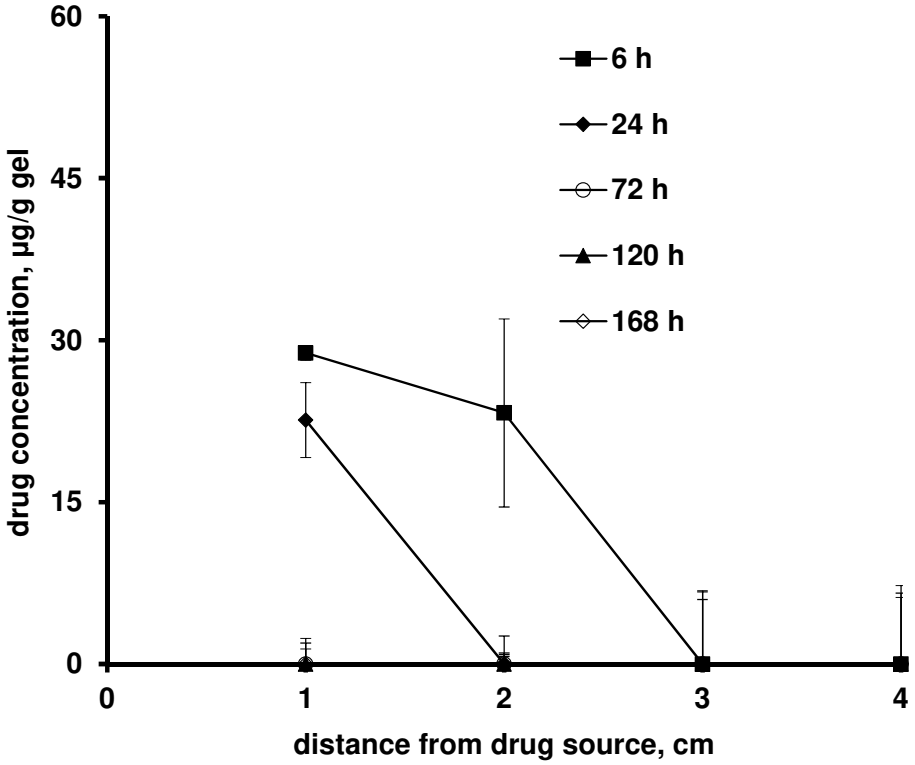

Ciprofloxacin released, mg/g prothesis
60
45
30
15
0
0
12
24
36
48
Time, h
USP 2
USP 4 Conventional
Agitated flask
USP 4 Modified

Figure 8

a)


60
45
30
15
0
drug concentration, µg/g gel
6 h
24 h
72 h
120 h
168 h
0
1
2
3
4
distance from drug source, cm

b)

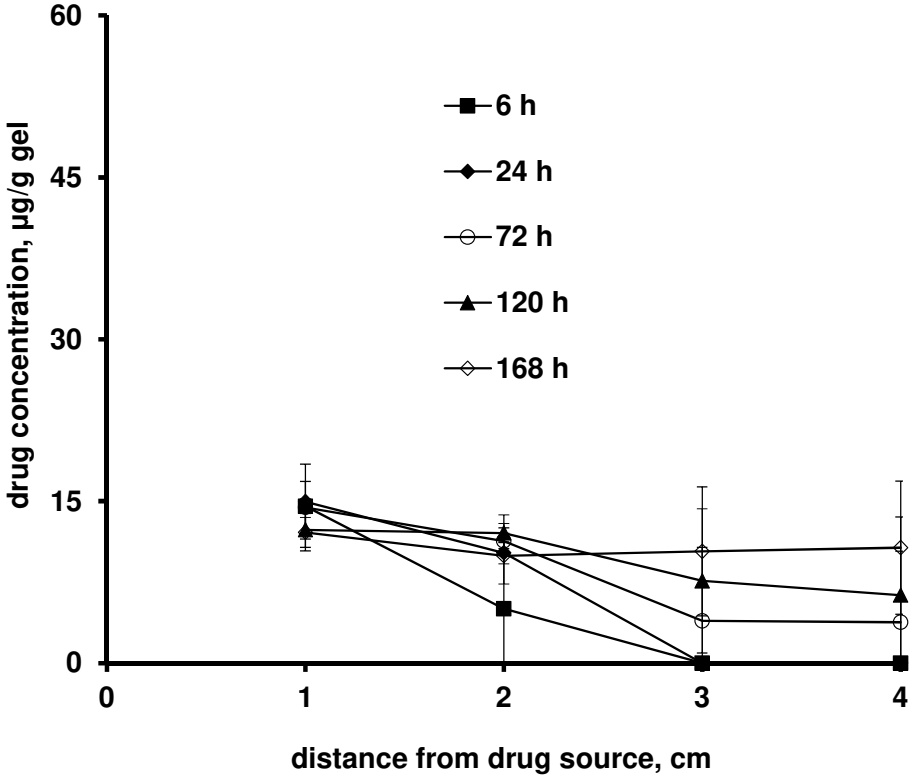
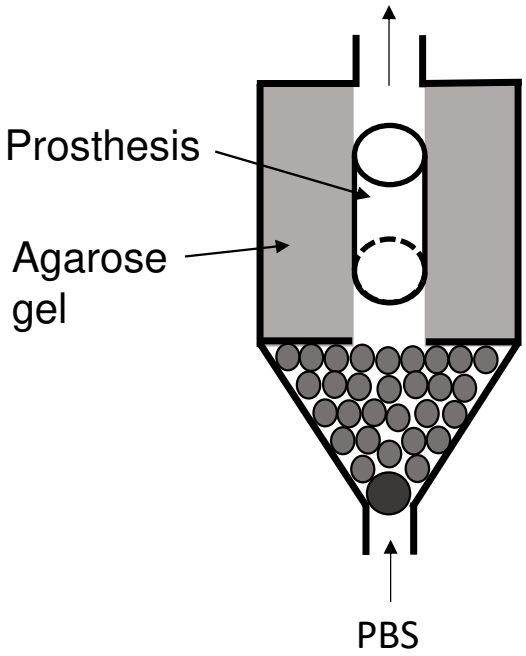
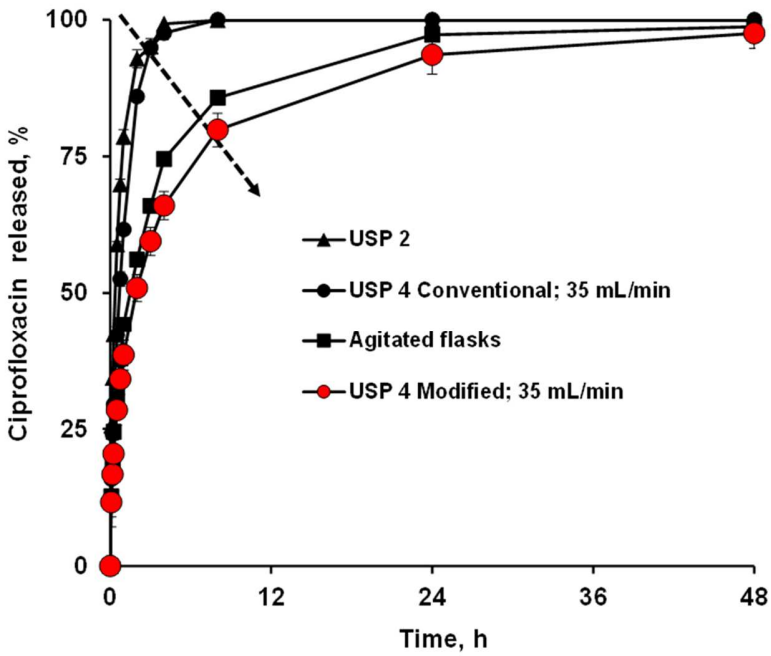
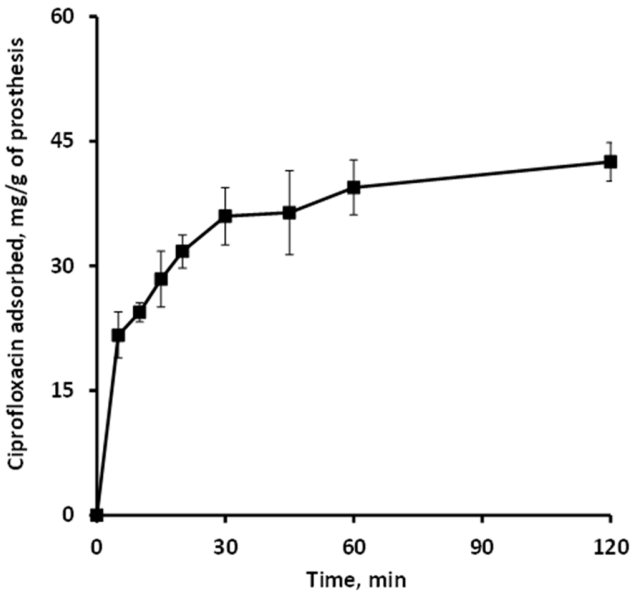
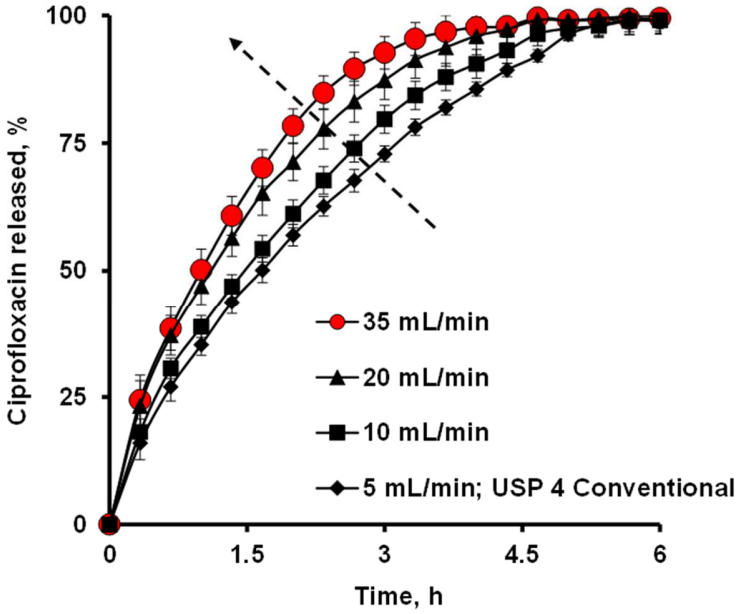

60
45
30
15
0
drug concentration, µg/g gel
6 h
24 h
72 h
120 h
168 h
0
1
2
3
4
distance from drug source, cm

Figure 9


Prosthesis
Agarose gel
PBS


Ciprofloxacin released, %
Time, h
USP 2
USP 4 Conventional; 35 mL/min
Agitated flasks
USP 4 Modified; 35 mL/min


Ciprofloxacin adsorbed, mg/g of prosthesis
Time, min


Ciprofloxacin released, %
Time, h
35 mL/min
20 mL/min
10 mL/min
5 mL/min; USP 4 Conventional