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Original Article

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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HIGHLIGHTS

- The set up pNaSS grafting process can be extended to a fibers complex structure.
- The pNaSS grafting improves cell proliferation and spreading.
- pNaSS-grafted PCL synthetic ligament can be successfully implanted in rat model.

GRAPHICAL ABSTRACT



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ABSTRACT

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

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

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

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

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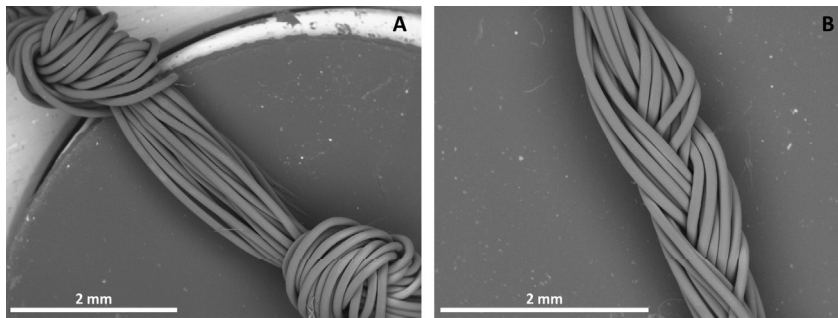


Fig. 1. SEM images of free fibers (A) and braided (B) prototypes.

1. Introduction

Injuries of knee anterior cruciate ligament (ACL) frequently happen during high demand sports and mainly affects young and feminine population [1]. Surgical reconstruction is most often required to treat the instability of the knee associated with ACL rupture and until now, the gold standard remains autograft [2]. Nevertheless, side effects as morbidity of autologous tissues, weak anchorage and recurrent pains of the patients led to artificial ligament solutions. The development of synthetic ligament began in the early 1980s with the use of inappropriate materials which lead to synovitis, prosthesis rupture and bone tunnel enlargement [3]. After several clinical fails, the use of poly(ethylene terephthalate) (PET) material was broadly accepted and the current prosthesis on the market, as the LARS ligament, are still made of polyester [4]. Nevertheless, nowadays these synthetic ligaments still encounter limitations such as the anchorage to the bone, the release of tear particles due to abrasion and a poor cell recolonization with limited tissue ingrowth [5]. Due to that, studies are conducted with the purpose of finding a material and design exhibiting similar mechanical properties than the native ACL and being well-integrated in the body avoiding adverse reactions thanks for instance to a surface treatment [6–8]. More recent researches focused on the development of biodegradable structures that can support mechanical stresses during the time that a neo-ligament is formed and recovers its first function of stabilizer. So that, the challenge is to find the best polymer to use. Poly(ϵ -caprolactone) (PCL) seems to be promising since it is FDA approved and widely used in different medical devices (MD) applications [9–12]. Moreover, our team has shown that the functionalization of PCL films by poly(sodium styrene sulfonate) (pNaSS) grafting can improve the cell response [13]. The development of a new pNaSS-grafted PCL synthetic ligament seems to be relevant as an innovative biodegradable and bioactive ligament prosthesis. In this context, this article aims to compare two different structures of PCL prototypes for the rat ACL replacement in order to find the best design for further *in vivo* assays. To achieve this goal, two different prototypes – braided or composed of free-fibers – were elaborated. After grafting pNaSS on the braided and free fibers prototypes, they were mechanically tested and *in vitro* assays were conducted. These preliminary data allowed us to determine which structure was the best to be implanted in rat. After one month of *in vivo* implantation the comportment of this new biodegradable and bioactive prosthesis was analyzed. It was expected that the braided pNaSS-grafted prototype was still present after one month implantation, induce cell colonization and be a support for rat walk.

2. Material and methods

2.1. Preparation of PCL prototypes

20 PCL fibers (diameter $110 \pm 15 \mu\text{m}$, length $75 \pm 5 \text{ mm}$) from Luxilon company (LUXILON Industries nv, Antwerpen, Belgium) are

associated and hand tied in order to achieve 20 fibers PCL bundles. PCL bundles are then used to elaborate to kinds of ligament prototypes: (i) a “braided prototype” made of fully braided bundles, (ii) a “free-fibers prototype” which is partially braided at its two end portions around a central free fiber part (see Fig. 1).

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

2.2. Grafting of poly(sodium styrene sulfonate) on PCL prototypes

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

2.3. Mechanical testing

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

2.4. Sterilization

Before biological experiments all the prototypes were packaged and sterilized as follows: two washing of 3 h in 1.5 M aqueous sodium chloride solution; one washing of 10 min in ultra-pure water; two washing of 3 h in 0.15 M aqueous sodium chloride solution; one washing of 10 min in ultra-pure water; one washing of 3 h in DPBS (Gibco); 20 min in 70% ethanol solution; following by

10 min ultra-pure water bath; then 15 min of UV irradiation were applied in both sides of the samples. All the washing was done under stirring. Prototypes were kept in sterile PBS solution at 4 °C until experiments.

2.5. Biological experiments

2.5.1. Cell isolation

Anterior cruciate ligaments (ACL) from sheep are collected thanks to the collaboration with the ENVA (Maisons-Alfort, France (Ligart protocol)). Tissues are cut into small pieces of 1 to 2 mm², washed three times in DPBS (Gibco) and incubated in a 0.1% (w/v) collagenase (Sigma-Aldrich) solution for 6 h at 37 °C under 5% CO₂. The mixture solution is centrifuged (3 min at 1500 rpm); the supernatant is withdrawn and the clot is suspended in DMEM (Gibco) complemented with 10% Bovine Calf Serum (Sigma-Aldrich), 1% Penicillin-streptomycin (Gibco), 1% L-glutamine (Gibco); fibroblasts are cultured in flask until confluence and prior seeding on samples.

2.5.2. In vitro study

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

2.5.3. In vivo study

A 37-weeks-old female Wistar rat of 360 g (approved supplier Janvier Labs, Saint Berthevin, France) was implanted with a pNaSS-grafted braided prototype. Anesthesia was performed by intraperitoneal injection of a mixture of dexmedetomidine/ketamine (0.2 mg/kg and 80 mg/kg respectively, Domitor® Orion pharma and Imalgene® Merial) and then maintained by inhalation of an isoflurane/O₂ mixture. The surgical procedure was then performed on the lower left limb. After exposition of the ACL by arthroscopy, the ligament was excised. Then, the knee was oriented in a hyper-flexion position and the femur and the tibia were drilled at a diameter of 1.5 and 1.1 mm respectively, the prototype was placed in the articulation through a 1.2 mm diameter needle, replacing the native ACL. Screws of 6 mm (femur) and 7 mm (tibia) length (references VS112.006 and VS112.007, Synthes) were placed for the fixation of the prototype into the bone. The prototype surplus was cut flush to not exceed the joint. The incision was closed in three stages: (i) closure of the joint capsule by interlocking with U-shaped stitches (Glycomère 631, Biosyn 1.5 decimal, Covidien); (ii) closure of the subcutaneous connective tissue by a simple suture (glycomère 631, Biosyn 1 decimal, Covidien); (iii) closure of the skin by intradermal overlock (glycomère 631, Biosyn 1 decimal, Covidien). No restriction on movements was applied. After one month implantation, the rat was sacrificed and the prototype was explanted going back through the osseous tunnel. The explant was fixed 1 h in 4% formaldehyde solution and observed with ESEM-Peltier at 4 °C.

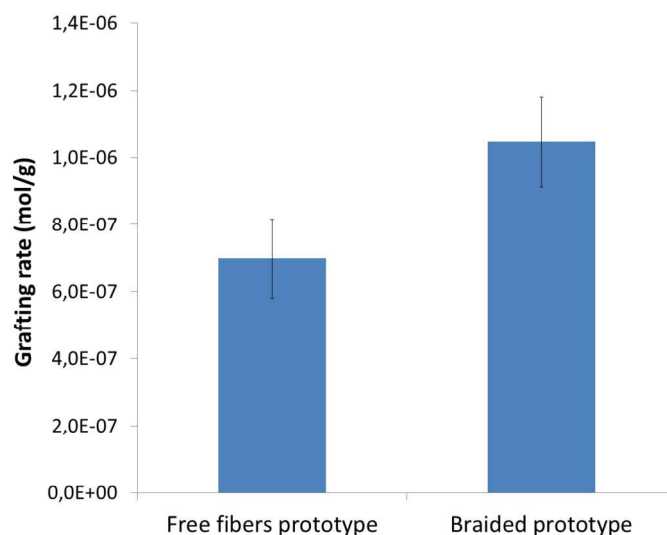


Fig. 2. Grafting rates of the two types of prototypes obtained with the toluidine blue colorimetric method.

3. Results

3.1. Elaboration of prototypes

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

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

3.2. Mechanical testing

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

The mechanical results and the location of the rupture are presented in the Table 1. The obtained results showed that the “free fibers” prototypes exhibit lower mechanical properties than braided 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 against 227.9 ± 5.4 MPa that can be endured. Moreover, the elongation of the braided prototypes is 1.5 time more important than the one of the free fibers prototypes going from $77.5 \pm 9.8\%$ to $115.6 \pm 4.6\%$. Finally, it is important to note that it was observed that the rupture always came above or below a knot and not in the middle of the implanted part of the prototype. The prototypes are therefore weakened by the presence of these knots.

3.3. Biological experiments

3.3.1. Cell culture in vitro assays

The observation of the cell cultures after 1 month revealed differences on the cells repartition when there are seeded onto the

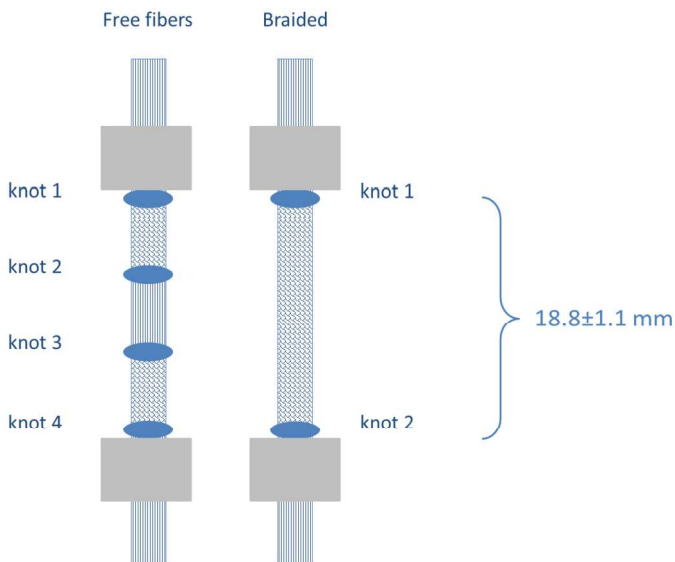


Fig. 3. Schematic representation of the placement of the prototypes in the jaws (in gray) of the traction machine.

Table 1
Mechanical properties of the two PCL prototypes.

	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	

free fibers prototypes when compared to the braided prototypes (see Fig. 4 and 5).

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

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

3.3.2. In vivo assay

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

Explant observations After cells and tissue fixation, the braided prototype was observed with ESEM. After 1 month implantation, we can see that the prototype has been recolonized by tissue (see Fig. 7). The recolonization is more important in the osseous part compared to the intra-articular part (see Fig. 7.A and 7.C), probably

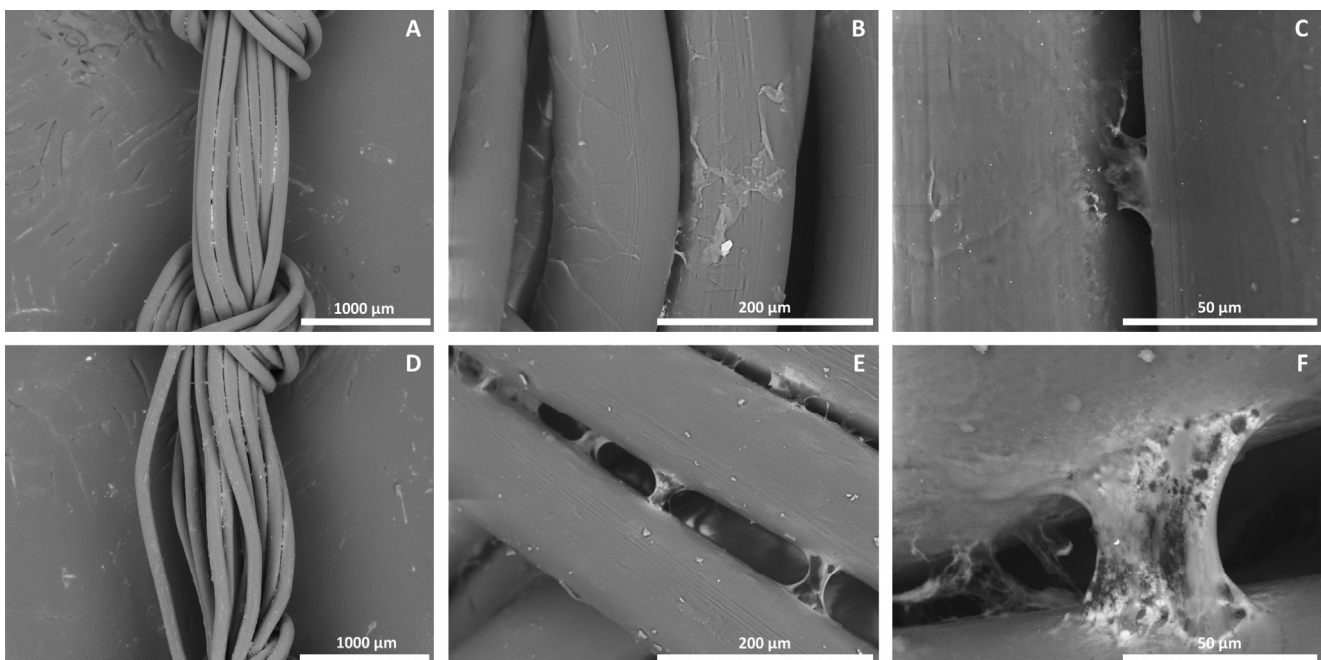


Fig. 4. ESEM images of ungrafted (A, B, C) and grafted (D, E, F) free fibers prototypes cultured with primary fibroblasts during 1 month.

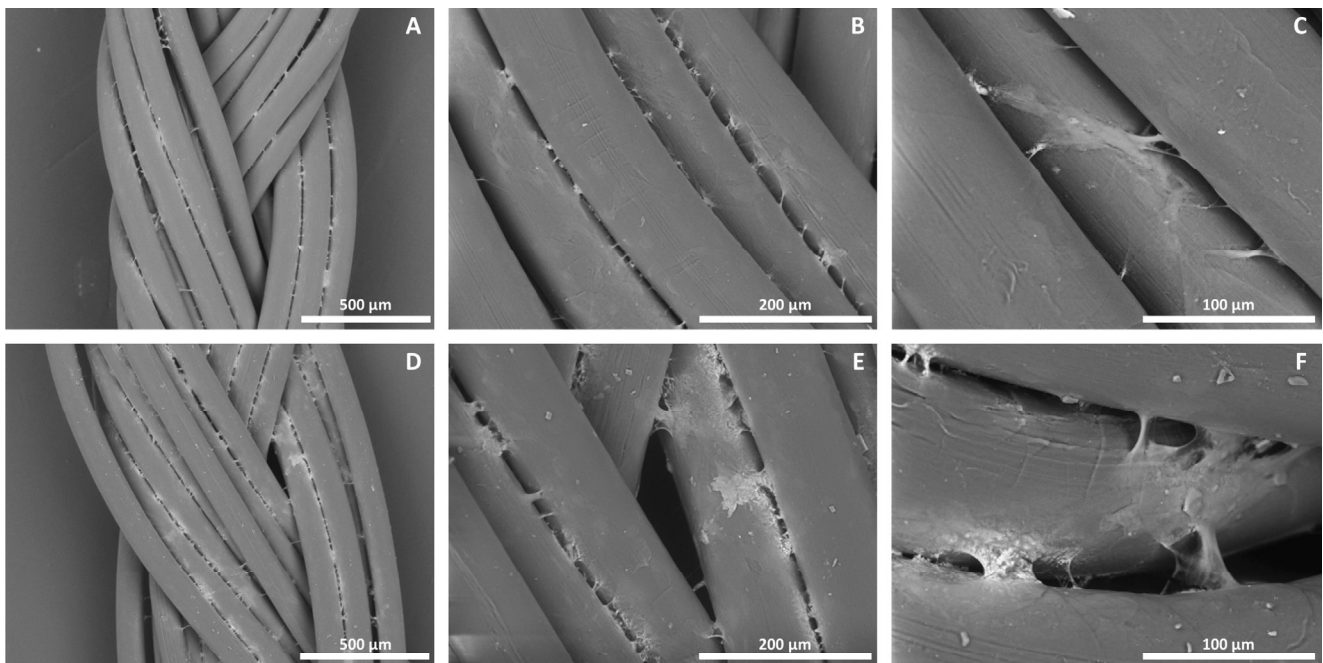


Fig. 5. ESEM images of ungrafted (A, B, C) and grafted (D, E, F) braided prototypes cultured with primary fibroblasts during 1 month.

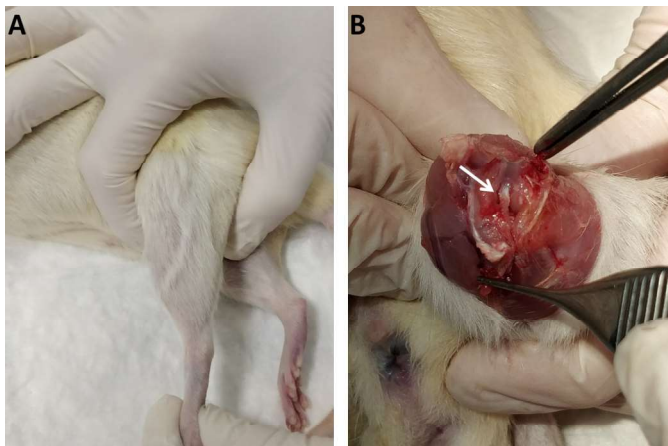


Fig. 6. Implanted limb of the rat (A) and prototype implanted (B) after 1 month.

thanks to the close proximity of cells. In both intra articular and osseous parts, the fibers are well surrounded by tissue. The image of the cross section of the prototype (see Fig. 7.B) shows the presence of tissue between the fibers confirming the good integration of the pNaSS-grafted braided prototype in the articulation.

4. Discussion

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

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

The mechanical results obtained for both prototypes clearly demonstrate the limit of handmade structure which required knots to stay united. In fact, these knots are going to concentrate stresses and so weaken the prototypes. The more knots there are, the more fragile is the structure. Moreover, in the case of the reconstruction of ACL in Wistar rat, the literature reports a failure load of the native ACL, for rats that weight between 300 g and 485 g, of 34.5–35.5 N depending of the weight but also of the age of the animal [18,19]. In our case, it was mechanically safer to choose to implant the PCL braided prototype which can endure a maximal force of 43.3 ± 1.0 N before failure.

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

Because this study was mainly conducted to evaluate the surgical feasibility to implant anterior cruciate ligament prosthesis in rat before starting an extensive *in vivo* study, only one rat was implanted. According to the previous results, it is obvious that our choice go to the pNaSS-grafted braided prototype. Despite the small size of the animal and the need of very precise material, the prototype was successfully implanted. After one month implantation, good clinical outcomes were noticed with among other no lameness, no inflammation and no anterior drawer. Finally, the observation of the explanted prototype demonstrates the potential of this implant which has been recolonized both at the intra-osseous and intra-articular level and around and along the fibers.

5. Conclusion

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

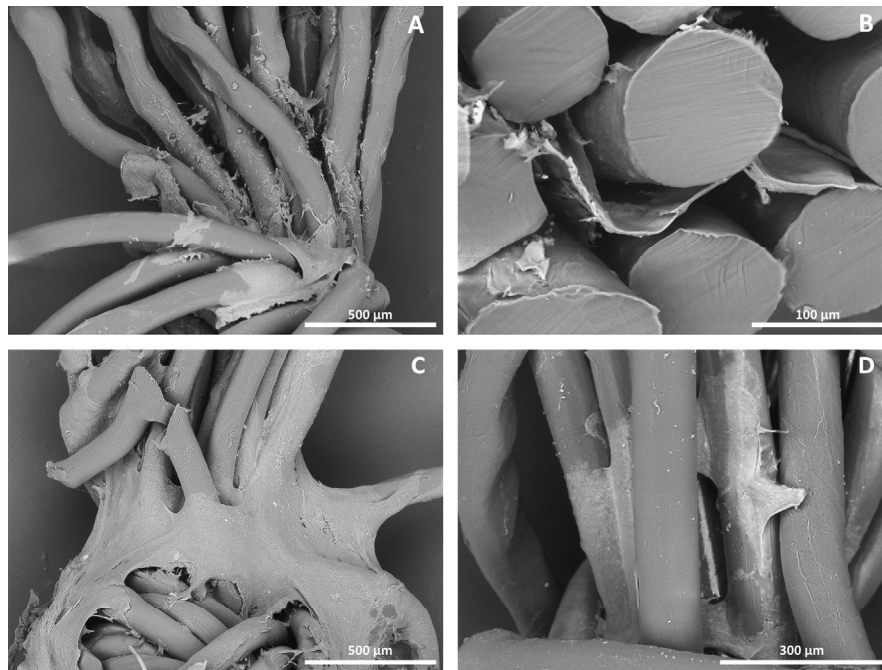


Fig. 7. ESEM images of the pNaSS-grafted braided prototype. A and B present the intra-articular part; C and D focus on the intra-osseous part.

will compare the grafted and the ungrafted prosthesis, will be conducted.

Human and animal rights

The authors declare that the work described has been carried out in accordance with the Declaration of Helsinki of the World Medical Association revised in 2013 for experiments involving humans as well as in accordance with the EU Directive 2010/63/EU for animal experiments.

Informed consent and patient details

The authors declare that this report does not contain any personal information that could lead to the identification of the patient(s).

Disclosure of interest

The authors declare that they have no known competing financial or personal relationships that could be viewed as influencing the work reported in this paper.

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Author contributions

All authors attest that they meet the current International Committee of Medical Journal Editors (ICMJE) criteria for Authorship.

CRediT authorship contribution statement

Amélie Leroux: Writing - original draft, Investigation, Formal analysis. **Emeline Maurice:** Investigation. **Véronique Viateau:** Methodology, Supervision. **Véronique Migonney:** Conceptualization, Writing - review & editing, Funding acquisition.

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