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Feasibility Study of the Elaboration of a Biodegradable and Bioactive Ligament Made of Poly(ϵ -caprolactone)-pNaSS Grafted Fibers for the Reconstruction of Anterior Cruciate Ligament: In Vivo Experiment

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4 Feasibility study of the elaboration of a biodegradable and bioactive ligament
5 made of poly(ϵ -caprolactone)-pNaSS grafted fibers for the reconstruction of
6 anterior cruciate ligament: in vivo experiment

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20 Abstract

21 *Background* - The anterior cruciate ligament rupture is a common injury which mainly affects young
22 and active population. Faced to this problem, the development of synthetic structures for ligament
23 reconstruction is increasing. The most recent researches focused on the development of
24 biodegradable structures that could be functionalized to enhance host integration. This work
25 describes the elaboration of different poly(ϵ -caprolactone) prototypes for the rat anterior cruciate
26 ligament replacement in order to found the best design for further *in vivo* assays.

27 *Methods* – According to the literature, it was decided to elaborate two different poly(ϵ -caprolactone)
28 prototypes: a braided one and a free-fibers one. A chemical grafting of a bioactive polymer –
29 poly(sodium styrene sulfonate) - was performed on both prototypes and mechanical and biological
30 testing were assessed. Based on these results, one rat was implanted with the best prototype.

31 *Results* – The mechanical and biological results demonstrated that the best prototype to implant was
32 the poly(sodium styrene sulfonate)-grafted braided prototype. After one-month implantation, no
33 inflammation was observable around the scar. The rat demonstrated good flexion and extension of
34 the lower limb without any anterior drawer. The prototype was highly anchored to the bone. ESEM
35 images of the explanted prototype showed the presence of cells and tissue ingrowth along and
36 around the fibers.

37 *Conclusion* - This work demonstrates the feasibility to implant a bioactive and biodegradable
38 synthetic ligament in the rat model without any inflammation and with a good tissue anchoring at a
39 short-term time. This will lead to an extensive *in vivo* assay.

40 *Keys words* – synthetic ligament; ACL reconstruction; bioactive surface; biodegradable polymer

41 Introduction

42 Injuries of knee anterior cruciate ligament (ACL) frequently happen during high demand sports and
43 mainly affects young and feminine population[1]. Surgical reconstruction is most often required to

44 treat the instability of the knee associated with ACL rupture and until now, the gold standard remains
45 autograft[2]. Nevertheless, side effects as morbidity of autologous tissues, weak anchorage and
46 recurrent pains of the patients led to artificial ligament solutions. The development of synthetic
47 ligament began in the early 1980's with the used of inappropriate materials which lead to synovitis,
48 prosthesis rupture and bone tunnel enlargement[3]. After several clinical fails, the use of
49 poly(ethylene terephthalate) (PET) material was broadly accepted and the current prosthesis on the
50 market, as the LARS ligament, are still made of polyester[4]. Nevertheless, nowadays these synthetic
51 ligaments still encounter limitations such as the anchorage to the bone, the release of tear particles
52 due to abrasion and a poor cell recolonization with limited tissue ingrowth[5]. Due to that, studies
53 are conducted with the purpose of finding a material and design exhibiting similar mechanical
54 properties than the native ACL and being well-integrated in the body avoiding adverse reactions
55 thanks for instance to a surface treatment[6]–[8]. More recent researches focused on the
56 development of biodegradable structures that can support mechanical stresses during the time that
57 a neo-ligament is formed and recovers its first function of stabilizer. So that, the challenge is to find
58 the best polymer to use. Poly(ϵ -caprolactone) (PCL) seems to be promising since it is FDA approved
59 and widely used in different medical devices (MD) applications [9]–[12]. Moreover, our team has
60 shown that the functionalization of PCL films by poly(sodium styrene sulfonate) (pNaSS) grafting can
61 improve the cell response[13]. The development of a new pNaSS-grafted PCL synthetic ligament
62 seems to be relevant as an innovative biodegradable and bioactive ligament prosthesis. In this
63 context, this article aims to compare two different structures of PCL prototypes for the rat ACL
64 replacement in order to find the best design for further *in vivo* assays. To achieve this goal, two
65 different prototypes - braided or composed of free-fibers - were elaborated. After grafting pNaSS on
66 the braided and free fibers prototypes, they were mechanically tested and *in vitro* assays were
67 conducted. These preliminary data allowed us to determine which structure was the best to be
68 implanted in rat. After one month of *in vivo* implantation the comportment of this new
69 biodegradable and bioactive prosthesis was analyzed. It was expected that the braided pNaSS-

70 grafted prototype was still present after one month implantation, induce cell colonization and be a
71 support for rat walk.

72 **Material and methods**

73 **Preparation of PCL prototypes**

74 20 PCL fibers (diameter $110\pm 15\mu\text{m}$, length $75\pm 5\text{ mm}$) from Luxilon company (LUXILON Industries nv,
75 Antwerpen, Belgium) are associated and hand tied in order to achieve 20 fibers PCL bundles. PCL
76 bundles are then used to elaborate to kinds of ligament prototypes: (i) a “braided prototype” made
77 of fully braided bundles, (ii) a “free-fibers prototype” which is partially braided at its two end
78 portions around a central free fiber part (see Fig1).

79 Whatever the prototype, it is divided in two parts: (1) an implanted part ($15\pm 2\text{ mm}$ long) which is
80 inserted in the knee articulation of the rat (intra articular and osseous parts) and (2) an extra part
81 ($30\pm 5\text{ mm}$ long) which allows the veterinarian to optimally implant the ligament into the knee joint
82 and straighten it for the best stabilization – this part is cut at the end of the implantation. Once the
83 prototypes are made, they are ultrasound washed with different solvents (10% (w/v)) in order to
84 remove the spin finish as following: hexane for 15 min, absolute ethanol for 5 min, water two times
85 for 5 min. Samples are then dried under vacuum and stored at 4°C until grafting process or
86 experiments.

87 **Grafting of poly(sodium styrene sulfonate) on PCL prototypes**

88 PCL prototypes are functionalized with poly(sodium styrene sulfonate) pNaSS using a grafting “from”
89 technique [14]. After the spin finish removal step, PCL samples are ozonized at 30°C in distilled water
90 under stirring for 10 min. Ozone is generated using an ozone generator BMT 802 N (ACW) with a gas
91 pressure of 0.5 bars and an oxygen flow rate of 0.6 L min^{-1} . Secondly, the ozonized PCL samples are
92 transferred into a degassed aqueous NaSS solution (15 % (w/v)) under argon and maintained 1 hour
93 at 45°C under stirring to allow the radical polymerization of the monomer. Then, samples are
94 extensively washed with distilled water for 48 hours and vacuum-dried. The evidence of the pNaSS

95 grafting is provided by toluidine blue colorimetric assay and the grafting rate is determined according
96 to Ciobanu et al[14].

97 **Mechanical testing**

98 The two prototypes have undergone tensile loading until rupture by using a Bose Electroforce 3230
99 equipment (Bose). The tests were carried out three times for each prototype - effective length of
100 18.8±1.1 mm - the jaws of the machine were placed after the implanted part to test the future
101 implant in its entirety. Stress strain curves are recorded and maximum force (F_{max}) in N, ultimate
102 tensile stress (UTS) in MPa and elongation (ϵ) in % are determined.

103 **Sterilization**

104 Before biological experiments all the prototypes were packaged and sterilized as following: two
105 washing of 3 hours in 1.5M aqueous sodium chloride solution; one washing of 10min in ultra-pure
106 water; two washing of 3 hours in 0.15M aqueous sodium chloride solution; one washing of 10min in
107 ultra-pure water; one washing of 3 hours in DPBS (Gibco); 20 minutes in 70% ethanol solution;
108 following by 10 min ultra-pure water bath; then 15 min of UV irradiation were applied in both sides
109 of the samples. All the washing was done under stirring. Prototypes were kept in sterile PBS solution
110 at 4°C until experiments.

111 **Biological experiments**

112 **Cell isolation**

113 Anterior cruciate ligaments (ACL) from sheep are collected thanks to the collaboration with the ENVA
114 (Maisons-Alfort, France (Ligart protocol)). Tissues are cut into small pieces of 1 to 2 mm², washed
115 three times in DPBS (Gibco) and incubated in a 0.1% (w/v) collagenase (Sigma-Aldrich) solution for 6
116 hours at 37°C under 5% CO₂. The mixture solution is centrifuged (3 min at 1500 rpm): the
117 supernatant is withdrawn and the clot is suspended in DMEM (Gibco) complemented with 10%

118 Bovine Calf Serum (Sigma-Aldrich), 1% Penicillin-streptomycin (Gibco), 1% L-glutamine (Gibco);
119 fibroblasts are cultured in flask until confluence and prior seeding on samples.

120 *In vitro* study

121 pNaSS-grafted and ungrafted “free-fibers” and “braided” prototypes are placed in a 6-wells plate
122 treated for low attachment cell cultures. A primary fibroblasts suspension (P3) of 300000 cells in
123 50µL are seeded onto the middle of the implanted part of the different prototypes and incubated 30
124 minutes at 37°C under 5% CO₂, then 200µL of medium are added for 1h to limit the drying. Each well
125 was then completed with 2mL of 10% serum supplemented DMEM and cultured for 1 month (33
126 days) at 37°C with a fresh medium replacement three times per week. After this period of time, the
127 prototypes were fixed 1 hour in a 4% formaldehyde solution, intensively rinse with ultra-pure water,
128 and observed in an environmental scanning electron microscope (ESEM). ESEM images were carried
129 out using a Hitachi TM3000 SEM operating at 15 kV and equipped with a Peltier cooling device
130 running at 4°C.

131 *In vivo* study

132 A 37-weeks-old female Wistar rat of 360g (approved supplier Janvier Labs, Saint Berthevin, France)
133 was implanted with a pNaSS-grafted braided prototype. Anesthesia was performed by
134 intraperitoneal injection of a mixture of dexmedetomidine/ketamine (0.2mg/kg and 80mg/kg
135 respectively, Domitor® Orion pharma and Imalgene® Merial) and then maintained by inhalation of an
136 isoflurane/O₂ mixture. The surgical procedure was then performed on the lower left limb. After
137 exposition of the ACL by arthrotomy, the ligament was excised. Then, the knee was oriented in a
138 hyper-flexion position and the femur and the tibia were drilled at a diameter of 1.5 and 1.1mm
139 respectively, the prototype was placed in the articulation through a 1.2mm diameter needle,
140 replacing the native ACL. Screws of 6mm (femur) and 7mm (tibia) length (references VS112.006 and
141 VS112.007, Synthes) were placed for the fixation of the prototype into the bone. The prototype
142 surplus was cut flush to not exceed the joint. The incision was closed in three stages: (i) closure of the

143 joint capsule by interlocking with U-shaped stitches (Glycomère 631, Biosyn 1.5 decimal, Covidien);
144 (ii) closure of the subcutaneous connective tissue by a simple suture (glycomère 631, Biosyn 1
145 decimal, Covidien) ; (iii) closure of the skin by intradermal overlock (glycomère 631, Biosyn 1 decimal,
146 Covidien). No restriction on movements was applied. After one month implantation, the rat was
147 sacrificed and the prototype was explanted going back through the osseous tunnel. The explant was
148 fixed 1 hour in 4% formaldehyde solution and observed with ESEM-Peltier at 4°C.

149 Results

150 Elaboration of prototypes

151 The free fibers and the braided prototypes were observed microscopically in order to validate that
152 the handmade elaboration did not alter the PCL fibers. As it can be seen in the Figure 1, the fibers do
153 not look impacted by the assembly.

154 Figure 1 : SEM images of free fibers (A) and braided (B) prototypes

155 The presence of grafted pNaSS molecules on PCL prototypes was determined according to the
156 toluidine blue assays process. The obtained average value of the grafting rate of the free fibers
157 prototypes (n=3) equals $0.70 \pm 0.12 \mu\text{mol.g}^{-1}$ whereas the grafting rate of braided prototypes (n=3)
158 reached $1.05 \pm 0.14 \mu\text{mol.g}^{-1}$ (see. Fig.2).

159 Figure 2 : Grating rates of the two types of prototypes obtained with the toluidine blue colorimetric method

160 Mechanical testing

161 Because the prototypes are handmade elaborated, the presence of some knots was necessary in
162 order to demarcate the braided areas from the free fibers areas, especially at the intra-articular part
163 level for free-fibers prototypes and for the limit between implanted and the external parts for both
164 prototypes. To avoid the compression of the knot in the jaws and so influence the test, it was
165 decided to evaluate the mechanical properties of the prototypes by placing the jaws after the
166 implanted area (see.Fig.3).

167 Figure 3 : schematic representation of the placement of the prototypes in the jaws (in gray) of the traction machine

168 The mechanical results and the location of the rupture are presented in the Table 1. The obtained
 169 results showed that the “free fibers” prototypes exhibit lower mechanical properties than braided
 170 prototypes with a maximal force of 38.8 ± 3.7 N against 43.3 ± 1.0 N and an UTS of 204.3 ± 19.4 MPa
 171 against 227.9 ± 5.4 MPa that can be endured. Moreover, the elongation of the braided prototypes is
 172 1.5 time more important than the one of the free fibers prototypes going from $77.5\pm 9.8\%$ to
 173 $115.6\pm 4.6\%$. Finally, it is important to note that it was observed that the rupture always came above
 174 or below a knot and not in the middle of the implanted part of the prototype. The prototypes are
 175 therefore weakened by the presence of these knots.

	F_{max} (N)	UTS (MPa)	ε (%)	Location of the rupture
Free fibers prototypes				
#1	40.95	215.45	88.22	above knot 1
#2	40.95	215.45	75.00	below knot 1
#3	34.57	181.88	69.15	below knot 1
Average	38.8±3.7	204.3±19.4	77.5±9.8	
Braided prototypes				
#1	42.24	222.24	110.29	below knot 2
#2	43.38	228.45	118.74	above knot 2
#3	44.29	233.02	117.74	above knot 2
Average	43.3±1.0	227.9±5.4	115.6±4.6	

176 Table 1 : Mechanical properties of the two PCL prototypes

177 Biological experiments

178 Cell culture *in vitro* assays

179 The observation of the cell cultures after 1 month revealed differences on the cells repartition when
 180 there are seeded onto the free fibers prototypes when compared to the braided prototypes (see
 181 Figure 4 and 5).

182 **Figure 4 : ESEM images of ungrafted (A, B,C) and grafted (D,E,F) free fibers prototypes cultured with primary fibroblasts**
 183 **during 1 month.**

184 **Figure 5 : ESEM images of ungrafted (A, B,C) and grafted (D,E,F) braided prototypes cultured with primary fibroblasts**
 185 **during 1 month.**

186 With the free fibers prototypes, it can be seen that the cells mainly grow between the fibers and that
 187 there are quite separated one from each other (see Fig.4). The ungrafted free fibers prototype
 188 presents fewer cells that the pNaSS grafted one (see Fig.4.B and 4.E) and the cells are better spread
 189 with the pNaSS grafting (see Fig.4.C and 4.F).

190 The ESEM images suggest that there are more cells that have grown onto the braided prototypes
191 when compared to the free fibers prototypes (see Fig.4.A and 5.A). Moreover, with the braided
192 prototypes, the cells also grow between the fibers but they seem to be more inter-connected
193 starting to form a cellular network. This fact is clearly enhanced by the presence of the pNaSS
194 grafting (see Fig.5.C and 5.F). When we compare the ungrafted and the grafted braided prototypes, it
195 can be observed that there are numerically more cells with the pNaSS is present on the fibers (see.
196 Fig.5.A and 5.D); in addition cells are better spread and they started to coat the fibers all around (see
197 Fig.5.C and 5.F).

198 *In vivo* assay

199 *Clinical evidences*

200 The day after the implantation, the rat was clearly using its limb, no evidence of lameness was noted
201 during the implantation period. After one month, the 360g-female Wistar rat was sacrificed. The rat
202 was weighted and an increase of 15g was noticed. The observation of the animal showed a slight
203 swelling of the implanted paw with respect to the opposite limb, accompanied by a slight edema but
204 without any sign of inflammation (see Fig.6). The range of motion of the joint was normal, with good
205 flexion and good extension, and with no anterior drawer, compared to the non-operated
206 contralateral limb. The joint was exposed and the implanted pNaSS-grafted braided prototype was
207 explanted. The prototype was difficult to withdraw because of its good stability and anchorage in the
208 osseous tunnels.

209 **Figure 6 : implanted limb of the rat (A) and prototype implanted (B) after 1 month**

210 *Explant observations*

211 After cells and tissue fixation, the braided prototype was observed with ESEM. After 1 month
212 implantation, we can see that the prototype has been recolonized by tissue (see Fig. 7). The
213 recolonization is more important in the osseous part compared to the intra-articular part (see Fig.7.A
214 and 7.C), probably thanks to the close proximity of cells. In both intra articular and osseous parts, the
215 fibers are well surrounded by tissue. The image of the cross section of the prototype (see. Fig.7.B)

216 shows the presence of tissue between the fibers confirming the good integration of the pNaSS-
217 grafted braided prototype in the articulation.

218 **Figure 7 : ESEM images of the pNaSS-grafted braided prototype. A and B present the intra-articular part; C and D focus on**
219 **the intra-osseous part.**

220 Discussion

221 Based on the literature, two types of prototypes were designed in order to found the best structure
222 for the reconstruction of the anterior cruciate ligament in the rat model: (1) a free fibers prototype
223 clearly inspired by the LARS ligament[15] and (2) a braided prototype which is an option that several
224 scientists try to explore[16], [17].

225 The covalent grafting of a bioactive polymer –poly(sodium styrene sulfonate) - has been developed
226 by our team onto PCL films and its bioactive effect has already been demonstrated[13]. In this study,
227 it is shown that the grafting process can be extent to fibers structure, with however a slightly better
228 grafting rate onto the braided prototypes.

229 The mechanical results obtained for both prototypes clearly demonstrate the limit of handmade
230 structure which required knots to stay united. In fact, these knots are going to concentrate stresses
231 and so weaken the prototypes. The more knots there are, the more fragile is the structure.

232 Moreover, in the case of the reconstruction of ACL in Wistar rat, the literature reports a failure load
233 of the native ACL, for rats that weight between 300g and 485g, of 34.5-35.5 N depending of the
234 weight but also of the age of the animal[18], [19]. In our case, it was mechanically safer to choose to
235 implant the PCL braided prototype which can endure a maximal force of 43.3 ± 1.0 N before failure.

236 The *in vitro* study on both grafted and ungrafted free fibers and braided prototypes was done. The
237 previously described bioactive feature can be found again in a prototype structure with a
238 proliferation enhancement and a more homogenous repartition of cells thanks to the presence of
239 pNaSS. The *in vitro* results demonstrated clearly that the pNaSS-grafted braided prototype is the best
240 candidate for bioactive and biodegradable ligament prosthesis in rat.

241 Because this study was mainly conducted to evaluate the surgical feasibility to implant anterior
242 cruciate ligament prosthesis in rat before starting an extensive *in vivo* study, only one rat was

243 implanted. According to the previous results, it is obvious that our choice go to the pNaSS-grafted
244 braided prototype. Despite the small size of the animal and the need of very precise material, the
245 prototype was successfully implanted. After one month implantation, good clinical outcomes were
246 noticed with among other no lameness, no inflammation and no anterior drawer. Finally, the
247 observation of the explanted prototype demonstrates the potential of this implant which has been
248 recolonized both at the intra-osseous and intra-articular level and around and along the fibers.

249 Conclusion

250 This study validates the feasibility to implant a bioactive and a biodegradable ligament to replace the
251 anterior cruciate ligament in the rat model, at least at the short-term. Our future works are going to
252 be concentrated to design a braided or knitted structure that does not imply the use of knots and
253 that can be standardized. Once this new structure will be operational, the implantation of numerous
254 rats in the context of an extensive *in vivo* study, which will compare the grafted and the ungrafted
255 prosthesis, will be conducted.

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