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In vitro evaluation of TiO₂ nanotubes as cefuroxime carriers on orthopaedic implants for the prevention of periprosthetic joint infections

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1 **In-vitro evaluation of TiO₂ nanotubes as cefuroxime carriers on**
2 **orthopedic implants for the prevention of periprosthetic joint**
3 **infections**

4
5 **Structured Abstract:**

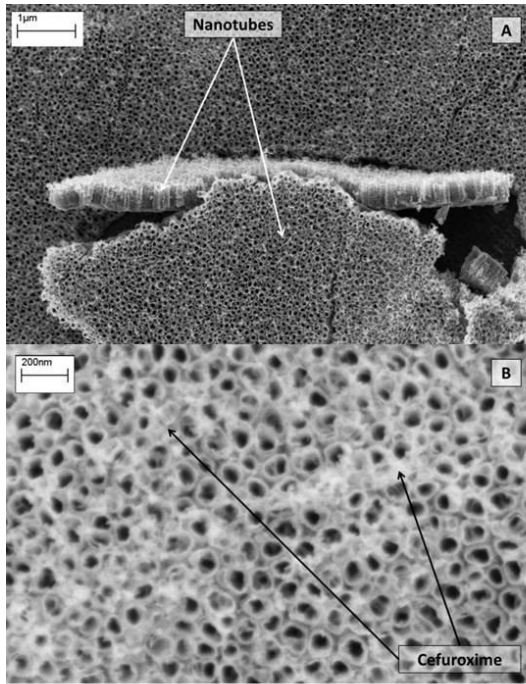
6 Context: The prevention of periprosthetic joint infections requires an antibiotic
7 prophylactic therapy, which could be delivered locally using titanium dioxide
8 nanotubes as novel reservoirs created directly on the orthopaedic implant titanium
9 surface.

10 Objective: In this study, the influence of several parameters that could impact the use
11 of titanium dioxide nanotubes as cefuroxime carriers was investigated.

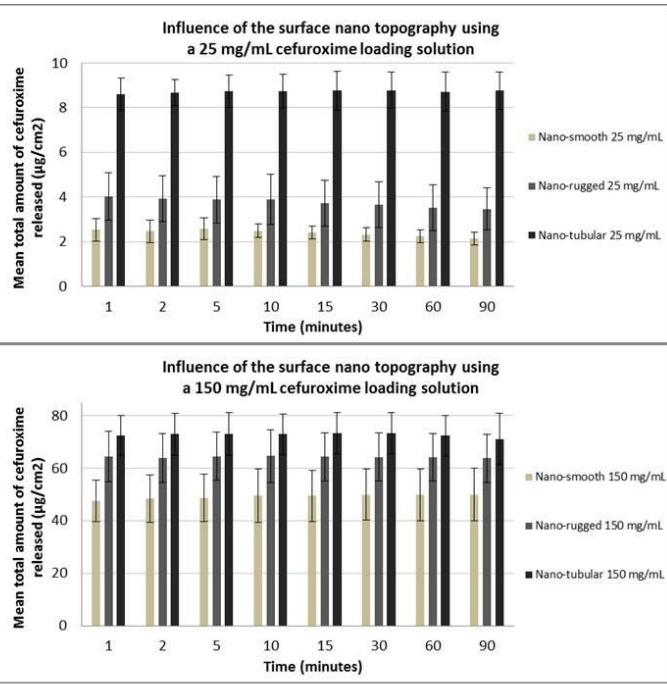
12 Method: Cefuroxime loading and release was studied for 90 minutes with three nano-
13 topography conditions (nano-smooth, nano-rugged and nano-tubular), two
14 cefuroxime loading solution concentrations (150 mg/mL and 25 mg/mL) and two
15 nano-tubular crystalline structures.

16 Results: In all tested conditions, maximum amount of cefuroxime was obtained within
17 two minutes. For both cefuroxime loading solution concentrations, nano-smooth
18 samples released the least cefuroxime, and the nano-tubular samples released the
19 most, and a six-fold increase in the concentration of the cefuroxime loading increased
20 the amount of cefuroxime quantified by more than seven times, for all tested nano-
21 topographies. However, the nano-tubes' crystalline structure did not have any
22 influence on the amount of cefuroxime quantified.

23 Conclusion: The results demonstrated that the surface nano-topography and loading
24 solution concentration influence the efficiency of titanium dioxide nanotubes as
25 cefuroxime carriers and need to be optimized for use as novel reservoirs for local
26 delivery of cefuroxime to prevent periprosthetic infections.



Surface topography of a nano-tubular anodized TiO_2 sample by SEM, before loading (A) and after loading with cefuroxime deposit and infiltration in the surface nanopores (B)



Total amount of released cefuroxime during 90 minutes for the different tested nano-topography and cefuroxime loading solution conditions

29

30

31 **In-vitro evaluation of TiO₂ nanotubes as cefuroxime carriers on**
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33 **infections**

34
35 P. Chennell^{1,2}, E. Fechet-Chassot¹, T. Devers³, K.O. Awitor¹, S. Descamps^{1,4}, V.
36 Sautou^{1,2}

37 ¹ EA 4676 C-BIOSENS, Clermont Université, Université d'Auvergne, BP 10448, F-
38 63000 Clermont-Ferrand, France

39 ² Service Pharmacie, CHU Clermont-Ferrand, F-63003 Clermont-Ferrand, France

40 ³ FRE 3520, CNRS - Université d'Orléans, 1b rue de la fêrolierie, 45071 Orléans
41 Cedex 2, France

42 ⁴ Service de Chirurgie Orthopédique, CHU Clermont-Ferrand, F-63003 Clermont-
43 Ferrand, France

44
45 **Corresponding author :**

46 Philip Chennell

47 pchennell@chu-clermontferrand.fr

48 C-BIOSENS, 4th R2, Faculty of Medicine and Pharmacy, Place Henri Dunant,
49 63000 Clermont-Ferrand cedex, France

50 Mobile : +33(0)6.38.13.79.11

51
52
53 **Keywords:**

54 Periprosthetic joint infections, Drug delivery systems, Titanium dioxide, Nanotubes,
55 Cefuroxime

56
57 **Introduction**

58 Total knee and hip arthroplasties (TKA and THA) are frequent orthopaedic
59 procedures, with over 4,400,000 TKA and 2,100,000 THA having been performed

60 between 1998 and 2008 in the United States (Memtsoudis et al., 2012).
61 Periprosthetic joint infection incidences vary notably with patient comorbidities, but
62 are estimated to range between 0.86% to 1.1% for TKA and between 0.3% to 1.63%
63 for THA (Dale et al., 2011; Jämsen et al., 2010; Ong et al., 2009; Phillips, 2006;
64 Pulido et al., 2008). With the number of TKA and THA predicted to grow by nearly
65 600% and more than 100% respectively between 2005 and 2030 in the United States
66 (S. Kurtz et al., 2007; Iorio et al., 2008), the absolute number of periprosthetic joint
67 infections is set to increase. The most prevalent etiological agents of orthopaedic
68 infections are gram positive opportunistic cocci, namely *Staphylococcus aureus* and
69 *Staphylococcus epidermidis*, responsible for more than two thirds of all infections
70 (Campoccia et al., 2006; Montanaro et al., 2011). If surgical debridement combined
71 with antibiotic therapy and implant retention is still possible in the early stages of
72 infections, in the other cases a surgical revision with implant ablation and several
73 weeks of intravenous antibiotic therapy is necessary to treat the infection completely
74 (Cuckler, 2005; Senthil et al., 2011).

75 A prophylactic systemic antibiotic treatment is recommended when using
76 implantable medical devices such as articular implants (AlBuhairan et al., 2008;
77 Meehan et al., 2009; Kuong et al., 2009; Gillespie and Walenkamp, 1996; Société de
78 Pathologie Infectieuse de Langue Française (SPILF), 2009). Regrettably, due to the
79 disturbed bone structure and local vascularity, caused by the trauma of the surgery,
80 as well as probable initial bad distribution of antibiotics into the bone (Smilack et al.,
81 1976), it is thought that the achieved *in situ* concentrations might not be enough to
82 exceed minimal inhibition concentrations (Schmidmaier et al., 2006; Zilberman and
83 Elsner, 2008), leading to a suboptimal efficiency.

84 Recently, the use of self-assembled titanium dioxide nanotubes (TiO₂ NT) as
85 reservoirs for localized antibiotic prophylactic therapy has been described (Ayon et
86 al., 2006; Gulati et al., 2011; Kang et al., 2007; Losic and Simovic, 2009; Macak et
87 al., 2007; Popat et al., 2007). Created directly on the titanium surface, TiO₂ NT, in
88 certain conditions, have been shown to improve osteoblast adhesion, reduce
89 bacterial colonization and serve as a reservoir for drugs (Losic and Simovic, 2009;
90 Gulati et al., 2011; Ghicov and Schmuki, 2009; Roy et al., 2011), possibly allowing an
91 *in situ* release of antibiotics after the prosthesis implantation.

92 In this work, Cefuroxime, a second generation cephalosporin, was chosen as
93 a model drug as its use is recommended by the American Academy of Orthopaedic
94 surgeons (Meehan et al., 2009) and by the French Society of Anaesthesia and
95 Intensive care (Société française d'anesthésie et de réanimation, 2011) for antibiotic
96 prophylactic treatment in orthopaedic surgery. Moreover, to our knowledge, there is
97 no data in the literature concerning the use of nanotubes as cefuroxime carriers.

98 We studied the influence of several parameters that could have an impact on
99 the use of TiO₂ NT as a cefuroxime carrier: the surface nano-topography (smooth
100 versus nano-rugged versus nanostructured titanium samples), the loading solution
101 concentration (by comparing the use of a saturated solution of cefuroxime (150
102 mg/mL) to a much lesser concentrated solution (25 mg/mL), for each nano-
103 topography condition) and the nanotube's crystalline structure (annealed nanotubes
104 versus non-annealed nanotubes).

105

106 **I. Materials and methods**

107 **A. Sample preparation**

108 **1. Titanium samples**

109 Unpolished titanium foil (99.6% purity, 0.1 mm thickness, Goodfellow, Lille France)
110 was first cut into rectangular samples (2.5 X 1.5 cm), which were then degreased by
111 successive 5 minutes sonications in trichloroethylene (VWR BDH Prolabo, Fontenay-
112 sous-Bois, France), acetone (Merck Millipore, Darmstadt, Germany) and methanol
113 (UCB Pharma France, Colombes, France), then rinsed with deionised water, dried in
114 the oven at 100 °C and finally cooled in a desiccator. The obtained samples are later
115 on referred to as nano-smooth samples.

116 To obtain nano-rugged surfaces, but without fully formed nanotubes, the as
117 previously described cleaned titanium foil samples (smooth samples) were anodized
118 by immersion to a height of 1.5 cm in a 0.4 wt. % HF aqueous solution with a
119 platinum cathode, as schematized in Figure 1. The anodizing voltage was
120 maintained at 20 V, for 80 seconds, at a constant temperature of 20 °C.

121 To obtain TiO₂ nanotubes (nano-tubular samples), the anodization was carried out in
122 the same conditions as described above but for 20 minutes.

123 All experiments were conducted on the obtained nano-smooth, nano-rugged or nano-
124 tubular titanium foil.

125 To convert the amorphous nanotubes into the mixed crystalline phases of anatase
126 and rutile, some samples were annealed at 500°C for 2 hours under oxygen, with a
127 heating and cooling rate of 5 °C min⁻¹.

128

129 **2. Cefuroxime loading solutions**

130 A 150 mg/mL cefuroxime solution was prepared by dissolving 1.5 g of cefuroxime
131 (Panpharma, Fougères, France, batch No 104179) with sterile deionised water
132 (VERSYLENE®, Fresenius Kabi, Louviers, France) to a total volume of 10 mL.

133

134 **3. Loading method and storage**

135 To load the samples with cefuroxime, an adapted soaking technique was used (Ayon
136 et al., 2006; Kim et al., 2008). Each sample was immersed to a height of 1.5 cm, in
137 either a 150 mg/ml or a 25 mg/mL solution of cefuroxime, for 30 minutes, in ambient
138 daylight, at room temperature (22-26°C). At the end of the loading time, the samples
139 were removed from the immersion solution and were immediately air blown to
140 remove excess solution on the surface and to dry them.

141 The samples were stored prior to loading and after loading, until cefuroxime
142 quantification, in a climate chamber (BINDER GmbH, Tuttlingen, Germany), in the
143 dark, at 25°C.

144

145 **B. Sample characterizations**

146 **1. Structural characterizations**

147 The crystalline structure and phase of the TiO₂ nanotube layers of a smooth, a non-
148 annealed and an annealed nano-tubular sample were determined by X-ray diffraction

149 (XRD) using a Scintag XRD X'TRA diffractometer with CuK α ($\lambda = 1.54^\circ$ radiation).
150 The CuK β radiation was filtered through a nickel filter. The diffraction pattern was
151 achieved between 20 and 80 $^\circ$ with a step angle of 0.05 $^\circ$ and a scanning speed of
152 0.01 $^\circ$ per second.

153 For the study of the surface nano-topography, structural characterization of a smooth,
154 a nano-rugged and a nano-tubular sample was performed before and after drug
155 loading with two different concentration cefuroxime solutions using a field emission
156 scanning electron microscope (SEM). It was performed using a Supra 55 VP SEM
157 (Carl Zeiss SMT, Nanterre, France) with secondary emission and in lens detector.
158 The accelerating voltage and the working distance were respectively 3 kV and either
159 5 or 6 mm (image dependent).

160 Images were acquired at different scan sizes from the top surface.

161 For the four different tested conditions of titanium foil nano-topography and crystalline
162 structure (smooth, nano-rugged, non-annealed nano-tubular and annealed nano-
163 tubular), surface wettability was investigated using a drop shape analysis system
164 (EasyDrop, Kruss, Hamburg, Germany). The contact angle was measured with a
165 deionized water sensile droplet of 3 μ L in ambient conditions. The measurement was
166 taken 5 seconds after the deposition of the water droplet on the substrate. After
167 measurement, the samples were cleaned, dried in nitrogen and stored in a
168 desiccator.

169

170 **2. Cefuroxime quantification**

171 Each sample was placed into a known volume of sterile deionised water (5 or 10 mL
172 depending on the estimated loaded quantity of cefuroxime), for 90 minutes. At
173 determined times (1; 2; 5; 10; 15; 30; 60 and 90 minutes) a determined volume (1000
174 μ L) of release solution was collected and was replaced with the same volume of
175 fresh sterile deionised water.

176 The cefuroxime present in the collected sample was then quantified by HPLC
177 composed of a PU-2080 Plus pump, and an AS-2055 Plus auto-sampler coupled with
178 an UV/VIS spectrophotometer (UV-2075 Plus detector), from Jasco France
179 (Bouguenais, France)

180 The HPLC separation column used was a 5 μm Lichrospher 100 RP 18 endcapped
181 column (125 \times 4.6 mm ID) (Macherey-Nagel EURL, Hoerd, France)

182 The HPLC mobile phase was composed of 85/15 phosphate buffer/acetonitrile (v/v)
183 mixture. The phosphate buffer used was a 0.1 mol/L solution of H_2KPO_4 (VWR
184 International Pessac, France). The flow rate through the column for the analysis was
185 set at 1 mL/min, with the column thermo regulated to a temperature of 35°C. The
186 injection volume was of 20 μL . The detection wavelength was set up at 273 nm.

187 Cefuroxime presents a retention time of 3.8 minutes. This chromatographic method is
188 linear for concentrations ranging from 0.25 $\mu\text{g/mL}$ to 20 $\mu\text{g/mL}$. The mean linear
189 regression equation obtained is $y = 35022x + 3014.7$ ($r^2 = 0.9998$), where x is the
190 cefuroxime concentration and y the surface area of the corresponding peak. This
191 method has acceptable accuracy and precision as the intra-assay and inter-assay
192 coefficients of variation are below 5%. The limit of quantification of this method is of
193 0.25 mg/mL.

194 Cefuroxime release results were expressed in μg of cefuroxime over the loaded
195 surface of anodized TiO_2 ($\mu\text{g/cm}^2$).

196

197 **C. Tested parameters**

198 In this work, several parameters that could have an influence on the use of
199 TiO_2 NT as cefuroxime reservoirs were investigated.

200 **1. Influence of the surface nano-topography and loading** 201 **solution concentration**

202 10 anodized (nano-tubular) non annealed Ti samples, 10 nano-rugged Ti samples,
203 and 10 smooth Ti samples were prepared. 5 samples of each surface nano-
204 topography (15 samples in total) were loaded by immersion into a 150 mg/mL
205 cefuroxime solution, as previously described, whilst the other 5 of each surface nano-
206 topography were loaded with a 25 mg/mL cefuroxime solution.

207 **2. Influence of the nanotube's crystalline structure**

208 To see if annealing (heat treating) the nanotubes could impact their use as
209 cefuroxime carriers as it modifies the nanotubes crystalline structure, annealed
210 nanotubes were compared to non-annealed nanotubes.

211 7 non-annealed anodized samples and 7 annealed anodized samples were loaded
212 by immersion in a 150 mg/mL cefuroxime solution, as previously described.

213

214 **D. Statistical considerations**

215 Statistical analysis was performed using a non-parametric Man-Whitney test. The
216 difference was considered significant for a p-value ≤ 0.05 .

217

218 **II. Results**

219 **A. Structural characterization and surface wettability**

220 Figure 1 shows the current density time curve for Ti anodization obtained in our
221 operating conditions, and illustrates the nanotube growth. SEM pictures of obtained
222 nano-rugged and nano-tubular samples before loading are presented in Figure 2.
223 Anodization occurred on both sides of the anodized samples.

224

225 Nano-smooth non anodized samples typically are not microscopically smooth, but do
226 not present any nano-scale ruggedness. Nano-rugged samples present nano-scale
227 modifications with the formation of what seems to be shallow nanopores, whilst
228 retaining their micro-scale topography. For the 20 minutes anodized samples, the
229 formed nanotubes have dimensions of between 300 to 400 nm high and 70 to 90 nm
230 in diameter.

231 The XRD results are depicted in Figure 3. The peaks characteristic of anatase and
232 rutile crystalline structure only appear after annealing at 500°C, and are not present
233 in Ti foil or as-anodized Ti .

234

235 Concerning the contact angle measurements (data summarised in Table I),
236 anodization times of 80 seconds and 1200 seconds greatly increase the contact
237 angle compared to smooth un-anodized titanium; however the contact angle

238 measured for an annealed nanotubular surface was lower than un-annealed
239 anodised surface (Figure 4), and even lower than for a nano-smooth surface.

240

241

242 **B. Cefuroxime quantification**

243 SEM pictures of loaded samples are shown in Figure 5, with light grey/white
244 colouring showing the titanium sample, and dark grey patches being cefuroxime
245 deposits. In Figure 5(A), a relatively large deposit of cefuroxime can clearly be seen,
246 with Figure 5(B) being a closer view of the phenomenon. Figure 5(C) and Figure 5(D)
247 show how cefuroxime infiltrates and sometimes covers the nano-rugged surface
248 nanopores, and in Figure 5(E) and Figure 5(F) the same can be said of the nano-
249 tubular surface.

250

251

252 Figure 6 (A) and (B) shows the total amount of cefuroxime released over a 90
253 minutes period from the different tested nano-surfaces with a 25 mg/mL and 150
254 mg/mL cefuroxime loading solution, and Figure 7 shows the total amount of
255 cefuroxime released over a 90 minutes period for annealed and un-annealed nano-
256 tubular surfaces. Table II summarise the statistical data of the different tested
257 conditions at 90 minutes.

258 Maximum cefuroxime release for the studied conditions was obtained within the first
259 one or two minutes, and no additional cefuroxime release was detected for the rest of
260 the release study period.

261 For the nanotube length tested, there is no statistical influence of the nanotube's
262 crystalline structure on the amount of cefuroxime quantified ($p = 0.28$). Cefuroxime
263 loading solution concentration does have a significant impact on cefuroxime
264 quantities released ($p = 0.001$) as does the surface nano-topography, but only
265 between the smooth samples and the nanotube samples for both tested loading
266 solution concentrations ($p = 0.04$). The difference between the nano-rugged surface
267 samples and the nanotubular surface samples is not statistically significant, for

268 neither loading solution concentrations ($p = 0.08$). The difference in cefuroxime
269 release is significant between the smooth and nano-rugged surface samples only for
270 a loading solution concentration of 25 mg/mL ($p = 0.02$).

271

272

273

274 **III. Discussion**

275 The aim of this work was to study several conditions which were hypothesised to
276 influence the use of TiO₂ nanostructures as cefuroxime carriers.

277 Firstly, to try and characterize whether nanotubes can be used as cefuroxime
278 reservoirs, 3 different nano-topography conditions were compared: smooth, nano-
279 rugged, nano-tubular. After loading, the smooth samples released the least
280 cefuroxime (median 49.89 $\mu\text{g}/\text{cm}^2$), and the nano-tubular samples released the most
281 (median 70.03 $\mu\text{g}/\text{cm}^2$). The difference between the smooth samples and
282 nanotubular samples was statistically different, for both loading solution
283 concentrations, but was much more pronounced for the 25 mg/mL loading solution
284 (4.4-fold increase) when compared to the 150 mg/mL loading solution (40%
285 increase). This could indicate either a loading of the nanotubes (penetration of
286 cefuroxime into the tubes), or an increased surface adsorption, mediated by the
287 nanotubes, or both. It could also be possible the cefuroxime penetrated the
288 interstices visible between the nanotubes. Despite the differences not being
289 significant between the nano-rugged samples and the smooth and nanotubular
290 samples, the quantities of cefuroxime released by the nano-rugged samples are
291 intermediate between the smooth and the nanotubular samples (67.89 $\mu\text{g}/\text{cm}^2$).

292 There is a statistical difference in released cefuroxime from samples loaded with a
293 150 mg/mL cefuroxime solution when compared to samples loaded with a 25 mg/mL
294 solution, for all tested surface nano-topographies, which indicates an influence of the
295 loading solution concentration, which is what was expected. However, despite there
296 being a fixed six-fold difference in concentration between the 25 mg/mL and the 150
297 mg/mL, the obtained difference in quantified cefuroxime was greater, and varied

298 depending on the nano-topography (24-fold, 20-fold and 7.7-fold for respectively the
299 nano-smooth, nano-rugged and nano-tubular topographies). Such a difference is
300 difficult to explain by just the results' variability. One explanation is that despite a
301 thorough air blowing cleaning procedure, surface adsorption of cefuroxime accounts
302 for an important percentage of total loaded and released drug.

303 TiO₂ exists naturally in 3 crystalline phases, anatase, rutile and brookite (Roy et al.,
304 2011), yet after their electrochemical formation, TiO₂ nanotubes are amorphous. By
305 annealing (heat treating), the nanotubes can be converted to anatase or rutile, which
306 changes notably the TiO₂ nanotubes electrochemical and photocatalytical properties,
307 but without notably altering the nanotube's morphological characteristics (Lin et al.,
308 2011; Yu and Wang, 2010). In this work, annealed nanotubes did release more
309 cefuroxime than non-annealed, but the difference was not statistically significant,
310 notably due to the observed large variations of cefuroxime release within the
311 annealed group. The annealed nano-tubular surfaces were however much more
312 hydrophilic than the un-annealed nano-tubular surfaces, with a contact angle of 42°
313 versus 120° for the un-anodized nano-tubular. In the conditions of this assay, it is
314 therefore hard to draw any conclusions on the real influence of the crystalline state.

315 The relatively high cefuroxime quantities quantified with the nano-smooth samples
316 could be linked to moderate surface wettability, as expressed by the measure of
317 water droplet contact angles, as the contact angle for such a surface was measured
318 to be 77°. By contrast, non-annealed anodized surfaces (nano-rugged and nano-
319 tubular) had contact angles measured to be around 120°, expressing a more
320 hydrophobic state, and yet released after loading more cefuroxime. Such results
321 could be in favour of nanotube loading and release of cefuroxime, independently of
322 the surfaces' wettability, at least for the tested conditions.

323 However, due to the small number of samples, the statistical power of the used test is
324 probably insufficient to identify small differences between certain tested conditions.

325

326 In our work, the drug release was nearly immediate, and is much faster than reported
327 in other studies. Popat et al report loaded bovine serum albumin (BSA) and lysozyme
328 using a pipetting method onto unpolished titanium samples with similar TiO₂

329 nanotubes and obtained maximum release times that varied between 25 and 110
330 minutes (Popat et al., 2007). Using a similar method, Aninwene et al loaded
331 penicillin/streptomycin or dexamethasone and obtained drug elution for 3 days
332 (Aninwene et al., 2008). The very fast release obtained here could be explained by
333 high Cefuroxime water solubility (150 mg/mL), or by a surface layer of Cefuroxime, or
334 possibly by both. Despite our cleaning method, SEM images showed significant
335 Cefuroxime deposit on the surface of the TiO₂ layer, on samples with different nano-
336 topography, possibly linked to the surface micro-topography. However, since nano-
337 tubular samples released significantly more Cefuroxime than smooth samples, it
338 seems quite plausible that Cefuroxime penetrated at least partially into the
339 nanotubes.

340 Longer release times have been reported. Peng et al, also using unpolished TiO₂
341 nanotube surfaces and a consistent cleaning technique, found that elution kinetics of
342 paclitaxel and BSA were influenced by nanotube height and pore diameter (Peng et
343 al., 2009), with nanotubes of 5 µm height and 100 nm of diameter releasing the most
344 drug for up to 3 weeks.

345 Therefore, in this study, the thickness of the nanotube layer could impact the
346 maximum loading capacity of the tubes, as the longer the tube, the more volume it
347 could contain. It could be possible that 400 nm of length is not enough to allow a
348 significant amount of drug into the nanotubes, with regard to the potential surface
349 adsorption. Therefore, any conclusions about the impact of the nanotubes' crystalline
350 structure might be premature, as the 400 nm high nanotubes could have been loaded
351 at maximum capacity regardless of the surface wettability.

352 In this work, the titanium foil used for the experiments was unpolished, and wasn't
353 microscopically smooth. This could have an impact on cefuroxime adsorption, as
354 SEM structural characterization of samples with different nano-topographies, loaded
355 with cefuroxime, showed an inhomogeneous cefuroxime spread. The microscopic
356 surface features could lead to the formation of "beds" of cefuroxime, protected from
357 the surface cleaning procedure. It has also already been hypothesized that in
358 previous studies at least a significant amount of drug stayed on the surface and did
359 not penetrate into the nanotubes (Peng et al., 2009). These microscopic features could
360 also account for the high variability between samples of the same series. Also,

361 surface adsorption could account for the instant burst-like release that was observed.
362 Therefore, it would seem that in order to more accurately measure the exact quantity
363 of drug actually loaded and then released by the nanotubes, microscopically smooth
364 samples are needed, as well as an adequate and validated surface cleaning method.

365

366 Several improvements could be made to this study. To achieve a microscopically
367 smooth surface, a polishing method (electro-chemical polishing or physical polishing)
368 could have been used. The effects of micro-scale ruggedness would therefore be
369 reduced. Also, higher nanotubes could offer improved loading volume and drug
370 storage capacity, and could also improve loading and elution kinetics.

371

372 The work presented here seems to indicate that some parameters, like nanotube
373 height and the loading solution concentration, have more influence on cefuroxime
374 release from TiO₂ nanotubes, whereas the crystalline structure of the nanotubes
375 didn't influence the amount of cefuroxime released. The nano-tubular samples
376 released more cefuroxime than nano-smooth or nano-rugged samples, for both 150
377 mg/mL and 25 mg/mL cefuroxime loading concentrations. However, cefuroxime
378 release kinetics were too fast for lasting local drug delivery, and need to be extended.
379 Longer nanotubes could increase the amount of cefuroxime loaded and release
380 times, but might also increase overall fragility, and thus need to be tested. Also, the
381 antibacterial efficacy of such a delivery method using cefuroxime still needs to be
382 investigated.

383

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387

388 **V. Bibliography**

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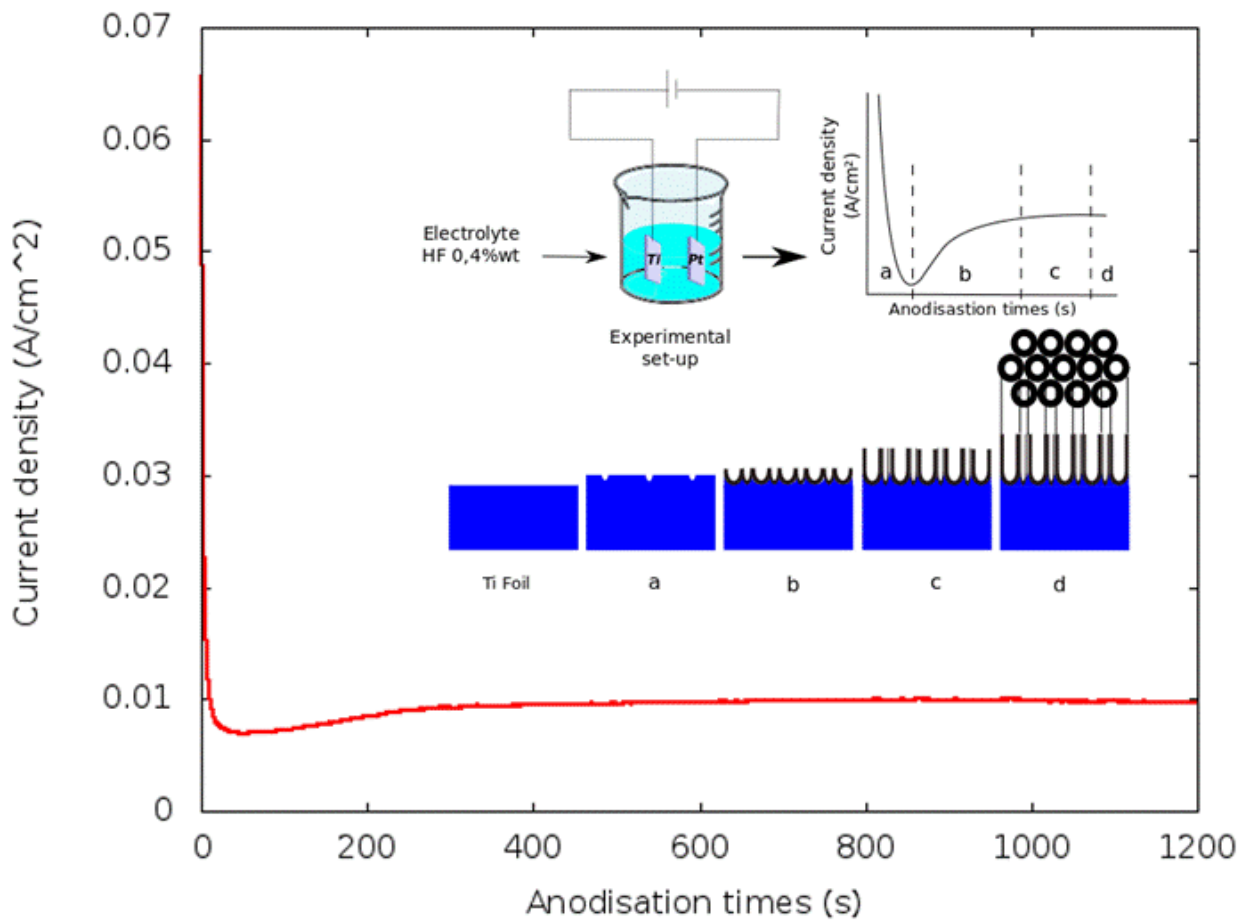
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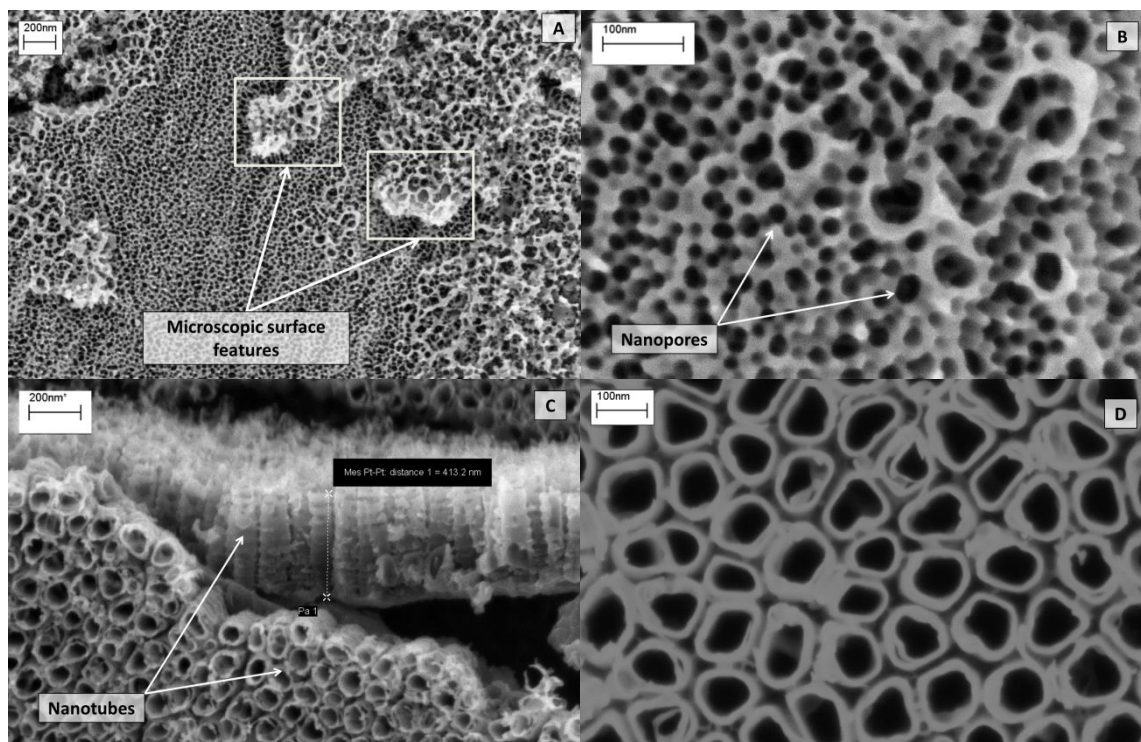
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483 **Figure 1:** Current density time curve with experimental setup. Nanotubes appear to
484 be fully formed as of 20 minutes (1200 seconds) of anodizing time (d). The nano-
485 rugged surface was obtained with an anodizing time of 80 seconds, which
486 corresponds to the low point of current density curve, between stage (a) and (b).

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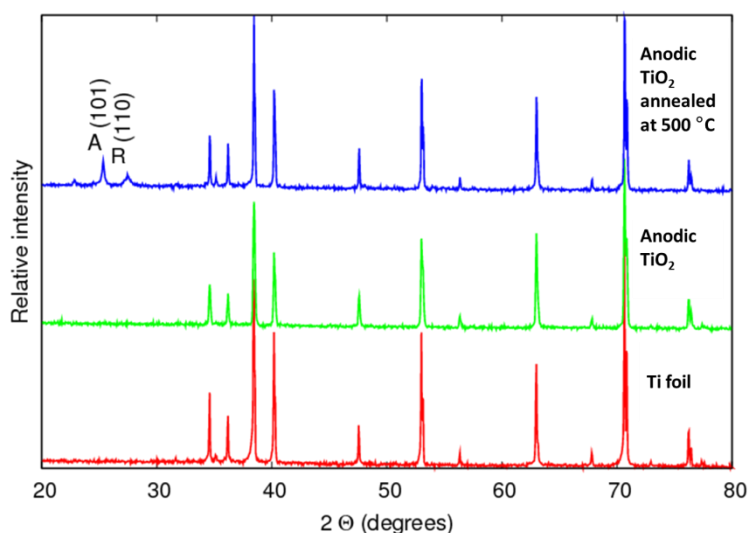
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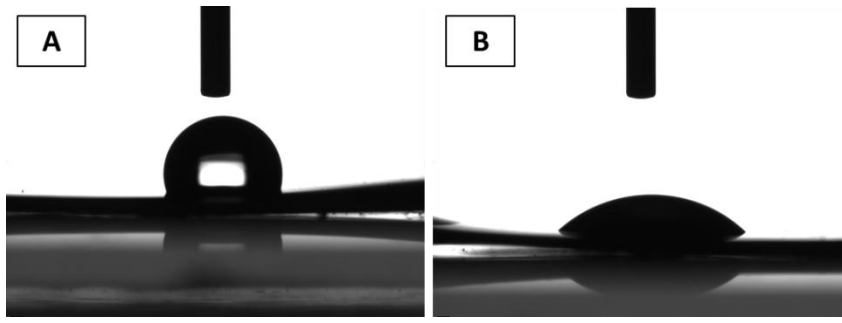
491 **Figure 2 (A), (B), (C) and (D):** Surface topography of a nano-rugged (A) and (B) and
492 nano-tubular (C) and (D) anodized TiO₂ sample by SEM, before loading.

493



494

495 **Figure 3:** X-ray diffractogram of smooth Ti foil (bottom graph), anodized (Anodic
496 TiO₂, middle graph) and 500°C annealed anodized titanium foil (top graph) with two
497 peaks (A and R) corresponding to respectively the anatase and rutile crystalline
498 phase.



499

500 **Figure 4:** (A) un-annealed nano-tubular sample and (B) annealed nano-tubular
501 sample

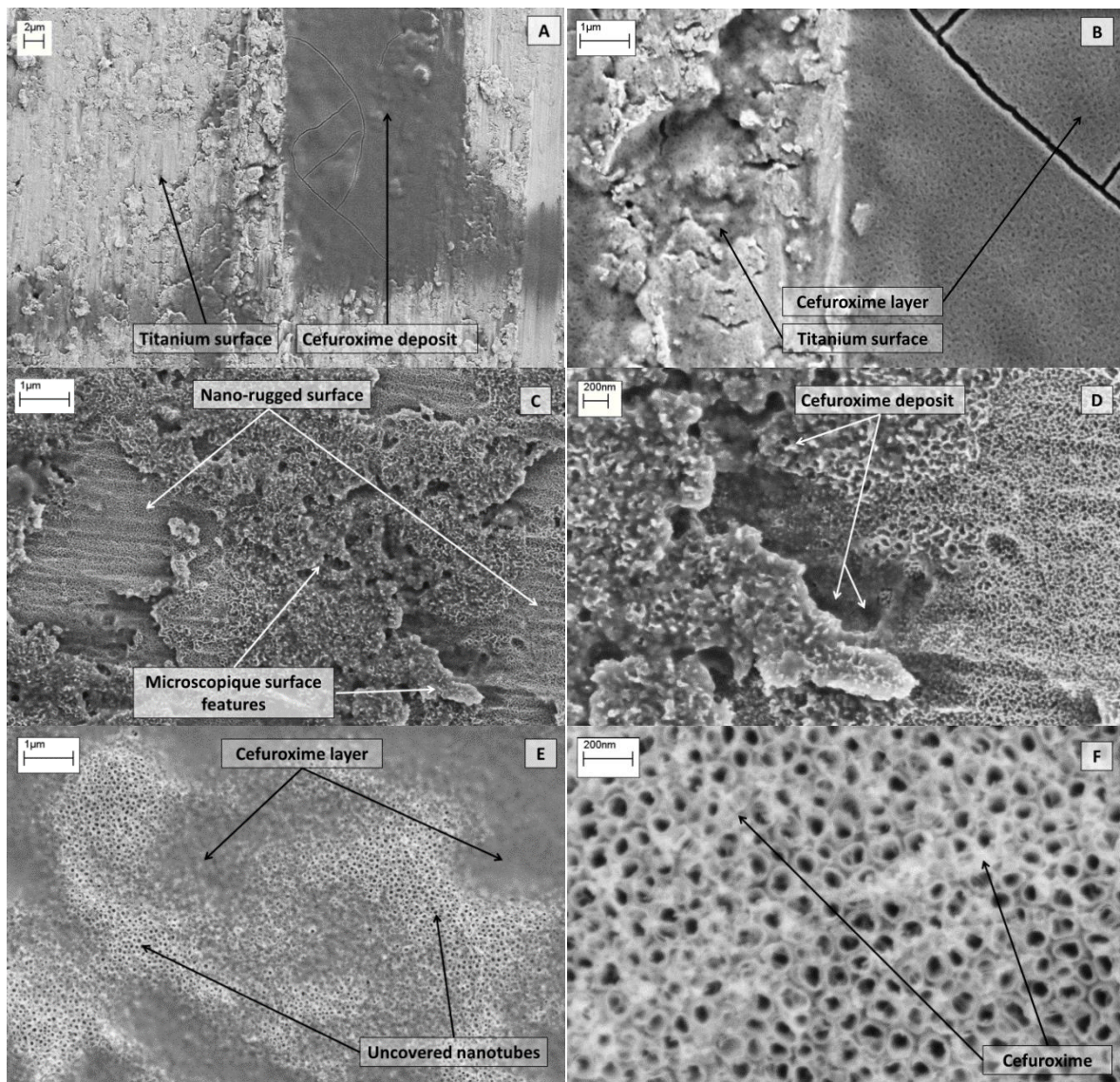
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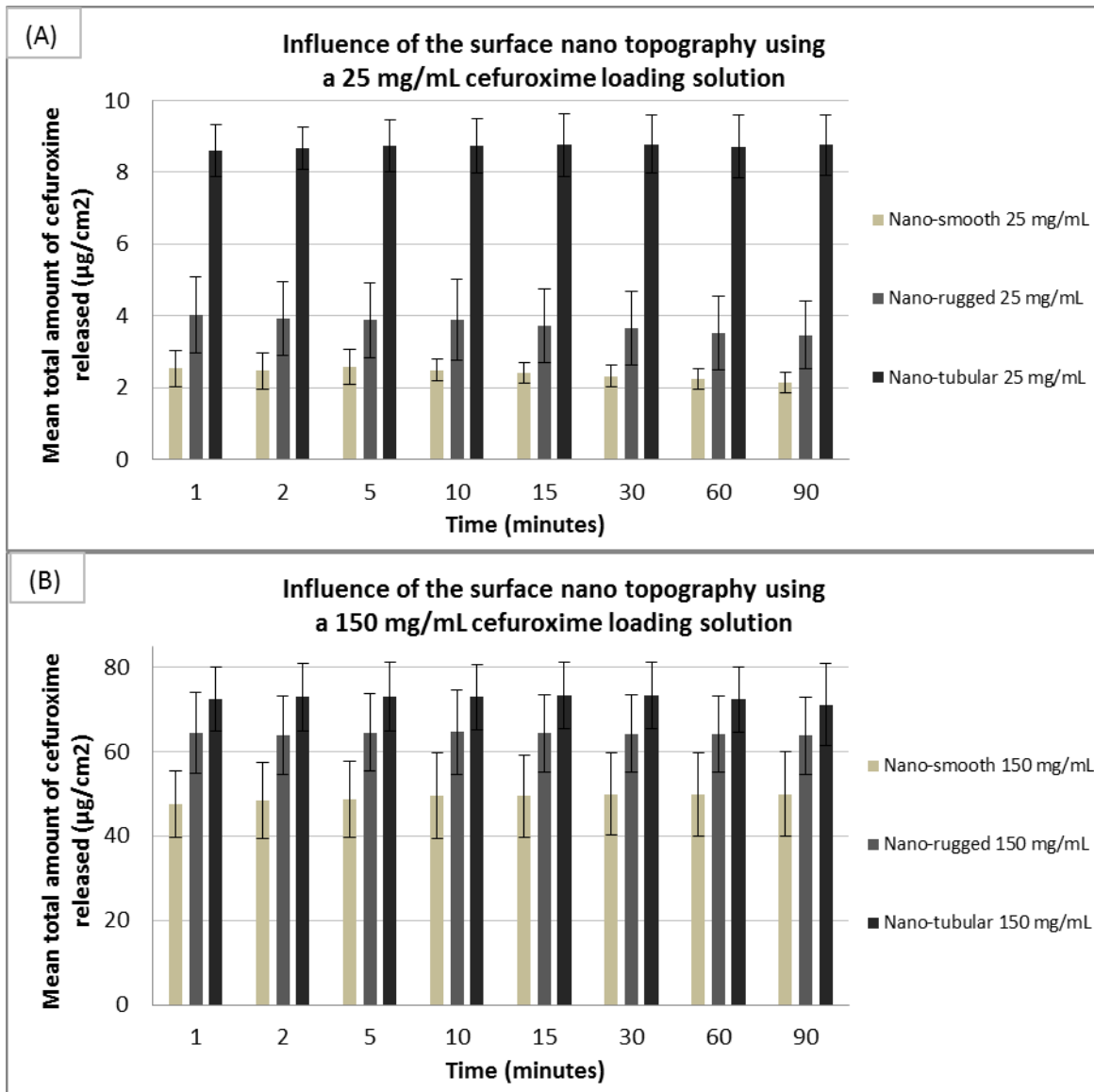
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507 **Figure 5:** (A) and (B): smooth titanium sample after cefuroxime loading, with (B)
508 being a close up view of (A). (C) and (D): nano-rugged titanium sample with
509 cefuroxime deposit and infiltration in the surface nanopores, with (D) being a close up
510 view of (C). (E) and (F): nano-tubular titanium sample with cefuroxime deposit and
511 infiltration in the surface nanopores.

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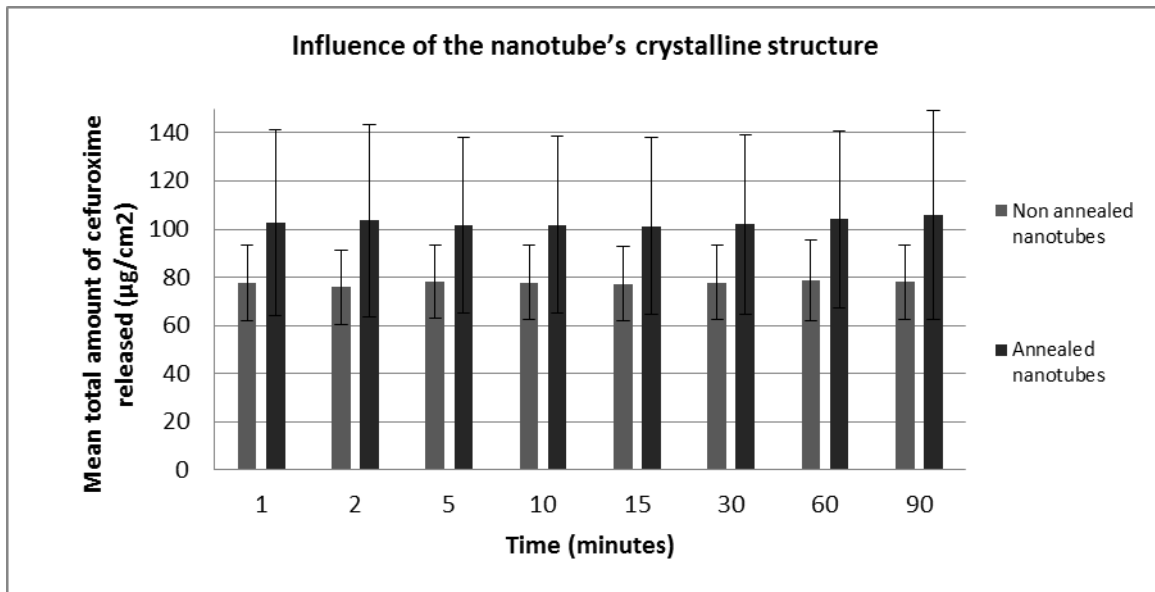


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515 Figure 6: Total amount of released cefuroxime during 90 minutes for the different
 516 tested nano-topography and cefuroxime loading solution conditions

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520 **Figure 7:** Total amount of cefuroxime released during 90 minutes for amorphous and
 521 crystalline structures

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524 **Table I :** Contact angle measurement data

Measured contact angle	Mean contact angle	Confidence intervall 95%
Nano-smooth surface	77,18	1,90
Non-annealed nano-rugged surface	122,00	1,95
Non-annealed nano-tubular surface	118,00	0,72
Annealed nano-tubular surface	41,73	0,67

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527 **Table II :** Statistical data for the different tested conditions at 90 minutes

Cefuroxime release at t = 90 minutes		Number of samples	Median total quantity of Cefuroxime (µg/cm ²)	Interquartile range	Mean total quantity of Cefuroxime (µg/cm ²)	Standard deviation	Variation coefficient (%)
Surface nano-topography and Loading solution concentration	25 mg/mL nano-smooth	3	2,07	0,55	2,13	0,28	13,2
	25 mg/mL nano-rugged	4	3,45	1,22	3,46	0,95	27,4
	25 mg/mL nano-tubular	4	9,09	1,58	8,77	0,84	9,6
Loading solution concentration	150 mg/mL nano-smooth	4	49,89	15,61	50,00	10,06	20,1
	150 mg/mL nano-rugged	4	67,89	17,84	63,86	9,07	14,2
	150 mg/mL nano-tubular	4	70,03	10,38	71,17	9,75	13,7
Crystalline structure	Non annealed	7	76,99	21,76	78,11	15,40	19,7
	Annealed	7	87,28	72,21	105,90	43,44	41,0

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