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Coline Pinese, Christian Gagnieu, Benjamin Nottelet, Capucine Rondot-Couzin, Sylvie Hunger, et al.. In vivo evaluation of hybrid patches composed of PLA based copolymers and collagen/chondroitin sulfate for ligament tissue regeneration. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, 2017, 105 (7), pp.1778-1788. 10.1002/jbm.b.33712 . hal-02387418

HAL Id: hal-02387418

<https://hal.umontpellier.fr/hal-02387418>

Submitted on 24 Jan 2024

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***In vivo* evaluation of hybrid patches composed of PLA based copolymers and collagen/chondroitin sulfate for ligament tissue regeneration**

Coline Pinese,¹ Christian Gagnieu,² Benjamin Nottelet,¹ Capucine Rondot-Couzin,³ Sylvie Hunger,¹ Jean Coudane,¹ Xavier Garric¹

¹Artificial Biopolymers Department, Max Mousseron Institute of Biomolecules (IBMM), UMR CNRS 5247, University of Montpellier, Faculty of Pharmacy, Montpellier 34093, France

²National Institute of Applied Science, MATEIS, UMR 5510, INSA de Lyon, Université Claude Bernard Lyon 1, 69100 Villeurbanne, France

³BIOM'UP, 69800 Saint Priest, France

Abstract: Biomaterials for soft tissues regeneration should exhibit sufficient mechanical strength, demonstrating a mechanical behavior similar to natural tissues and should also promote tissues ingrowth. This study was aimed at developing new hybrid patches for ligament tissue regeneration by synergistic incorporation of a knitted structure of degradable polymer fibers to provide mechanical strength and of a biomimetic matrix to help injured tissues regeneration. PLA-Pluronic[®] (PLA-P) and PLA-Tetronic[®] (PLA-T) new copolymers were shaped as knitted patches and were associated with collagen I (Coll) and collagen I/chondroitine-sulfate (Coll CS) 3-dimensional matrices. *In vitro* study using ligamentocytes showed the beneficial effects of CS on ligamentocytes proliferation. Hybrid patches were then subcuta-

neously implanted in rats for 4 and 12 weeks. Despite degradation, patches retained strength to answer the mechanical physiological needs. Tissue integration capacity was assessed with histological studies. We showed that copolymers, associated with collagen and chondroitin sulfate sponge, exhibited very good tissue integration and allowed neotissue synthesis after 12 weeks *in vivo*. To conclude, PLA-P/CollCS and PLA-T/CollCS hybrid patches in terms of structure and composition give good hopes for tendon and ligament regeneration. © 2016 Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater*, 105B: 1778–1788, 2017.

Key Words: soft tissues reinforcement, ligament, biomaterials, PLA-Pluronic, collagen

INTRODUCTION

With >800 000 cases per year in the US, ligament and tendon ruptures are a health public problem.¹ The most common ligament ruptures are anterior cruciate ligament (ACL), rotator cuff and acromioclavicular joint ligament ruptures with a respective occurrence in France of 37, 30, and 25%.² These injuries can range from simple fiber rupture to a complete rupture and can cause short-term and long-term complications engaging the functional prognosis. Various surgical procedures are available for the repair of ligament and tendon tears, such as allograft and autograft of tendon. Limited supplies of donors, disease transmission of allografts and high morbidity of autografts created new demands in tissue regeneration area such as a treatment of ligament failures.³ Polymeric ligament patches or polymeric 3D total

ligament reinforcement are, depending on anatomic localization, promising treatments for rotator cuff, acromioclavicular joint ligament or ACL ruptures.⁴

Soft tissue regeneration with degradable scaffolds combines two aspects. The first one is a need of scaffold degradation implying intrinsic properties loss. Indeed, immediately after implantation, degradable scaffolds should provide physiological and mechanical properties to meet tissue physiological requirements. Moreover, during the healing process the scaffold should progressively degrade at a rate that allows new tissue to receive the appropriate level of load without danger of rupture. The scaffold combined to new tissue should present at every moment adequate mechanical properties. The second aspect concerns the promotion of rapid cell colonization and neotissue synthesis.

Correspondence to: X. Garric; e-mail: xavier.garric@univ-montp1.fr
Contract grant sponsor: Contract grant sponsor: ANRT (Biom'up)

Scaffold shape, structure, and composition are key parameters that can influence the regenerative properties of the implant by allowing tissue ingrowth.

To provide mechanical properties, artificial ligament were firstly composed of nondegradable synthetic materials.⁵⁻⁹ However, their breaks and debris were responsible of inflammation *in vivo*.¹⁰ It was also shown that, as they do not provide tissue integration, their goal is limited to reinforcement.⁶ The emergence of degradable materials allowed the development of reinforcements which degrade to let space to the neotissue. The challenge is to elaborate reinforcements with initial mechanical properties close to tissue mechanical properties. Although particularly popular, thanks to its degradable properties and good biocompatibility, poly(lactide)s (PLA) do not meet optimal properties to replace soft tissues like ligaments, tendons, cartilage, or blood vessels.^{11,12} Our previous studies showed that PLA copolymerization with Pluronic[®] or Tetronic[®] modifies PLA mechanical properties.¹³ Indeed, compared to PLA, copolymers' Young moduli are lower and copolymers yield at failure is higher. We also demonstrated that, even though 200 kg mol⁻¹ copolymers showed a significant molecular weight decrease after 7 weeks of degradation, these copolymers kept a higher mechanical integrity with maintained Young's moduli in the range 400–600 MPa.¹⁴ Those changes are interesting for a biomaterial dedicated to a tissue under solicitation. Copolymers knitted textiles have been developed to present interesting mechanical properties close to mechanical properties of cruciate anterior ligament. It has been shown that PLA degradation time can be >5 years after implantation in bone tissues.¹⁵ For a temporary application, it can cause inflammation and disturb neotissues production.¹⁰ Therefore, it is essential to study *in vivo* polymers degradation to obtain the best balance between mechanical properties and degradation time.

During scaffold degradation, mechanical properties of reinforcements will be provided by scaffolds but also by the presence of neotissues produced thorough scaffolds. It is therefore essential to facilitate cell colonization and synthesis of extra-cellular matrix to enhance neotissue production. The native extracellular matrix (ECM) of ligament and tendon tissues is a complex structure mainly composed of a collagen fibers network. Therefore, collagen is a potential biomaterial for ligament and tendon regeneration.¹⁶⁻²⁰ However, collagen alone cannot be used in load bearing applications because of its low mechanical strength. Accordingly, to enhance tissue integration and provide mechanical support, composite, or hybrid structures containing collagen and synthetic polymer, such as polylactide (PLA) or poly(lactide-co-glycolide) (PLGA) have been developed.²¹ Moreover, ligament ECM contains fundamental substance mainly composed of glycosaminoglycans such as chondroitin sulfates (CS). The addition of CS in collagen material to mimic ECM is a very interesting way to enhance tissue formation and has already been studied.²²⁻²⁵ It was shown that depending on different physical and chemical parameters as collagen origin, pH solubilization, CS concentration or solubilization technique, CS addition has different effects on cell behavior

and on collagen synthesis.^{24,26} Indeed, size of neocollagen fibers and rate of synthesis can be enhanced or inhibited by CS.

The aim of this work is to develop and study different hybrid patches containing a biomimetic material to promote neotissue formation and a synthetic material to answer the need in mechanical properties. Two new PLA-based copolymers were used in order to study the balance between mechanical properties and degradation time (PLA-P and PLA-T). Because of their elasticity and low density, we hypothesized that knitted hybrid patches could present an optimal scaffold structure for tendon and ligament regeneration application. These knitted patches were associated with two biomacromolecular matrices based on collagen: fibrous collagen 1 alone (Coll) or a mixture of fibrous collagen 1 associated with chondroitin sulfate (CollCS) to evaluate the benefit on tissue production. We firstly evaluated *in vitro* ligamentocytes proliferation on hybrid patches. We secondly carried out an *in vivo* subcutaneous study of hybrid patches. After 4 and 12 weeks of subcutaneous implantation, a histological study was performed to study tissues integration. Copolymers properties were also studied to evaluate the balance between degradation and mechanical properties.

MATERIAL AND METHOD

Materials

Poloxamer (Pluronic[®] F-127; 12,600 g/mol), tin(II)2-ethylhexanoate (Sn(Oct)₂, 95%), dichloromethane, diethyl ether, and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (St-Quentin Fallavier, France). Poloxamine (Tetronic[®] 1107; 15,000 g/mol) was purchased from BASF (Levallois Perret, France). DD-Lactide (DD-LA), LL-lactide (LL-LA), and D, L-lactide (D, L-LA) were purchased from Purac (Lyon, France). Dulbecco's Modified Eagle Medium (DMEM) Nut Mix F-1212, calf fetal serum, L-glutamine, phosphate-buffered saline (PBS), penicillin, streptomycin, amphotericin B, and non-essential amino acid were purchased from Gibco (Saint Aubin, France). 12-well non adhesive culture plates and 12-well adhesive culture plates were purchased from Becton Dickinson (Le Pont de Claix, France). CS (AB00300) was purchased from Solabia (Beauvais, France). Collagen was extracted from pig tendon with acid extraction process of Biom'Up (Saint Priest, France).

Fabrication of copolymer knitted scaffolds

PLA₉₄ copolymerizations were performed from two central blocks: Pluronic[®] (PLA₉₄-P) and Tetronic[®] (PLA₉₄-T) as described in our previous work.¹³ 400 kg mol⁻¹ copolymers and a 200 kg mol⁻¹ PLA polymer reference were obtained. Polymer spinning was carried out with a pilot spinning machine (Centexbel, Gent, Belgium). Polymers were heated at 200°C during 10 min and then pressurized to cross a 1.5 mm diameter die with a 10 rpm spinning speed. Obtained monofilaments were drawn out with a drawing frame to a draw ratio of 4. Linear knitted textiles were achieved from PLA-P, PLA-T, and PLA multifilaments. Monofilaments were first coated with batching silicon oil to protect them from important friction forces in the knitted process.

Then, three monofilaments were twisted to create a multifilament yarn. This multifilament yarn was knitted with a Silver Reed SK270 knitted machine to obtain linear knitted textiles of PLA-P, PLA-T, and PLA. Silicon batching oil has been removed by 3×15 min baths of surfactant in ultrasound.

Collagen matrix fabrication

Two different collagen matrixes were produced: a collagen matrix (Coll) and a collagen/CS matrix (CollCS). Fibrillar collagen I was extracted from pork tendons and solubilized in water (0.7% w/v). Crosslinking was performed with oxidized glycogen. Aldehyde groups of glycogen react on amine groups of collagen lysine.²⁷ The quantity of oxidized glycogen was adjusted to have 0.8 mol of glycogen aldehyde per mol of amine of lysine either in Coll and CollCS solution. For collagen-CS samples, 10% of CS (w/v) was added in the basic collagen solution (containing ammonia to bring solution to pH 10.9) to obtain a homogenous viscous suspension. To create hybrid patches, 25 mL of collagen or collagen/CS solution were poured into a 80 cm² rectangular metallic mold. Five textiles were set down in the solution then 25 mL of collagenic solution were poured onto textiles. To activate the crosslinking of collagen-CS hybrid patches, molds were placed in a ventilate enclosure (0.02 m³) with 60 mL of citric acid and 60 mL of purified water for 24 h. To activate the crosslinking of collagen hybrid patches, molds were placed in a sealed enclosure (0.02 m³) with 320 μ L of ammonia (32% w:v) for 4 h. Then, samples were freeze-drying to obtain collagen and collagen/CS sponges. Molds were placed on a metallic plate at -20°C freezer at 4.10^{-4} mBar for 24 h.

All hybrid patches were sterilized by beta-ray between 25 and 27 kGy (Ionisos, France).

Ligamentocyte adhesion and proliferation

Primary ligamentocytes, extracted from rat ACL, were cultured in DMEM Nut Mix F-12 supplemented with 10% of decompartmented calf fetal serum, 4 mM of L-glutamine, 1.5 g L⁻¹ of NaHCO₂, 5000 U mL⁻¹ penicillin/5000 mg mL⁻¹ streptomycin, 2.5 μ g mL⁻¹ of amphotericin B and 1% (v/v in DMEM/F12) of nonessential amino acid. Ligaments were obtained from another *in vivo* study that includes the taking of ligament. All animals were treated according to institutional guidelines of laboratory and animal treatment and care. All experiments were approved by the animal research committee. Samples were cut (3.9 cm²) from PLA-P film, Coll, and collCS sponges. After sterilization with beta-ray radiations, samples were placed in 12-well nonadhesive polystyrene culture plates. Second-passage rat ligamentocytes were seeded at 25,000 cells per well in triplicate. The same seeded have been done in well treated for cell culture polystyrene (TCPS) as a control. For ligamentocyte adhesion study, cells were seeded and after 4 h of incubation at 37°C and 5% CO₂, then cell quantification was performed. For ligamentocyte proliferation study, cells were seeded and culture medium was added to immerse all samples after 2 h of cells adhesion. Cell quantification was performed by

measurement of viability with MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) after 2, 6, and 12 days of culture. Typically, the medium was aspirated, samples were rinsed with PBS and 1.5 mL of PBS solution containing 0.5 mg mL⁻¹ of MTT was added. After 3 h of incubation, formazan blue crystals were dissolved in 1 mL of DMSO and after 40 min of shaking, absorbance was measured at 600 nm.

In vivo implantation

Six hybrid patches of PLA-P/Coll, PLA-P/CollCS, PLA-T/CollCS, and PLA/CollCS were subcutaneously implanted in 325–349 g Sprague Dawley rats. To distinguish easily textile, all textiles were individualized using a nondegradable blue polypropylene suture yarn. Every textile has a size of $1 \pm 0.1 \times 2 \pm 0.1$ cm. Incisions of 1.5 mm were performed in parallel of each side of the spine (at 0.7 mm), on the upper back. Two independent compartments were created in dorsal subcutaneous tissues. Composites previously immersed in physiological serum for 1 min, were then implanted into compartments according to a randomization. Surgical controls without sample were also achieved for each implantation time. Finally, sutures were made to close the implantation sites (2-0 silk suture yarn). After 4 and 12 weeks, degradation of polymers from PLA-P/CollCS, PLA-P/Coll, PLA-T/CollCS, and PLA/CollCS were studied by evaluating molecular weight of the copolymers, and mechanical properties of the whole explant.

Histological study

Specimens were fixed, dehydrated, and embedded within paraffin blocks. Histological sections (5 mm) were prepared using a microtome, and subsequently deparaffinized with xylene, dehydrated using decreasing concentrations of ethanol, and then stained with hematoxylin and eosin. A semi-quantitative evaluation of inflammation and tissue integration was performed. The inflammation score was estimated by the quantity of each inflammatory cells (polynuclear, eosinophil, mast cells, lymphocytes, macrophages/monocytes, giant cells) present in tissues around implanted composites as 0:Absent - 1:Limited - 2:Moderated - 3:High - 4:Important. Total inflammation scores is the average of each cell score. Number of fibroblast-like cells and number of blood vessels within the newly formed tissue were quantified with a tissue integration score as 0:Absent - 1:Limited - 2:Moderated - 3:High - 4:Important.

Degradation study

To evaluate degradation of polymers, number average molecular weight (M_n) and dispersity (D) of polymers were determined by size exclusion chromatography (SEC) using a Viscotek GPCMax autosampler system fitted with two Viscotek LT5000L Mixed Medium columns (300 \times 7.8 mm). Polymer solution (10 mg mL⁻¹ in THF, sigma) was filtered through a 0.45 μ m Millipore filter before injection of 20 μ L of filtered solution. M_n was expressed according to calibration using Polystyrene standards. Thermal properties evolution was evaluated with differential scanning calorimetry

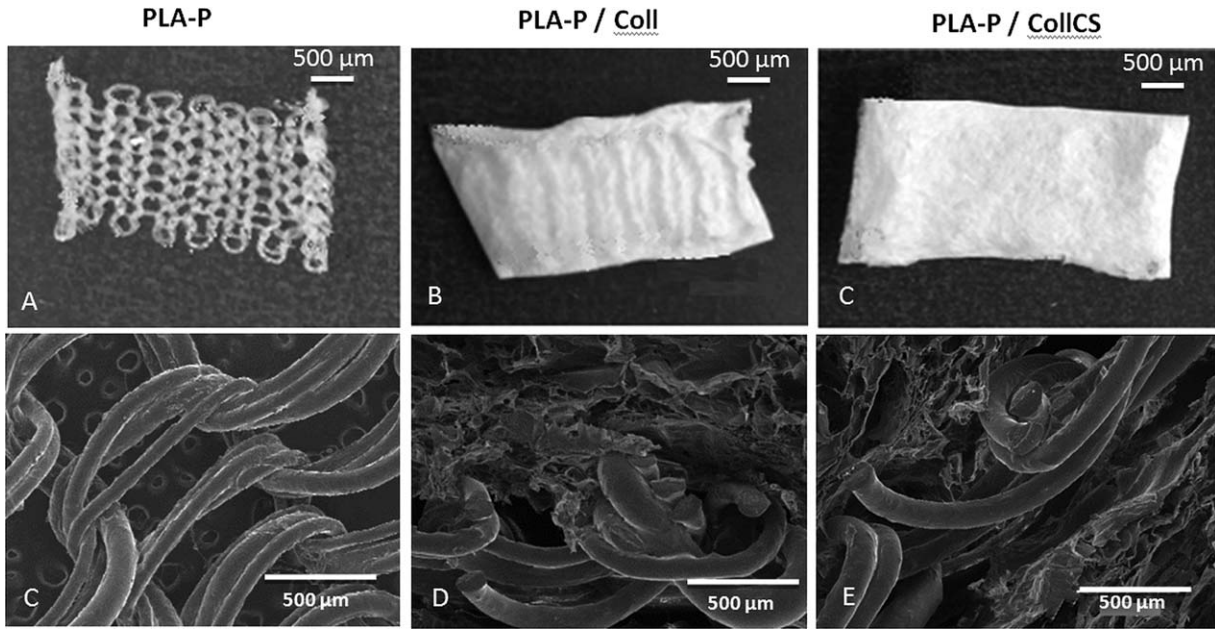


FIGURE 1. Macroscopic and SEM illustrations of PLA-P knitted fabric alone (A, D), PLA-P knitted fabric associated with a collagen matrix (PLA-P/Coll; B, E), and a collagen/CS matrix (PLA-P/CollCS; C, F)

(DSC). Measurements were carried out under nitrogen on a Perkin Elmer Instrument DSC 6000 Thermal Analyzer. Samples were submitted to a first heating scan to 200°C followed by a cooling (10°C min⁻¹ from 200 to 100°C, and 7°C min⁻¹ from 100 to -30°C) and a second heating scan to 200°C (10°C min⁻¹). Glass transition temperature (T_g), cold crystallization temperature (T_{cc}), melting temperature (T_m), and melting enthalpy (ΔH_m) were measured on the second heating ramp.

Mechanical properties evaluation

Three samples were used for the mechanical properties studies. Tissues surrounding hybrid textiles were removed cautiously with a scalpel, caring to let tissues produced within the knitted scaffold. Samples were analyzed with an Instron 4444 at a crosshead speed rate of 5 mm min⁻¹ and each sample being loaded to failure. Each sample was analyzed at 37°C in triplicate and stiffness (N mm⁻¹), maximal load (L_{max}) and strain at failure (ϵ_f , %) were expressed as the mean value of the three measurements. Stiffness was calculated using the initial linear portion of the load/strain curve.

Statistical analyses

Results corresponding to separate experiments done in triplicate and values are given as mean \pm SD. Statistical analyses were performed with the SigmaStat software. Comparison between several groups used a nonparametric Wilcoxon test (a level of $p < 0.05$ was considered statistically significant).

RESULTS

Knitted hybrid patches were developed as a scaffold for ligament regeneration. Figure 1 shows a knitted textile, alone and embedded in a Coll and CollCS freeze drying matrix.

SEM observation of the hybrid patches demonstrates that collagen matrix covered the interstices of the knitted scaffold creating a denser surface.

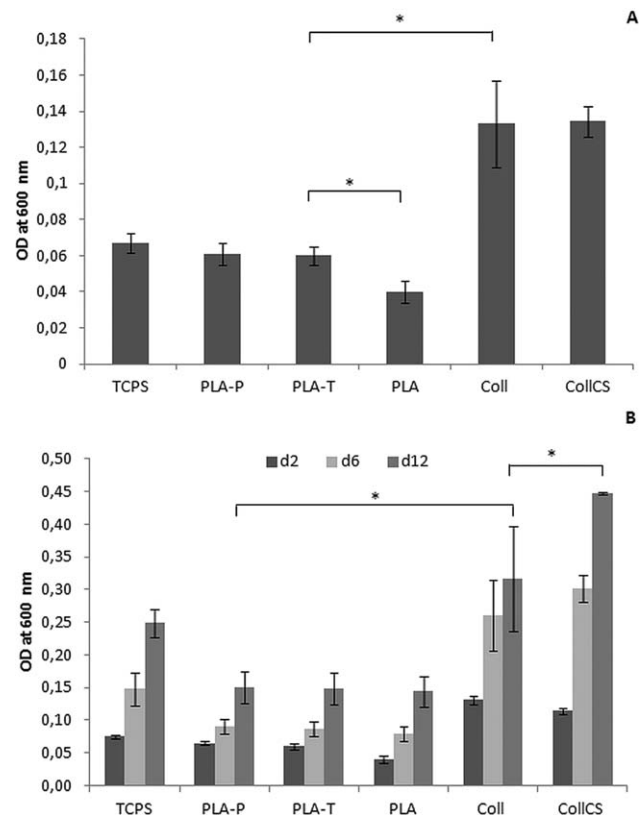


FIGURE 2. Primary rat ligamentocyte adhesion (A) and proliferation (B) on treated for cells culture polystyrene (TCPS) control, PLA-P, PLA-T, PLA, collagen (Coll), and collagen-CS (CollCS) matrix.

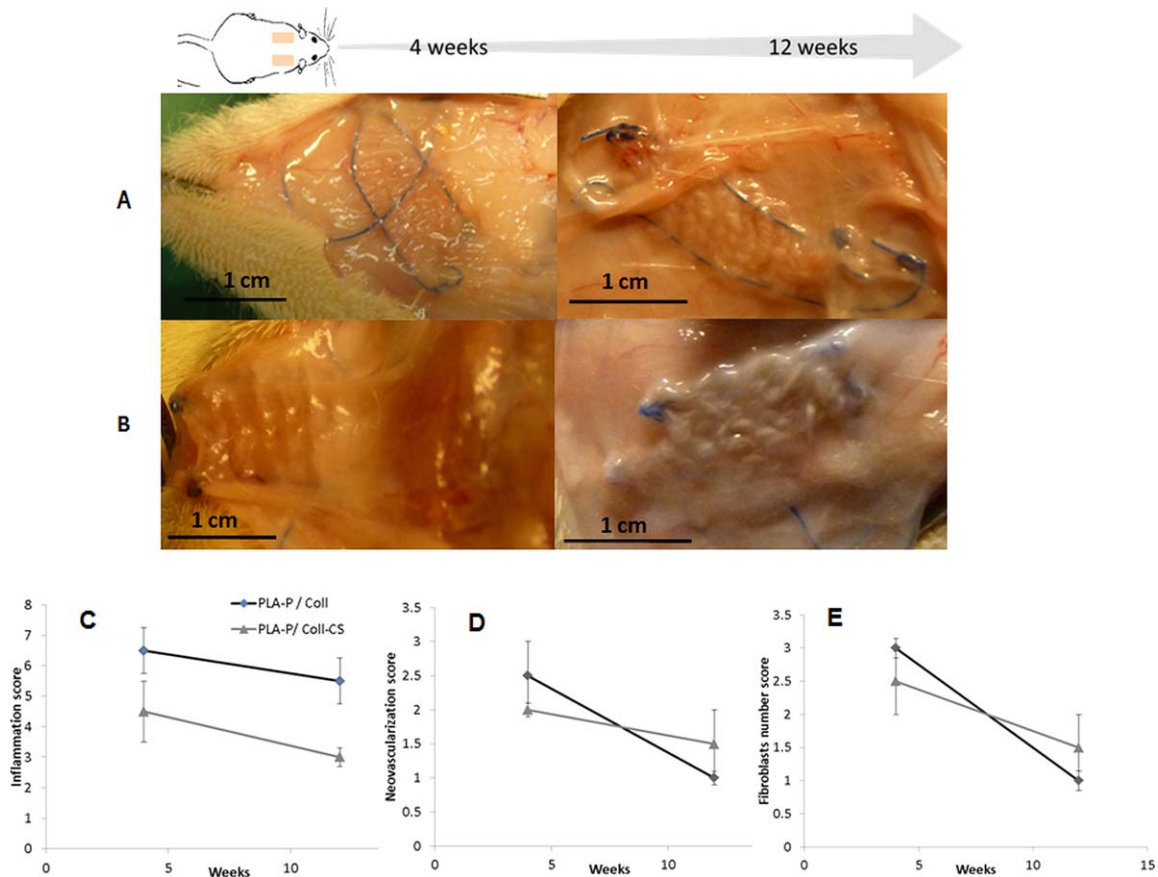


FIGURE 3. Tissue integration of hybrid patches after subcutaneous implantation: Illustration of PLA-P/CollCS (A) and PLA-P/Coll (B) integration after 4 weeks (left) and 12 weeks (right) and histological scores of inflammation (C), neovessel (D), and fibroblast quantities (E).

***In vitro* ligamentocyte adhesion and proliferation**

We developed a way of solubilization of CS in basic collagen solution. One goal of this work is dedicated to studying the influence of CS on ligamentocyte adhesion and proliferation. Ligamentocyte adhesion on materials was assessed on freeze-dried collagen, collagen-CS, PLA-P, PLA-T, and PLA plates [Figure 2(A)]. The optical density (OD) of solubilized formazan blue crystals reflects the amount of adherent cells on materials. With an OD of 0.06, compared with 0.04, PLA-T and PLA-P show a slight better cell adhesion than on PLA alone, similar to TCPS plates control. However, with the same OD of 0.06, no influence of the Pluronic® or Tetricon® block on ligamentocytes adhesion was observed. This figure highlights the beneficial effect of collagen and collagen-CS on cell adhesion compared to PLA-P, PLA-T, or PLA. With an OD of 0.13, cell adhesion is significantly higher on the freeze-dried collagen than on copolymers plates (OD value = 0.06 and p values < 0.001). However, OD values for Coll and CollCS samples are both of 0.13. The presence of CS in freeze-dried collagen products has no effect on ligamentocytes adhesion, compared with collagen sample alone.

Ligamentocytes proliferation on biomaterials after 2, 6, and 12 days of culture is shown in Figure 2(B). This figure highlights several results. First, there is a significant difference of OD values between tested days ($p_{\text{value}} < 0.001$) for

each sample, showing a ligamentocytes proliferation on each sample. Secondly, after 12 days, ligamentocytes proliferation on copolymers and polymers samples is lower than on TCPS plates control (OD value around 0.15). However, OD values reflecting cells quantity obtain on Coll samples after 6 and 12 days are significantly higher than OD values obtain on copolymers, suggesting that ligamentocytes proliferation is promoted by collagen. Finally, at day 12, the amount of cells on CollCS samples is significantly higher (OD = 0.448) than on Coll samples (OD = 0.316; $p_{\text{value}} = 0.003$). The structure and composition of collagen matrix with CS appears to improve ligamentocytes proliferation after 12 days of culture.

Effect of composite materials on tissue regeneration

After 4 and 12 weeks of subcutaneous implantation in rats, effect on tissue regeneration of both collagenic matrices was assessed (Figure 3). Hybrid patches of PLA-P associated with collagen or collagen-CS were implanted (PLA-P/Coll and PLA-P/CollCS). After 4 and 12 weeks of subcutaneous implantation in rats, good connective tissue infiltrations without any signs of infection or encapsulation were macroscopically observed on surfaces of composites [Figure 3(A,B)]. Cell colonization and tissue formation of each sample were evaluated by histological staining. Overall, despite

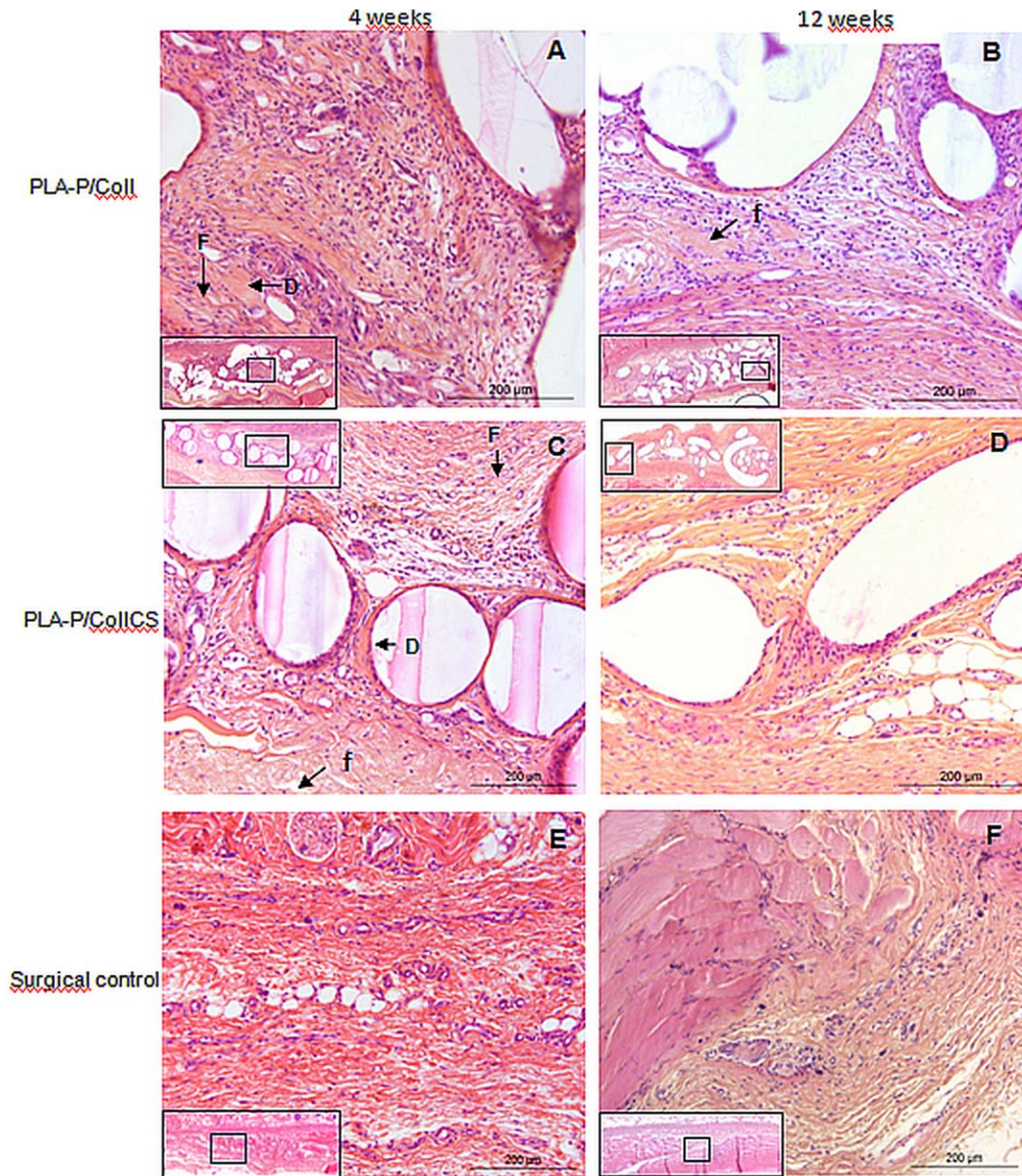


FIGURE 4. H&S staining of PLA-P/Coll (A, B), PLA-P/CollCS (C, D), and surgical control (E, F) after 4 and 12 weeks of subcutaneous implantation, where F is organized fibrillar neomatrix, D is unfibrillar dense neomatrix, and f is loose fibrillar neomatrix

a few inflammatory areas, no major sign of inflammation or encapsulation that would isolate composite textile was observed after 4 and 12 weeks *in vivo*.

After 4 weeks of *in vivo* subcutaneous implantation, inflammation is low and mainly peripheral. However, two layers of inflammatory cells containing macrophages, monocytes, and giant cells were observed around polymers fibers. No polynuclear, eosinophils, mast cells, lymphocytes, and necrosis were observed. The histological scores of inflammation [Figure 3(C)] indicate that neotissues in collagen matrix with a score of 6.5 (PLA-P/Coll) present a significantly more important inflammation than in the collagen-CS matrix (PLA-P/CollCS) with a score of 4.5. After 12 weeks of *in vivo* subcutaneous implantation, neotissue inflammation

decreased to reach the score of, respectively, 5.5 and 3 for PLA-P/Coll and for PLA-P/CollCS matrix samples. No eosinophils, mast cells, or lymphocytes were detected. Inflammation is mainly localized around the polymers fibers.

A moderate neovascularization was observed in all composite samples. With neovascularization scores of 2.5 and 2 for PLA-P/Coll and PLA-P/CollCS samples, respectively [Figure 3(D)], no significant difference between the two matrixes after 4 weeks *in vivo* was observed. After 12 weeks *in vivo*, vessel quantity decreases to reach the neovascularization score of 1 and 1.5 in collagen and collagen-CS samples respectively. However, neovessel diametric size evolves to a larger and usual vessel diametric size meaning that tissues need less blood supply to fight against inflammation.

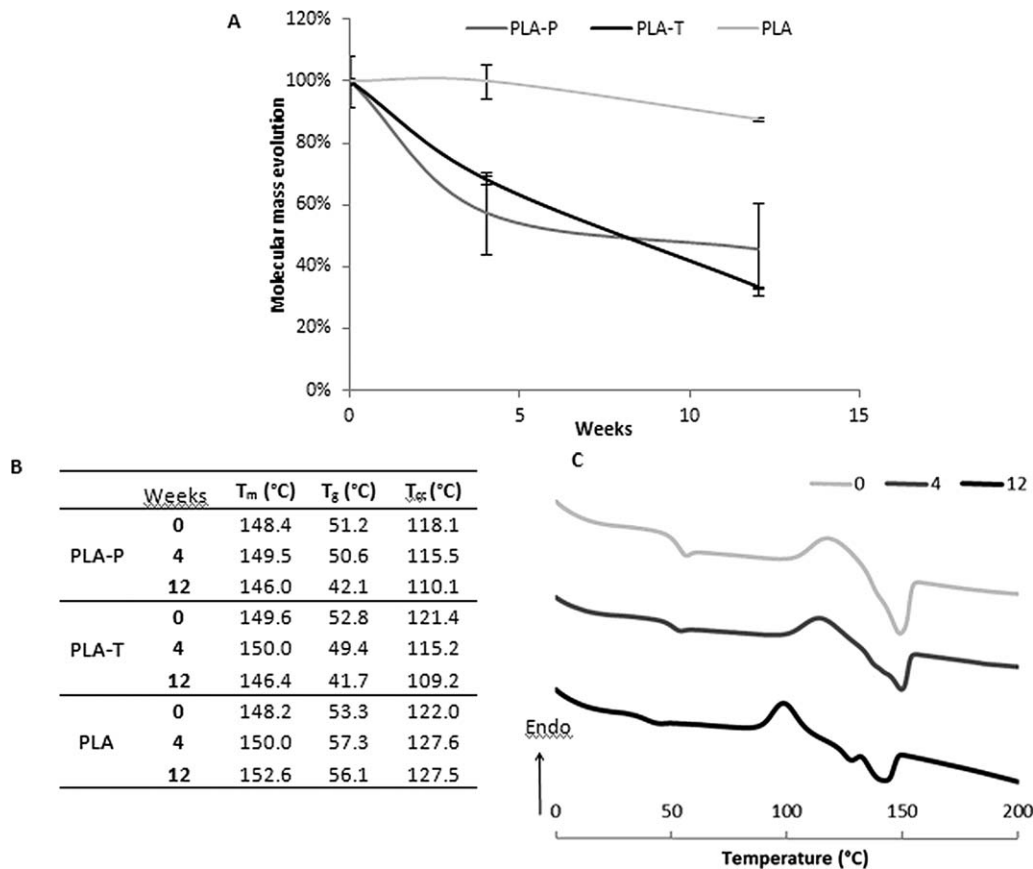


FIGURE 5. PLA-P, PLA-T and PLA degradation after 4 and 12 weeks of subcutaneous implantation: copolymers molecular weight evolution (A), main copolymers thermal properties evolution (B) and illustration of PLA-P thermal properties evolution. Melting temperature (T_m), melting enthalpy (ΔH_m), glass transition temperature (T_g), and cold crystallization temperature (T_{cc}) determined by DCS.

With a fibroblast number score of 2.5 for PLA-P/Coll and of 3 for PLA-P/CollCS [Figure 3(E)], the amount of fibroblasts is similar in all composite samples after 4 weeks *in vivo*. Their distribution is homogeneous in samples and allows a neomatrix synthesis. After 12 weeks *in vivo*, fibroblast quantity decreases to reach the fibroblast number score of 1 and 1.5 in PLA-P/Coll and PLA-P/CollCS samples, respectively.

After 4 weeks, a neomatrix was synthesized in the entire composite, from its periphery to its center and between polymer fibers. The main difference between PLA-P/Coll and PLA-P/CollCS comes from the structure of neomatrix in the center of the composite, as illustrated in Figure 4. Composite samples with a Coll matrix present two neomatrix structures [Figure 4(A)]: mostly a fibrillar neomatrix, mainly formed by long individualized neosynthesized collagen fibers (F) (fibers are aligned and arranged in samples) and a dense neomatrix formed by unfibrillar collagen (D). Samples with a CollCS matrix present three neomatrix structures [Figure 4(C)]: a fibrillary (F), a loose fibrillary (f) and a dense collagen neomatrix (D). The very fibrillar neomatrix (F) composed of long aligned fibers is mainly visible on samples periphery. The loose fibrillar neomatrix of small collagen fibers (f) is mainly visible on samples periphery and between polymer fibers. Finally, the dense collagen

neomatrix (D) is visible between polymer fibers. In comparison, surgical control sample [Figure 4(E)] presents a neomatrix with collagen fibers, which are short and moderately organized.

After 12 weeks *in vivo*, PLA-P/CollCS patches are fully filled by oriented long collagen fibers [Figure 4(D)]. PLA-P/Coll patches [Figure 4(B)], also present a fibrillar collagen neomatrix. However, fibers organization is less compact due to persisting inflammation and the presence of loose fibrillar neomatrix.

Evolution of textile properties after *in vivo* implantation

To study the evolution of copolymer textile properties, composites of PLA-P, PLA-T, and PLA associated with a collagen-CS and collagen matrices were subcutaneously implanted in rats for periods of 4 and 12 weeks.

Molecular weight evolution. Figure 5(A) shows the evolution of macromolecules molecular weight during the implantation time, expressed in percentage of initial molecular weight. PLA homopolymer molecular weights decrease gradually to 88% of the initial molecular weight after 12 weeks *in vivo*. In opposition, copolymers of PLA-P and PLA-T degrade more rapidly and reach 46 and 33% of their initial molecular weight after 12 weeks *in vivo*. Evolution of

A

PLA-P/ CollCS	0	6.5 ± 3.73	14.85 ± 2.33	324 ± 60
	4	6.13 ± 1.62	6.4 ± 4.26	257 ± 107
	12	11.76 ± 4.84	3.88 ± 1.91	321 ± 70
PLA-T/ CollCS	0	5.19 ± 2.26	14.16 ± 3.07	256 ± 99
	4	11.06 ± 0.67	11.11 ± 0.4	395 ± 24
	12	10.54 ± 3.89	2.45 ± 0.19	168 ± 47
PLA/ CollCS	0	4.16 ± 0.93	7.33 ± 0.56	198 ± 53
	4	7.20 ± 1.38	5.51 ± 1.56	361 ± 44
	12	7.55 ± 0.13	3.64 ± 1.29	205 ± 33
PLA-P/ Coll	0	6.5 ± 3.73	14.85 ± 2.33	324 ± 60
	4	3.15 ± 0.99	5.47 ± 1.5	105 ± 109
	12	8.41 ± 3.68	2.5 ± 1.1	88 ± 89

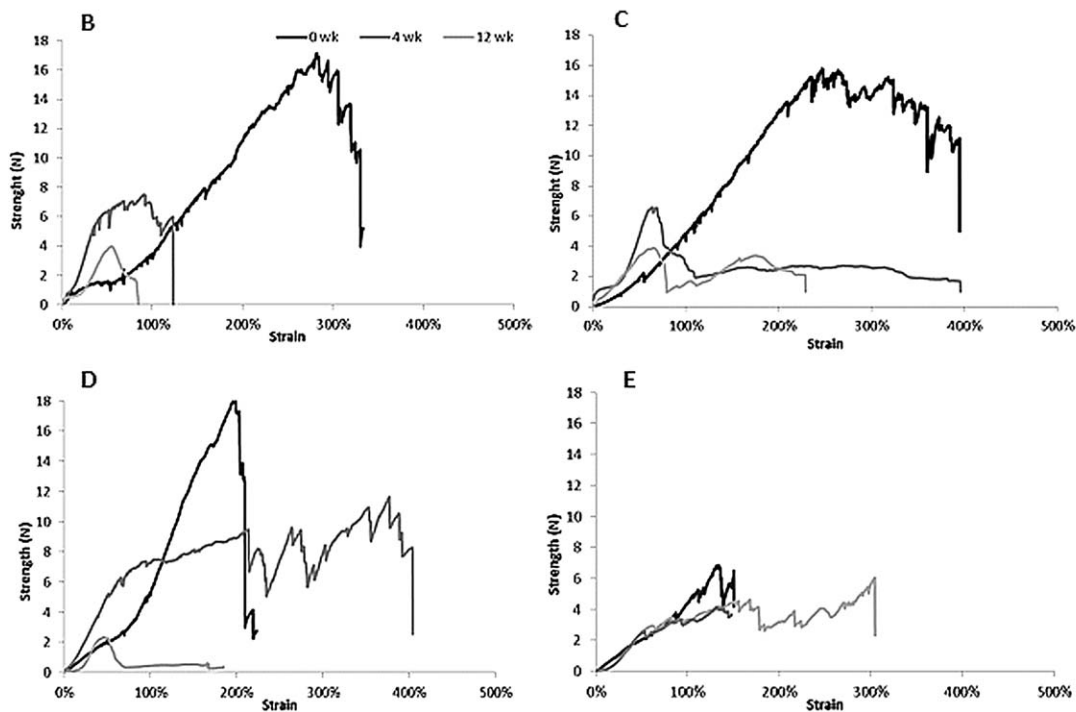


FIGURE 6. Evolution of hybrid patch mechanical properties after 4 and 12 weeks of subcutaneous implantation: main values of stiffness, maximal load (L_{max}), and strain at failure (ϵ_f ; A) and strength and strain curve of PLA-P/Coll (B), PLA-P/CollCS (C), PLA-T/CollCS (D), and PLA/CollCS (E).

thermal properties [Figure 5(B,C)], also show signs of copolymer degradation. First, a decrease of glass transition temperatures (T_g) for both copolymers was observed, reflecting the degradation of amorphous regions. Indeed, initially of 51.2 and 52.8°C for PLA-P and PLA-T, respectively, T_g drops to 42.1 and 41.7°C after 12 weeks *in vivo*. In opposition, T_g of PLA increases from 53.3 to 56.1°C after 12 weeks *in vivo*. A second indication of the degradation is the change of copolymers melting profile. Originally, monomodal, the melting profile becomes bimodal. PLA-P and PLA-T recrystallization temperature decreases from 118 to 110°C and 121 to 109°C respectively after 12 weeks *in vivo*. Value for PLA increases from 122 to 127.5°C.

Mechanical properties evolution. Figure 6 shows mechanical properties evolution during implantation time. After 4 and 12 weeks of *in vivo* implantation, it can be observed an increase of stiffness from 6.5 to 11.8 $N\ mm^{-1}$ for PLA-P/CollCS, from 5.2 to 10.5 $N\ mm^{-1}$ for PLA-T/CollCS and from 4.2 to 7.5 $N\ mm^{-1}$ for PLA/CollCS. Maximum tensile loads recorded during the test decrease gradually with time. PLA-P/CollCS maximum tensile loads initially at 14.85N, drop to 6.4N after 4 weeks of implantation and to 3.9N after 12 weeks (37 and 17% of the initial maximum tensile loads, respectively). This decrease is delayed for PLA-T/CollCS samples whose maximum tensile loads value is 14.16N initially, 11.1N after 4 weeks of implantation, and 2.5N after

12 weeks of implantation (78 and 17% of the initial maximum tensile loads respectively). PLA-T mechanical properties decrease is delayed after 4 weeks compared with PLA-P. However, this difference is no longer visible after 12 weeks.

This progressive loss of strength is moderate for the PLA homopolymer with a maximum tensile load of 7.3N initially, 5.5N after 4 weeks of implantation, and 3.6N after 12 weeks of implantation (75 and 50% of the initial maximum tensile loads, respectively).

Strain at failure of PLA-P associated with collagen matrix (PLA-P/Coll) decreases during implantation time from initially 324% to, respectively, 105 and 88% after 4 and 12 weeks *in vivo*. In comparison, PLA-P/CollCS keeps a constant strain at failure. Figure 6 shows material strain-strength curve for each implantation time. This figure shows that failure of PLA-P/Coll [Figure 6(A)] samples abruptly happens and then no more strength is recorded. However, when associated with collagen CS matrix (PLA-P/CollCS-6B) copolymers strength is still recorded after copolymers failure. This recorded strength is likely due to the presence of neotissue within knitted textiles which oppose a resistance to traction.

DISCUSSION

Main objectives of ligament reinforcement conception are 1/to provide a temporary mechanical support, strong enough to answer physiological strains of injured tissues, and also degradable to let space for neotissue formation; 2/to allow a qualitative and quantitative tissue neosynthesis, which will offset loss of mechanical properties during scaffold degradation.

The goal of this study was to develop hybrid patches comprising a natural component to promote neotissue formation and a synthetic component to answer the need in mechanical properties.

As a unique method of material processing, knitting has shown the potential to provide tissue engineering with many kinds of knitted scaffolds, or participate in the construction of tissue-engineered scaffolds.²¹ Indeed, knitted scaffold possesses highly ordered loop structures and versatile mechanical properties²⁸ that can provide sufficient internal connective space for tissue ingrowth.²⁹ Consequently, we hypothesized that our copolymeric knitted scaffold could be an optimal structure for tendon and ligament regeneration application.

To promote cell colonization, collagen or collagen-CS matrices were associated to copolymers knitted scaffolds. *In vitro* experimentations show that the presence of collagen enhances ligamentocyte adhesion and proliferation compared with polymers and copolymers used alone. Effects of CS on cell adhesion were not observed but we showed a positive influence of CS on ligamentocytes proliferation, which is significantly higher on collagen-CS matrix than on collagen matrix only. The effect can be induced by the matrix structure and by the CS themselves. The CS-6 and CS-4 are the most common categories of CS and have different properties. Some literature data³⁰ describe an improvement of cell

proliferation by these two types of CS but an antagonist action on cells adhesion: CS-4 are responsible for cell adhesion inhibition whereas CS-6 promote it. The CS used in this study is a mixture (50/50) of CS-6 and CS-4. Therefore, this seems to indicate that the effects of two types of CS on cell adhesion were offset.

After 4 and 12 weeks of subcutaneous implantation in rats, no systemic or local toxicity was observed and inflammation was moderate. ECM synthesis and cell colonization of hybrid patches are scored as moderate to high (score = 2.5–3) after 4 weeks *in vivo* for all hybrid patches. Our results show that the neo-ECM structure is different depending on the sample: on collagen-CS samples, fibroblasts synthesize two kinds of fibrillar matrix (organized and unorganized) and a part of unfibrillar matrix. On collagen sample without CS, fibroblasts synthesize an organized fibrillar matrix and an unfibrillar matrix. Many studies correlate the effects of CS with collagen fibril diameter and fibrogenesis rate.²⁴ CS appears to allow the additional production of unorganized fibrillar matrix, absent on collagen samples after 4 weeks. Moreover, after 12 weeks, maturation phase of tissues seems to be more advanced on collagen-CS matrix than on collagen matrix. It is illustrated by the strength recorded during mechanical tests. Failure of PLA-P/Coll samples happened abruptly when strength due to neotissues is still recorded after failure of PLA-P/CollCS samples. Therefore, these results suggest that collagen-CS matrixes are promising material for soft tissue engineering.

The second aim of this work is to provide a temporary mechanical support for ligament regeneration. Degradable copolymers were synthesized and tailored as a textile to meet ligament mechanical strength requirement.¹³ We showed in this study that the presence of bloc (Pluronic and Tetronic) modifies the *in vivo* degradation rate of PLA. Indeed, copolymers keep after 12 weeks 46 and 33% of their initial molecular weight for PLA-P and PLA-T, respectively (88% for PLA). The degradation is confirmed by thermal properties. A decrease of glass transition temperature was observed in copolymers, reflecting the degradation of amorphous regions and consequently an increase of copolymers melting enthalpies. Moreover, copolymers melting profiles are modified to become bimodal. These phenomena have their origins in the crystallinity of polymers. Copolymers degradation results in PLA chains hydrolysis, which involves a release of small PLA chains. These small chains may be entrapped in materials and present a faster crystallization kinetic, which allows polymer crystallization at lower temperatures. As a result, PLA-P and PLA-T recrystallization temperatures decrease while PLA recrystallization temperature increases. Degradation of the two copolymers is faster and this can be explained by the presence of hydrophilic center blocks, which increases materials hydrophilicity. One of the main consequence, is a raise of water penetration, which leads to an increase of hydrolytic degradation rate.³¹

The direct consequence of polymer degradation is a progressive loss of mechanical properties. During 12 weeks of *in vivo* implantation, a progressive increase of polymers

stiffness was observed. This phenomenon can be explained by the increase of crystallinity during degradation caused by the degradation of amorphous areas. Then, copolymer strengths at failure progressively decrease with implantation time. After 12 weeks, copolymer textiles conserve 26% of their initial maximal strength for PLA-P and 17% for PLA-T. PLA-T copolymers show a slower loss of mechanical properties compare to PLA-P copolymers. Homopolymer textiles maintain 50% of their initial maximal strength after a few weeks. Despite the fact that copolymers are significantly degraded, they keep interesting mechanical properties. These results suggest that an equilibrium between polymers degradation time and mechanical properties can be glimpsed. To compare with what happens *in vivo* in case of surgery, transplants used in gold standard surgical technic also lose their properties after *in vivo* implantation and become brittle. Ballock et al.³² showed that patellar grafts used for ACL reconstruction keep only 15% of the initial breaking strength after 6 months of implantation as rabbit's reinforcement ligament.

Our copolymer knitted patches provide good mechanical properties after 12 months of *in vivo* implantation even though the quantity of copolymers per cm² is low. The knitted structure fit in with the ligament regeneration application. Cell penetration and tissue integration on scaffolds are promoted by the knitting shape that provides adequate space for cell colonization. In comparison, a study of the tissue integration of a PLA braid implanted in rabbit knees³³ shows after 4 weeks the presence of a fibrous tissue and a very low cell penetration due to lack of space within the scaffold. Very few neomatrix is therefore synthesized in the braid after 4 weeks *in vivo* and, after 12 weeks, a loose and anarchic neo-collagen synthesis is visible within the PLA braid. As a consequence, degradable knitted shapes seem to be more suitable for soft tissue regeneration than degradable braid shapes. In our study, we also showed that knitted scaffold is gradually degraded to let space to neotissues. The result of our study is that Coll-CS patches allow interesting tissues integration and a start of tissue organization. Associating good tissue regeneration and mechanical strength, hybrid patches seems to be really interesting to enhance soft tissues regeneration.

Interesting perspectives of this work would be a study of hybrid patches in a suitable and more realistic *in vivo* model. It is well known that mechanical stresses are benefic on neotissue production and organization.³⁴ In a more realistic model, polymer degradation would be offset in term of mechanical resistance by a strong and organized neotissue. These results would show that hybrid patches are suitable for this application. Moreover, the adjustment of textile design can provide various mechanical properties to textiles. Varying knitted design is also an additional perspective to obtain the ideal balance between mechanical properties and degradation.

CONCLUSION

The aim of this work was to develop and study different hybrid patches for ligament regeneration comprising a

biomimetic material to promote neotissue formation and a synthetic component to answer the need in mechanical properties. The results of this study demonstrate the capacity of hybrid patches to allow soft tissue regeneration. We showed the influence of the introduction of CS in a collagen sponge on *in vitro* cell behavior and *in vivo* ECM synthesis. The collagen-chondroitin sulfate sponges enhance cell proliferation and collagen fibrillation *in vivo*. We showed that Pluronic[®] PLA and Tetronic[®] PLA copolymers are promising biomaterials for temporary application in ligament healing due to their mechanical properties and faster degradation time compared to PLA homopolymer. We showed that PLA-based copolymers associated with collagen and CS sponge exhibit a very good tissue integration and allows neotissue synthesis after 12 weeks *in vivo*. Finally, PLA-P/CollCS and PLA-T/CollCS hybrid patches give good hopes for tendon and ligament regeneration in terms of structure and composition and should be evaluated in an animal model of ligament healing.

ACKNOWLEDGMENTS

The authors also thank Synbio3 platform (IBMM, Montpellier) for SEC and DSC experiments.

Contract grant sponsor: ANRT (Biom'up).

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