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1

2 **Interactions between surfactants in solution and electrospun protein fibers -**
3 **effects on release behavior and fiber properties.**

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15 **Abstract**

16 Intermolecular interaction phenomena occurring between endogenous compounds such as proteins and
17 bile salts and electrospun compounds applied for biomedical purposes is so far unreported, despite the
18 exposure of fibers to such biorelevant compounds when applied for e.g. tissue engineering, wound
19 healing and drug delivery. In the present study, we present a systematic investigation of how surfactants
20 and proteins, as physiologically relevant components, interact with insulin-loaded fish sarcoplasmic
21 protein (FSP) electrospun fibers (FSP-Ins fibers) and thereby affect fiber properties such as accessible
22 surface hydrophilicity, physical stability and release characteristics of an encapsulated drug. Interactions
23 between insulin-loaded protein fibers and five anionic surfactants (sodium taurocholate, sodium
24 taurodeoxycholate, sodium glycocholate, sodium glycodeoxycholate, and sodium dodecyl sulfate), a
25 cationic surfactant (benzalkonium chloride) and a neutral surfactant (Triton X-100) were studied. The
26 anionic surfactants increased the insulin release in a concentration-dependent manner, whereas the
27 neutral surfactant had no significant effect on the release. Interestingly, only minute amounts of insulin
28 was released from the fibers when benzalkonium chloride was present. The FSP-Ins fibers appeared
29 dense after incubation with this cationic surfactant, whereas high fiber porosity was observed after
30 incubation with anionic or neutral surfactants. Contact angle measurements and staining with the
31 hydrophobic dye 8-anilino-1-naphthalenesulfonic acid indicated that the FSP-Ins fibers were
32 hydrophobic, and showed that the fiber surface properties were affected differently by the surfactants.
33 Bovine serum albumin also affected insulin release *in vitro*, indicating that also proteins may affect the
34 fiber performance in an *in vivo* setting.

35

36 Keywords: Electrospinning, proteins, surfactants, drug delivery, intermolecular interactions, tissue
37 engineering

38 **Introduction**

39 Electrospun nanofibers may be applicable for a large range of biomedical applications within e.g tissue
40 engineering, regenerative medicine and drug delivery¹⁻⁵. The nanofibers can be functionalized in order
41 to improve the performance *in vivo*, for example by addition of proteins to increase biocompatibility^{6,7},
42 addition of a polymer to strengthen the mechanical properties⁷, addition of drugs such as antibiotics in
43 wound dressing⁸⁻¹⁰ or growth factors or DNA for tissue engineering¹¹⁻¹⁵. Even cells can be electrospun
44 to promote regeneration of new tissue¹⁶⁻¹⁸. During optimization of the fiber properties, special attention
45 should be paid to the hydrophilicity of the fibers, fiber stability and drug release profiles (in case of
46 encapsulated compounds)¹². Drug release and stability properties are often studied *in vitro* in water^{19,20},
47 phosphate buffer²¹, phosphate-buffered saline (PBS)^{8,22-33} or other similar buffers not containing specific
48 physiologically occurring compounds^{34,35}. Depending on the application of the electrospun fibers, they
49 will be exposed to different physiological environments, which contain compounds such as bile salts,
50 proteins, and salts. However, the effects of physiologically occurring components on fiber stability and
51 drug release characteristics are rarely taken into account, for which reason the fiber performance *in vitro*
52 may deviate from the fiber performance *in vivo*. In a previous study we found that release of insulin from
53 protein fibers was increased in simulated small intestinal fluid containing lipids and sodium taurocholate
54 (ref; FSP-Ins paper). The study also demonstrated that transport of insulin across a Caco-2 cell monolayer
55 was increased due to interactions between the cells and fibers. In a study by Alborzi *et al.* release of folic
56 acid from alginate-pectin/polyethylene oxide electrospun fibers was studied in simulated intestinal fluid
57 containing bile extract, and the authors found that the release was decreased in the presence of bile
58 extract³⁶. Sofokleous *et al.* studied release of amoxicillin from poly(D,L-lactide-co-glycolide) (PLGA)
59 for wound healing purposes, and found the drug release from the PLGA dressings to be different in the

tested media (water, simulated body fluid and PBS)¹⁰. Moreover, Maretschek *et al.* found release of cytochrome C from poly(L-lactide) fibers to be increased when polysorbate was present in the release medium³⁷. Despite of those observations, and to the authors knowledge, no study has been dedicated to detailed investigations on the potential effects in relation to the performance of electrospun fiber scaffolds caused by interactions with biorelevant compounds. In the current study, we investigated how biorelevant compounds affect the properties of electrospun fibers by systematically studying the interactions between insulin-loaded electrospun protein fibers and four different bile salts present in the human intestinal fluid (sodium taurocholate (TC), sodium taurodeoxycholate (TDC), sodium glycocholate (GC) and sodium glycodeoxycholate (GDC)). In order to further elucidate the mechanism accounting for the drug release induced by biological surfactants, different synthetic small molecular size surfactants were included in the study: the anionic sodium dodecyl sulfate (SDS), the cationic benzalkonium chloride (BC) and the neutral Triton X-100. The unpredictable effects of the different surfactants on the insulin release profile, fiber porosity and fiber surface properties are reported.

Materials and methods

Materials

Cod (*Gadus morhua*) from the North Sea was obtained from Hanstholm Fisk (Hanstholm, Denmark). Human insulin was kindly provided by Sanofi-Aventis Deutschland (Frankfurt, Germany). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) was acquired from AppliChem (Darmstadt, Germany). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 2-(N-morpholino)ethane sulfonic acid (MES), Hank's balanced salt solution (HBSS), TC, TDC, GC, GDC, Triton X-100, SDS, 8-anilinonaphtalene-1-sulfonic acid (ANS), and benzoylacetone (BZA) were obtained from Sigma-Aldrich (St. Louis, MO,

81 USA), and BC was obtained from Nomeco (Copenhagen, Denmark). All other reagents were obtained
82 commercially at analytical grade or HPLC grade for solvents for chromatography, and used without
83 further purification. Ultrapure water from Barnstead NanoPure Systems (Thermo Scientific, Waltham,
84 MA, USA) was used throughout the studies.

85 **Preparation of FSP-Ins fibers**

86 Fish sarcoplasmic proteins (FSP) were isolated from fresh cod and fibers were prepared as previously
87 described³⁸ (Supplementary data S1, S2). A concentration of 125 mg/mL FSP was used together with 20
88 mg/mL insulin (Ins) for production of the FSP-Ins fibers. Electrospinning was conducted at room
89 temperature, and samples were stored at -20°C until further analysis.

90 ***In vitro* release studies**

91 The *in vitro* release of insulin from FSP-Ins fibers was performed in HBSS buffer supplemented with 10
92 mM MES (MES-HBSS, pH 6.5) containing varying amounts (1.5 mM, 3 mM or 6 mM) of surfactant
93 (TC, TDC, GC, GDC, Triton X-100, SDS or BC) during 5 h at 37°C with continuous orbital mixing.
94 FSP-Ins fibers were placed in the pre-warmed buffers at a concentration of 3.5 mg/mL and aliquots of
95 30 µl were taken from the supernatant at regular time points and replaced with buffer. The samples were
96 analyzed for content of insulin by using reverse phase high-pressure liquid chromatography (RP-HPLC)
97 as described below. Each experiment was carried out at least in triplicates.

98 **Insulin quantification**

99 Insulin concentrations were analyzed by RP-HPLC using a Prominence system (Shimadzu, Kyoto,
100 Japan) equipped with an Aeris WIDEPORE XB-C18 column (100×2.10 mm, 3.6 µm) (Phenomenex,
101 Allerød, Denmark) and a PDA detector (SPD-M20A, Shimadzu). The mobile phase A consisted of 95%

102 H₂O/5% acetonitrile (AcCN)/0.1% trifluoroacetic acid (TFA) (v/v/v) and mobile phase B consisted of
103 95% AcCN/5% H₂O/0.1% TFA (v/v/v). The autosampler temperature was 4°C. Insulin was eluted using
104 a linear gradient of mobile phase B from 20-50% over 3.5 min at a constant flow of 0.8 mL/min and with
105 a column temperature of 40°C. The amount of insulin was quantified as the peak area obtained at 218
106 nm (retention time 2.7 min). Limit of detection and limit of quantification were 0.2 µg/mL and 0.8 µg/mL
107 (n=3), respectively.

108 **Internal fiber porosity**

109 The internal porosity of FSP-Ins fibers was studied by using cryo-scanning electron microscopy (cryo-
110 SEM) using a Quanta FEG 3D (FEI, Hillsboro, OR, USA) system equipped with a Leica cryo stage and
111 the EM VCT100 Cryo transport system, in combination with the MED020 freeze fracture and
112 Platinum/Carbon or Carbon coating unit (Leica, Wetzlar, Germany). The samples were incubated in
113 MES-HBSS (pH 6.5) with 6 mM surfactant (TC, SDS, Triton X-100, BC), and subsequently placed on
114 an aluminum stub that was mounted on a transport shuttle, and fixed to the stub with carbon/contact
115 medium. The sample was frozen in liquid nitrogen in order to fracture the fibers with a cooled knife
116 inside the MED020. Subsequently, the sample sublimated for 3 min and sputter-coated with platinum
117 (coating thickness 5 nm).

118 **Critical micelle concentration**

119 The critical micelle concentration (CMC) of the different surfactants in MES-HBSS (pH 6.5) was
120 determined according to the method described by Dominguez *et al.*³⁹. Briefly, BZA was added to a final
121 concentration of 0.14 mM, together with varying concentrations of each of the surfactant (TC, GC, TDC,
122 GDO, Triton X-100, SDS and BC) in MES-HBSS. A total volume of 200 µl was added to a clear flat-
123 bottomed 96-well UV-transparent plate (clear acrylic copolymer plate) (Corning Inc., Corning, NY,

124 USA), and absorption was measured at 313 nm at 37°C using a Safire² plate reader (Tecan Group,
125 Männedorf, Switzerland).

126 **Contact angle**

127 The contact angle between FSP-Ins fibers and aqueous solutions of surfactants (6 mM TC, Triton X-100,
128 SDS or BC in MES-HBSS, pH 6.5) was measured using an optical tensiometer (Theta, Attension, Espoo,
129 Finland) with a highspeed camera (3000 fps, MotionXtra N3, IDT, Tallahassee, FL, USA). A volume of
130 7.5 µl of buffer was deposited on the fiber surface, and images were immediately recorded with 59 frame
131 per sec the first 3.4 sec, and thereafter 1 frame per second until 34 sec. The contact angle was derived
132 from the images as the angle between the tangent to the drop at the surface-liquid interface and the fiber.
133 The contact angle, calculated as the mean of the left and right contact angle, was plotted as a function of
134 time, and the initial contact angle was found by extrapolation.

135 **Confocal microscopy**

136 FSP-Ins fibers were placed in MES-HBSS (pH 6.5) with and without surfactants (6 mM TC, Triton X-
137 100 or BC) and incubated for 3 h. After 2 h, ANS was added to a final concentration of 25 µM and the
138 incubation continued for 1 h. The fibers were transferred to a coverslip and images were obtained by
139 using a laser scanning confocal microscope (Zeiss LSM 780, Jena, Germany) applying excitation at 405
140 nm and emission measured at 489 nm.

141 **Statistics**

142 Statistical analysis was performed using GraphPad (GraphPad, La Jolla, CA, USA). Unpaired Student's
143 *t* test was used for assessment of statistically significant differences.

144 **Results and discussion**

145 **Release of insulin from FSP-Ins fibers was affected by surfactants in solution**

146 Insulin release from FSP-Ins fibers was studied in MES-HBSS in the absence or presence of surfactants
147 (Figure 1). The chosen surfactants were divided into four groups, i) biologic anionic surfactants (TC,
148 TDC, GC, GDC), ii) synthetic anionic surfactant (SDS), iii) cationic surfactant (BC) and iv) neutral
149 surfactant (Triton X-100) (for the chemical structures see Supplementary information S3). The release
150 was studied at increasing surfactant concentrations, up to 6 mM, and the presence of bile salts increased
151 the release of insulin in a concentration-dependent manner (Figure 1 A–D). No additional effect on the
152 release rate and the released amount of insulin was observed with bile salt concentrations higher than 6
153 mM (data not shown). The fibers were physically stable in the buffer during long periods of time, as no
154 change in internal structure was observed even after 2 months of incubation (Supplementary data S4),
155 and the fibers remained non-dissolved even longer. To investigate if the effect on the insulin release
156 could be caused by surfactants in general, insulin release upon exposure to synthetic surfactants (SDS,
157 BC and Triton X-100) was analyzed in a similar manner (Figure 1 E–H). SDS induced a similar behavior
158 as the bile salts, with increasing SDS concentrations leading to increased insulin release. However, fibers
159 that were exposed to high concentrations of SDS were degraded, as observed visually, and accordingly
160 the release of insulin continued to increase, contrary to what was observed in the presence of the bile
161 salts. The synthetic SDS is a well-known denaturing agent, and as a rule of thumb it takes 1.4 g SDS to
162 denature a gram of protein to a completely unfolded structure. Although the FSP-fibers were not saturated
163 with SDS at 6 mM SDS, degradation of the fibers clearly occurred. Further increasing the SDS
164 concentration up to 18 mM led to complete release of the entrapped insulin due to extensive fiber
165 degradation (data not shown). Complete drug release allowed for quantification of the insulin

166 encapsulation efficiency, which was found to be $98.6 \pm 2.9 \%$ ($n = 3$) (Ref FSP-Ins). Insulin release in 3
167 mM SDS was similar to the release in MES-HBSS, but it was noticeable that when lowering the SDS
168 concentration to 1.5 mM SDS, the insulin release decreased. The presence of the neutral surfactant, Triton
169 X-100, did not alter the insulin release profile as compared to that observed with the MES-HBSS (Figure
170 1F), and no concentration-dependency in the presence of SDS was observed. Interestingly, almost no
171 insulin was released from the FSP-Ins fibers upon incubation with the cationic surfactant BC (Figure 1
172 G). Approximately 1 % of the encapsulated insulin was released in the 3 mM and 6 mM BC solution as
173 burst release, but after 2 h no insulin was detectable. The strength of the interactions between BC and
174 FSP-Ins fibers was investigated by transferring the FSP-Ins fibers to 12 mM SDS, after five h of
175 incubation in 3 mM BC, and surprisingly no insulin was released after addition to the SDS solution (data
176 not shown). This strongly indicated that the interaction between BC and the FSP-Ins fibers resulted in a
177 significant stabilization of the fiber structure, preventing insulin release. The differences in the way the
178 surfactants affected insulin release, suggested that i) the surfactants interacted differently with the FSP-
179 Ins fibers, and ii) the interaction, and the results of the interaction, strongly depend on the properties of
180 the surfactants. The CMC of the surfactants in MES-HBSS was determined to investigate if there was
181 any relation between the CMC and the effect on release (Figure 1 H), however, no correlation to the
182 CMC was found, which indicated that the organization of the surfactant molecules in solution did not
183 affect the behavior of the surfactants with respect to fiber interactions. In our previous study, the
184 simulated intestinal fluid also contained lipids, but the presence of lipids were found not to affect insulin
185 release (data not shown). Bovine serum albumin (BSA) is a common protein in the human body, and for
186 that reason the effect of BSA on insulin release was briefly touched upon. Boegh *et al.* have developed
187 a biosimilar mucus, which contained 3.1 % (w/v) BSA, and with that amount of BSA in the MES-HBSS

188 buffer, the release of insulin was increased (Supplementary information S5) indicating that interactions
 189 with fibers and thereby altering fiber properties is not limited to traditional small molecules surfactants.

191 **Figure 1.** Insulin release from FSP-Ins fibers *in vitro* in MES-HBSS with varying concentrations of TC (A), TDC (B), GC (C), GDC
 192 (D), SDS (E), Triton X-100 (F) and BC (G); 0 mM (black), 1.5 mM (green), 3 mM (blue) and 6 mM (red). CMC values of the
 193 surfactants in the buffer is presented (H). Data represent mean $\pm$ SD, $n \geq 3$.

194

195 **Fiber porosity was affected by interactions with surfactant**

196 *In vitro* release of a compound encapsulated in insoluble fibers has previously been correlated to the
197 porosity of the fibers^{19,20,40}. From those studies it was found that the release was facilitated by a two-step
198 mechanism; desorption of the compound from the nanoporous surface of the fibers, as the rate limiting
199 step, followed by diffusion from the fiber matrix into the buffer. As a result, only compounds situated at
200 the surface of the fibers were released, as long as the fibers were intact. Modulating the porosity, and
201 thus the surface area, for instance by addition of porogens, such as poly(ethylene glycol), or varying the
202 polymer nature, concentration or molecular weight, modified the maximum released amount, and also
203 the compound release profile^{19,40,41}. In Figure 1 the maximum release changed according to the type and
204 the amount of surfactant present in the MES-HBSS buffer. To investigate if the observed tendencies were
205 associated to changes in fiber porosity, the inner structure (cross-sections) of the FSP-Ins fibers after
206 incubation in MES-HBSS, MES-HBSS with 6 mM TC or MES-HBSS with 6 mM BC, was analyzed
207 with cryo-SEM, and compared to the inner structure of the fibers before incubation (Figure 2). Before
208 incubation in a buffer, a small degree of porosity was observed throughout the fibers, and in some fibers
209 the porosity in the core was increased, which most likely is caused by fast evaporation of the solvent
210 (Figure 2D). After incubation in MES-HBSS and MES-HBSS with TC (Figure 2 A and B) the inner
211 structure of the FSP-Ins fibers became more porous, whereas the fibers that had been exposed to BC
212 became dense (Figure 2C). The trend in porosity correlated with insulin being released in MES-HBSS
213 and MES-HBSS with TC, whereas release in MES-HBSS containing BC was negligible. The fact that
214 the burst release in BC was almost nonexistent (Figure 1 G), suggested that BC instantaneously interacted
215 with the surface of the fibers, and induced a stabilizing effect thus precluding insulin release and causing

216 the inner fiber structure to become dense. The inner structure of fibers exposed to Triton X-100 or SDS
 217 were also investigated (data not shown), and porosity was observed in both cases. As with MES-HBSS
 218 in the presence and absence of TC, porosity after exposure to Triton X-100 and SDS correlated with
 219 insulin being released from FSP-Ins fibers in both Triton X-100 and SDS solutions. It was not possible
 220 to study a potential connection between extend of insulin release and degree of porosity, since the
 221 porosity made the fibers fragile and sensitive to beam damage, which resulted in partly closing of the
 222 pores, which thereby prohibited quantitative analysis of the porosity.

223
 224 **Figure 2. Cryo-SEM images of FSP-Ins fibers after incubation in MES-HBSS (A), MES-HBSS with 6 mM TC (B), MES-HBSS with**
 225 **6 mM BC (C) and before incubation (D). Scale bars are 1 μ m.**

226 **Interactions between surfactants and FSP-Ins fiber led to changes in the contact angles**

227 The interactions between surfactants (TC, SDS, Triton X-100 and BC) in MES-HBSS and FSP-Ins fibers
 228 were further evaluated by contact angle measurements (Figure 3). MES-HBSS without surfactants

229 displayed a large contact angle of 110 ± 8 degr., which indicated that the FSP-Ins fibers were
 230 hydrophobic. Addition of SDS caused no significant change in the contact angle (117 ± 7 degr.), whereas
 231 addition of TC increased the contact angle to 130 ± 6 degr. The presence of BC decreased the contact
 232 angle to 84 ± 21 deg. And Triton X-100 decreased the value even further to 37 ± 13 degr. The decreased
 233 contact angle caused by Triton X-100 and BC suggested that interactions between Triton X-100, or BC,
 234 and the fibers, facilitated increased surface hydrophilicity. The rate by which the liquid drop was
 235 absorbed into the fibers, varied in the order Triton X-100 > BC > SDS > TC > MES-HBSS. The
 236 differences in contact angle and time for complete liquid drop absorption emphasized that the surfactants
 237 interacted with the fibers in different ways, dependent on the properties of the surfactants. The effect of
 238 the surfactant concentration was investigated by comparing the contact angle for 3 mM and 6 mM TC or
 239 SDS, but no dose-dependency was observed in this concentration range (Figure 3 B)

240
 241 **Figure 3. Contact angles between FSP-Ins and MES-HBSS with or without surfactants (A) and comparison of contact angles**
 242 **between FSP-Ins fibers and MES-HBSS containing either 3 mM or 6 mM surfactant (B). Data represent mean value $\pm$ SD, $n > 3$.**
 243 **Representative images of a drop of buffer (MES-HBSS with or without surfactants) on FSP-Ins fibers (C).**

Surface properties of the FSP-Ins fibers were affected by interactions with surfactants

The hydrophobic nature of the FSP-Ins fibers was studied by ANS fluorescence (Figure 4). ANS is widely used to visualize hydrophobic regions in e.g. fibrillar aggregates and fibers, as its fluorescence is enhanced when ANS interacts with hydrophobic environments^{42,43}. Increase in fluorescence intensity can thus be ascribed to the exposure of hydrophobic pockets to ANS molecules favouring the binding. Figure 4 A shows the ANS fluorescence in fibers after incubation with MES-HBSS. The visualization of the FSP-Ins fibers by the application of ANS fluorescence verified that the surface of the FSP-Ins fibers were hydrophobic. The homogeneity of the fluorescence throughout the fiber matrix furthermore suggested that the properties of the surface were consistent, despite of the complexity of the FSP matrix. The presence of 6 mM TC in the MES-HBSS buffer did not alter the ANS fluorescence (Figure 4 B), indicating that a similar amount of hydrophobic pockets were still available for interactions with ANS. Interestingly, exposure of the FSP-Ins fibers to 6 mM Triton X-100 or 6 mM BC caused a decrease in ANS fluorescence, as shown in Figure 4C and D, respectively. In both cases the ANS fluorescence decreased significantly, compared to fibers exposed to MES-HBSS without surfactants, indicating that the amount of accessible hydrophobic regions was decreased. This was in agreement with the contact angle measurements, where an increased hydrophilicity was caused by BC and Triton X-100, and hydrophobicity was observed for MES-HBSS in the presence and absence of TC. The loss in the amount of hydrophobic regions available for ANS binding can be explained by changes in the FSP-Ins fiber surface properties, and here we propose two possible explanations. In the electrospun fibers, the protein entanglements may cause the proteins to be in an energetically unfavorable state. Addition of surfactants to the buffer may cause a relaxation of the proteins, which results in small rearrangements in the protein structure, and thus shielding of hydrophobic regions. Another hypothesis rely on the strong interactions between surfactants and fibers, which will compete with and prevent the ANS molecules from interacting

267 with the fibers. Irrespective of the exact mechanism, changes in the surface properties of the fibers upon
268 incubation with neutral or cationic surfactants were evident.

269

270 **Figure 4. Cross-sectional confocal images of fibers displaying ANS fluorescence after incubation with FSP-Ins fibers in MES-HBSS**
271 **(A), MES-HBSS with 6 mM TC (B), MES-HBSS with 6 mM Triton X-100 (C) and MES-HBSS with 6 mM BC (D). All images were**
272 **recorded with the same settings.**

273 Conclusion

274 The results presented in this study clearly indicate that biorelevant compounds interact with electrospun
275 fibers. The interactions between surfactants in solution and electrospun protein fibers were found to
276 significantly influence the release of entrapped cargo, the fiber stability, porosity and the fiber surface
277 properties. Anionic surfactants were shown to have a concentration-dependent effect on insulin release,
278 whereas addition of Triton X-100 did not cause a significant change in the amount released, and in MES-
279 HBSS with BC, only negligible amounts of insulin were released. Cryo-SEM images of the fibers after
280 incubation with the surfactants showed a correlation between release of cargo and increased porosity of
281 the fibers, in the sense that when insulin was released, fiber porosity was observed. Contact angle

measurements revealed that the surfactants had a significant influence on wetting of the fibers; for anionic surfactants a small increase in the contact angle was observed, whereas the presence of the cationic surfactant, and especially the neutral surfactant, decreased the contact angle considerably. Interactions between fibers and surfactants were further elucidated, by decreased levels of interactions with the hydrophobic pocket fluorophore, ANS, after fiber incubation with neutral or cationic surfactants, as compared to anionic or no surfactants. Overall, this study emphasizes the importance of taking into account biologically relevant components, such as bile salts and proteins, with which electrospun fibers may be exposed to when used for tissue engineering, wound healing, drug delivery and other biomedical applications.

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