Design of a Bioabsorbable Multilayered Patch for Esophagus Tissue Engineering

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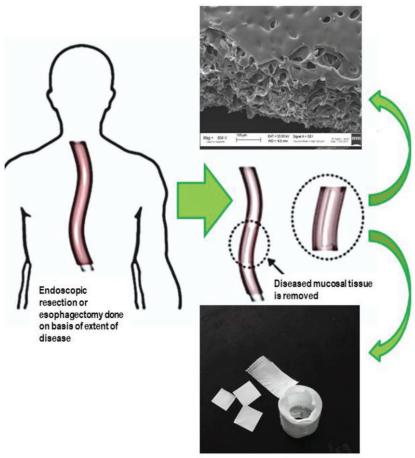
A gold standard for esophagus reconstruction is not still available. The present work aims to design a polymer patch combining synthetic polylactide-*co*-polycaprolacton and chitosan biopolymers, tailoring patch properties to esophageal tissue characteristics by a temperature-induced precipitation method, to get multilayered patches (1L, 2L, and 3L). Characterization shows stable multilayered patches (1L and 2L) by selection of copolymer type, and their M_w. In vitro investigation of the functional patch properties in simulated physiologic and pathologic conditions demonstrates that the chitosan layer (patch 3L) decreases patch stability and cell adhesion, while improves cell proliferation. Patches 2L and 3L comply with physiological esophageal pressure (3–5 kPa) and elongation (20%).



1. Introduction

Currently, the ideal approach in tissue regeneration and repair should be minimal invasive, therefore, not involving autologous tissue grafts. In these terms, polymer devices promoting cell proliferation, and able to guide tissue regeneration, are particularly interesting; their principal objective is to recapitulate extracellular matrix (ECM) function in a temporarily coordinated and spatially organized structure.[1–4]

Esophagus anatomy and its pathologies such as gastroesophageal reflux and Barrett's esophagus, or pediatric surgical conditions, like long-gap esophageal atresia, highlight different acquired or congenital states where esophagus substitution is needed.[5] Moreover, treatment of severe esophageal burns or cancer can require partial esophagus substitution or its complete reconstruction. Commonly used surgical techniques, such as gastric and colonic interpositions, are frequently associated with complications such as stricture formation and the potentially carcinogenic effect of acidic reflux, leading to high morbidity. Therefore, studies on regenerative approaches aimed at improving esophageal reconstructive techniques are of great interest, and important issues still need to be addressed.[6–8]

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Decellularized matrices derived from different organs and tissues have been widely studied. Main advantage of this approach is the proven atoxicity of matrix and its degradation products. However, studies in the literature show heterogeneous results depending on the decellularized matrix source. Biologically derived materials such as collagen are attractive for their biocompatibility and they have been widely studied biomaterials with some controversial results.[9,10]

A biomaterial-based scaffold provides unique advantage of being a standardized matrix giving reproducible response in implanted host tissues. Therefore, the importance of biomaterial nature has been highlighted in the literature, both regarding biocompatibility needed to avoid host rejection and biodegradation, useful to achieve scaffold resorption. The latter property should be attentively evaluated in order to get a dynamic scaffold whose degradation rate was synchronized to new tissue in-growth rate. Moreover, biomaterial degradation products should not be recognized as toxic, and possibly they should be eliminated by metabolic pathways. Knowledge of esophagus anatomy and physiology is important in order to design a polymer scaffold. Esophagus is a muscular tube, of 18–26 cm length, with a complex structure and composed of four tissue layers: mucosa, muscularis mucosa, submucosa, and adventitia. Mucosa, the limiting esophagus lumen, is composed of nonkeratinized stratified squamous epithelium and connective tissue (lamina propria); muscularis mucosa is mainly made of smooth muscle fibers and is just beneath the lamina propria; submucosa is a layer of connective tissue beneath the muscularis mucosae; muscularis externa, underneath submucosa is a muscle layer combining smooth and striated muscle fibers; adventitia is the inner esophagus wall layer made of loose connective tissue with blood vessel and nerves. Due to its physiologic role, esophagus is a highly elastic organ withstanding food friction and pressures ranging between 3 and 5 kPa. Hence, the biomaterial selected as the scaffold component should possess elastic properties, or the scaffold should be composed of different biomaterials giving suitable elasticity to the structure. Synthetic biodegradable polymers such as polyglycolic acid, polylactide (PLA), poly(lactide-*co*-glycolic acid), polycaprolactone (PCL), and PLA–PCL copolymers have been investigated in these years with good results in organ reconstruction including esophagus.[11–14] Moreover, synthetic polymeric scaffolds, made of these materials, can be grafted with ECM proteins via surface modification techniques, in order to promote cell attachment.[14,15]

Since currently a gold standard for esophagus reconstruction is still not available, the present work is a preliminary investigation addressed to design a polymer patch combining synthetic and natural biopolymers in order to suitably tailor patch properties according to esophageal tissue characteristics. Attention has drawn to polymer selection and their combination in suitable ratios, taking into account the fact that patches should be resorbable, biodegradable, biocompatible, able to support cell migration and proliferation from the surrounding tissue, and resistant to mechanical traction. In this work, multilayered patches made of polymer blends and with diverse polymer compositions and concentrations were prepared by the temperature-induced precipitation (TIP) method. The patches were in vitro characterized for their morphology, chemical properties such as degradation behavior, and physical properties such as tensile strength. Moreover, the patches were in vitro incubated with cell culture to test cell adhesion and proliferation, and a preliminary ex vivo test on porcine excised esophagus was carried out.

2. Experimental Section

2.1. Materials

Polylactide-*co*-polycaprolacton Purasorb PLC 85:15 L-lactide/caprolactone, weight average molecular weight (M_w) 181 492 Da and number average molecular weight (M_n) 135 165 Da, and polylactide-*co*-polycaprolacton PLC 70:30, L-lactide/caprolactone, M_w 160 241 Da and M_n 119 405 Da, copolymers were purchased from Purac (Purac Biomaterials Gorinchem, Netherlands). Chitosan Protosan CL113 Batch no. 310-490-01, M_w 110 000 Da, was purchased from PRONOVA (Pronova Biopolymer a.s. Gaustalleen 21, Olso, Norway).

Agarose CAS no. 9012-36-6, sodium tripolyphosphate (TPP), M_w 367.86 g mol^{-1}, sodium nitrate ($NaNO_3$, M_w 84.99 g mol^{-1}) CAS no. 7631-99-4, dimethylsulfoxide (DMSO, C_2H_6OS, M_w 78.13 g mol^{-1}), sodium azide (NaN_3), M_w 216.04 g mol^{-1}, water solubility 41.7 g/100 mL at 17 °C, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), M_w 414.33 Da, and collagen from veal skin CAS no. 9007-34-5 were purchased from Sigma Aldrich (Sigma Aldrich SpA, Milan, Italy).

0.1 M 36% (M_w 36.46 g mol^{-1}) chlorhydric acid (HCl) Spectrosol was from BDH Chemical, Ltd, Poole, England.

Tetrahydrofuran (THF), 99.5%, CAS no. 109-99-9, was purchased from Carlo Erba, Milano, Italia.

Dulbecco modified Eagle's medium (DMEM), pH 7.8, was purchased from Lonza (Lonza, Verviers, Belgium).

Phosphate-buffered saline (PBS), pH 7.4. 10×10^{-3} M, was prepared according to Italian Official Pharmacopeia (FUI XII ed) specifications. Homemade distilled water was filtered through a 0.22 μm membrane filter.

All solvents and reagents used were of analytical grade.

2.2. Methods

One layered, two layered, and three layered patches were prepared by the TIP method. The patch preparation procedure was carried out in clean sanitized environment, under laminar flow hood.

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Table 1. Composition, thickness, wettability, and porosity of patches.

Patch code	Type	Patch composition			Thickness [μm]	Wettability [(θ) ± SD]	Porosity [(E%) ± SD]
		PLC 85:15 [mg]	PLC 70:30 [mg]	Chitosan CL113 [mg]			
1L	Monolayered ed composite	120	–	–	130 ± 4.50	42.02 ± 5.50	85.5 ± 1.7
2L	Bilayered composite	120	80	–	200 ± 10.2	41.63 ± 5.63	83.8 ± 2.1
3L	Three layered composite	120	80	20	250 ± 9.90	0 ± 0	81.9 ± 2.6

Polymers and their combinations were selected with the aim to provide suitable patch characteristic as reported in the literature.[15–17] Layer 1 made of PLC 85:15 should be impermeable with tensile strength, corresponding to esophagus layer closest to the lumen (mucosa). The high polylactide content in PLC 85:15 should provide these characteristics. Layer 2 made of PLC 70:30 should be stiffer, simulating submucosa and muscularis layer beneath mucosa. The higher polycaprolacton content in PLC 70:30 improves polymer stiffness while decreasing typical PLA brittleness. Layer 3 made of chitosan is added with the aim of making the outer layer more hydrophilic, improving cell adhesion.[18] The three layered patch should combine the properties of each polymer, giving to the patch suitable characteristics in order to (i) resist to mechanical stress and acidic pH values that can arise into esophagus in some physiologic and pathologic situations; (ii) offer the suitable support for cell attachment and proliferation. In the case of three layered patches, chitosan layer position was designed in order to get the chitosan layer turned to the inner side of esophagus channel. The patch composition and codes are reported in Table 1.

2.3. Scaffold Preparation

One layered patch (1L, Table 1) preparation protocol was as follows. 15% w/v PLC 85:15 was dissolved in dioxane by magnetic stirring at 500 rpm, at room temperature for 60 min. The viscous polymer solution was sonicated for 3 min in order to eliminate the entrapped air bubbles, and 800 μL of the solution was dropped in a Teflon mold (2.5 cm width × 2.5 cm length × 4 mm height). The polymer solution was then freezed at −25 °C for 5 h and lyophilized (Freeze dryer Lio-5P, Cinquepascal, Italy) at −48 °C and 0.4 mbar for 12 h. The two layered patches (2L, Table 1) were prepared following the same protocol: 800 μL of 10% w/v polylactide-*co*-polycaprolacton (PLA–PCL, 70:30) dioxane solution was dropped on the already lyophilized PLC 85:15 layer at 5 °C, and immediately lyophilized under the same conditions as explained above in order to prevent dioxane to solubilize PLC 85:15 lyophilized layer. The PLC 70:30 layer concentration was set at 10% w/v in order to achieve a layer more porous than layer 1 (PLC 85:15 15% w/v). In the case of three layered patch preparation, 800 μL of 2.5% w/v chitosan hydrochloride (CL 113) aqueous solution was dropped at first in the Teflon mold covered with an aluminum foil, freezed, and lyophilized as explained before. Afterward, 10% w/v PLC 70:30 dioxane solution was dropped on the lyophilized chitosan layer, freezed, and lyophilized, and at last 15% w/v PLC 85:15 dioxane solution was dropped on the two layered lyophilized patch and immediately lyophilized. The setup protocol took care of preventing chitosan layer detachment. The lyophilized three layered patches were extracted from the Teflon mold and soaked for 10 min in 10% w/w TPP solution in order to crosslink the chitosan layer.

The rationale of selecting 1,4-dioxane as polymer organic solvent for PLC copolymers was its high boiling temperature (101 °C) that holds back its evaporation during the polymer dissolution step, keeping the polymer concentration constant. Moreover, 1,4-dioxane freezing temperature is 12 °C and permits complete freezing of the solvent during the lyophilization freezing step, with following complete sublimation. Organic solvent elimination is an important issue in order to get a safe product for biomedical use.

2.4. Scaffold Characterization

2.4.1. Morphologic Characterization

Morphologic characterization is important since it permits us to directly visualize the scaffold microstructure. Analyses were performed on six square samples (2 mm × mm) for each patch type, cut from different positions in the original patch, in the middle and on edges, respectively. Samples were mounted on aluminum stages, sputter-coated with gold for 1 min, and observed by scanning electron microscopy (SEM) (Zeiss EVOMA10 electron microscope (Carl Zeiss Oberkochem)) at an accelerated voltage and different magnifications. SEM analysis was used to get results about thickness, pore size, and structure of patches.

2.4.2. In Vitro Evaluation of Cell Attachment and Proliferation

The study evaluated both cell attachment and five days long-term proliferation of fibroblasts seeded and cultured on scaffolds.

Adult dermal fibroblasts, purchased as primary cells from International PBI (Milan, Italy), were cultured in DMEM containing 10% Foetal Bovine Serum (FBS) and 1% antibiotic solution (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) and detached for the biological experiments after expansion. Cells used were always in the 13th to 14th passage. All patch samples were sanitized by exposing them to a UV lamp for 12 h (overnight) before incubation with cell cultures.

Samples (1L, one layered patch; 2L, two layered patch; and 3L, three layered patch) were fixed in six well culture plates, by means of a drop of 4% w/v agarose aqueous solution, and

conditioned with 3 mL of DMEM supplemented with 10% FBS for 4 h. After conditioning, 1×10^5 cells were seeded onto each sample and incubated at 37 °C, and 5% of CO_2 for 3 h, and up to 5 d to assess their cell seeding capacity and long-term cell growth, respectively. Culture medium was refreshed every 48 h. Cells cultured without patches were used as positive control (CRT+), patches incubated in wells without cells were used as negative control (CRT–). At scheduled times (3 and 24 h, and 5 d), samples were collected and cell mitochondrial activity was determined by MTT assay as follows. The culture medium was withdrawn and substituted with DMEM without FBS; afterward, 30 µL of MTT solution (5 mg mL^{-1} in DMEM) was added into the wells. Cells were incubated for 2.5 h at 37 °C to allow MTT reduction by viable cell mitochondrial dehydrogenase. After this time, samples were removed from wells, and a suitable detergent was added to dissolve resulting blue formazane crystals. 1 mL THF was used to dissolve formazane crystals into cells adhered and entrapped into/onto patches, while 2 mL DMSO was added to each well to dissolve formazane crystals of cells adhered to the bottom of the well and of control. The performed procedure was an MTT-modified test, as reported in a previous work of the same authors.[19] Results were analyzed by a multiwell scanning spectrophotometer (Microplate Reader Model 680, Bio-Rad Laboratories, USA), and optical density was measured at 595 nm (solutions obtained in THF) and at 570 nm (solutions obtained in DMSO), always with 655 nm as reference wavelength. Results of cell viability are expressed as UV–vis absorbance of incubated samples and compared to absorbance of cells incubated without patches (CRT+). CRT– is due in order to verify patch biomaterial does not interfere with UV–vis absorbance. Six replicate for each type of patch were performed and data are presented as mean±standard deviation (SD).

2.4.3. In Vitro Evaluation of Patch Degradation in Simulated In Vivo Physiologic Conditions

An in vitro test was set up in order to assess the patch degradation behavior in simulated in vivo physiologic conditions. The following parameters are considered: esophageal tissue pH is 7.4, meanwhile the esophageal lumen pH value can change during the day time and/or fasting or feeding conditions depending on the esophageal portion (proximal or distal). The proximal esophageal lumen pH value can vary between 6.5 and 7.4 under physiologic fasting conditions, and it can temporarily lower up to pH 4 when the lumen is in contact with acidic food. Even lower pH values can be temporarily detected in lumen of distal esophageal portion (the portion closer to stomach).

The test was performed as follows: each patch sample was incubated for 28 d in 7 mL of PBS, pH 7.4, at 37 °C, supplemented with 0.01% w/v NaN_3 in order to prevent microbiologic contamination. The incubation medium was refreshed every 48 h.

At 3, 5, 14, 21, and 28 d incubation, patches were withdrawn and the following parameters were evaluated: water uptake, mass loss, and polymer M_w, M_n, and polydispersity index (PI), as reported below.

2.4.4. Water Uptake Determination

Freeze dried polymeric patches were weighted (M_0) on an analytical balance (Mettler Toledo, Mod AG245, Switzerland) before starting the in vitro degradation test. At scheduled time points (3, 5, 14, 21, and 28 d), patches were withdrawn from the incubation medium and weighted (M_t). Percentage of water uptake was determined by subtracting patch weight in wet state (M_t) from that of same patch in dried state (M_0), according to Equation (1)

$$\text{Water uptake } (\%) = (M_t - M_o)/M_o \times 100 \quad (1)$$

The test was performed in triplicate for each patch type. Results are reported as water uptake percentage average.

2.4.5. Mass Loss Determination

At scheduled time points (3, 5, 14, 21, and 28 d), patches were recovered, lyophilized (–50 °C, for 24 h), and weighted (M_x), and their weight was compared to the initial weight of the same patch in dried state (M_0). Mass loss percentage was calculated using Equation (2)

$$\text{Mass loss} = (M_o - M_x)/M_o \times 100 \quad (2)$$

The test was carried out on all patch types, and in triplicate on each patch. Results are expressed as percentage of polymer remaining after each time point tested.

2.4.6. Polymer Molecular Weight Determination

Patch degradation performances were evaluated through PLC 85:15 and PLC 70:30 polymer M_w), M_n, and PI variations. The M_w and M_n were determined by gel permeation chromatography (GPC) for all patches withdrawn at the scheduled incubation times and lyophilized. Samples for GPC analysis were prepared by dissolving them in THF at a concentration of 1–2 mg mL^{-1}.

In the case of patches 1L and 2L, a polymer solution in THF was obtained; in the case of patch 3L, a polymer suspension was obtained, since chitosan is not soluble in THF. In both cases, THF solutions or suspensions were filtered through a 0.45 mm filter (Millipore, MA, USA) and injected in the GPC apparatus; GPC eluent was tetrahydrofuran at a flow rate of 1 mL min^{-1}. GPC apparatus consisted of a guard column (Phenogel 10E 4 Å µm, 300 × 7.8 mm, Phenomenex, Milan, Italy) and two Phenogel 10E 3 Å µm and 500 Å columns connected in series, a pump (Varian 9010, Milan, Italy), a Prostar 355 RI detector (Varian Milan, Italy), and software for M_w distribution computing (Galaxie Ws, ver. 1.8 Single-Instrument, Varian Milan, Italy). Resulting data are expressed as average of five parallel samples.

2.4.7. In Vitro Evaluation of Patch Degradation in Simulated In Vivo Pathologic Conditions

In pathologic conditions such as esophageal reflux, dramatic changes of esophageal pH can occur, even for a short time. In order to evaluate if pH changes affect patch degradation, an in vitro degradation study in standardized pH change conditions was set up and performed on all patches.

Each patch was incubated for 28 d in 7 mL of PBS, pH 7.4, at 37 °C, supplemented with 0.01% w/v NaN_3. The incubation medium was substituted every 48 h with 7 mL solution of

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PBS supplemented with 500 µL 0.1 M HCl, pH 1. Samples were soaked for 30 min; pH was measured during time interval (pH meter 827 pH lab; Metrohom, Switzerland) and afterward they were withdrawn, dripped and soaked in 7 mL PBS, pH 7.4, for 47 h before repeating the pH change procedure. At scheduled incubation times (3, 5, 14, 21, and 28 d), patches were withdrawn and water uptake, mass loss, and polymer M_w, M_n, and PI were evaluated as reported above. Moreover, the incubation medium pH was monitored all along the test.

2.4.8. Patch Hydrophilicity (Wettability)

Wettability was assessed by static water contact angle measurements at room temperature using the Lorentzen–Wettre apparatus (Lorentzen & Wettre, Sweden). The test measures the contact angle (θ) that a droplet of water has with material surface. The liquid used was PBS, pH 7.4, and the drop volume was 45 µL. 2 cm × 2 cm patch samples were tested in triplicate for each patch type.

2.4.9. Patch Porosity Evaluation

1L, 2L, and 3L patch porosity was measured by a modified liquid displacement method: ethanol was chosen as the displacement liquid because it penetrates easily into the pores; it is a chitosan and PLC nonsolvent and it does not induce polymer shrinkage or swelling.[19]

Briefly, a weighted patch sample was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The sample was kept in the nonsolvent for 10 min, and then a set of evacuation–repressurization cycles were carried out to force ethanol into the pore structure. Cycling was continued until no air bubbles were observed from the patch surface. Total volume of ethanol and ethanol-soaked patch was then recorded as V_2. Volume difference, ($V_2 - V_1$), corresponds to patch skeleton. The ethanol-soaked patch was then removed from the cylinder and residual ethanol volume was recorded as V_3. The volume ($V_1 - V_3$), corresponding to ethanol volume retained into patch porosity, was defined as patch pore volume. Total patch volume was calculated as follows

$$V = (V_2 - V_1) + (V_1 - V_3) = (V_2 - V_3) \quad (3)$$

Patch porosity (E) expressed in percentage (%) was calculated by

$$E(\%) = \frac{(V_1 - V_3)}{(V_2 - V_3)} \times 100 \quad (4)$$

The porosity values were determined from three samples for each patch type and expressed as mean ± SD.

2.4.10. In Vitro Permeability Study

Permeability test was performed on all patches in order to evaluate (i) patch permeability with respect to cell nutrients, i.e., small molecules such as glucose (M_w =180 Da); (ii) patch permeability toward water.

Franz diffusion cells (Standard Franz cells, Permegear, Inc., Hellertown, PA 18055, USA) were used on purpose and a cellulose membrane of 0.22 µm porosity (Millipore S.p.A. Filtration Technologies, Vimodrone, Milan, Italy) was used as positive control. Pullulan standard, M_w 180 Da, was used as the model molecule to represent the behavior of small nutrient molecules such as glucose.

The donor compartment of Franz cells was filled with 1 mL of a 2 mg mL^{-1} pullulan solution in PBS; the acceptor compartment was filled with 5 mL PBS, pH 7.4, and maintained continuously under magnetic stirring at 50 rpm. A battery of six Franz cells thermostated at 37 °C was used for each type of patch experiment. At scheduled times (10, 20, 30, 60, 80, 100, 120, 140, 160, and 200 min), 500 µL of solution was withdrawn from the acceptor compartment and analyzed by GPC; each 500 µL withdrawn was refilled with 500 µL of fresh PBS.

GPC apparatus consisted of a guard column PL-AQUAGEL-OH (50 × 75 mm, Phenomenex, Milan, Italy) and two Phenomenex YARRA columns 3 and 2 µm, 150 × 7.8 mm, respectively, and connected in series, a pump (Varian 9010, Milan, Italy), a Prostar 355 RI detector (Varian Milan, Italy), and software for M_w distribution computing (Galaxie Ws, ver. 1.8 Single-Instrument, Varian Milan, Italy).

2.4.11. Mechanical Properties

Uniaxial tensile tests were performed with an electromagnetic MTS Insight System 10 kN (MTS System Corporation) equipped with an NG Video Extensometer (Messphysic Material Testing) and a load cell of 250 N. To avoid patch damages due to the high closing pressure of the pneumatic machine grips, PLA grips (Figure 1a) were 3D printed using a 3NTR A4v2 fused deposition modeling printer. Patches were glued using cyanoacrylate between two grips at each side, immediately before the test, and fixed in the machine gripping system. In order to track the elongation of the central part of the patch through the video extensometer, two black and white strips were used as markers and placed at a fixed distance in the middle of each patch (Figure 1a). A tensile test was carried out at a speed of 1 mm min^{-1}, up to patch breaking (Figure 1b). Data were acquired at a constant sampling rate of 10 Hz.

Five patches of each type were tested, and results are reported as average within each patch group (1L, 2L, and 3L).

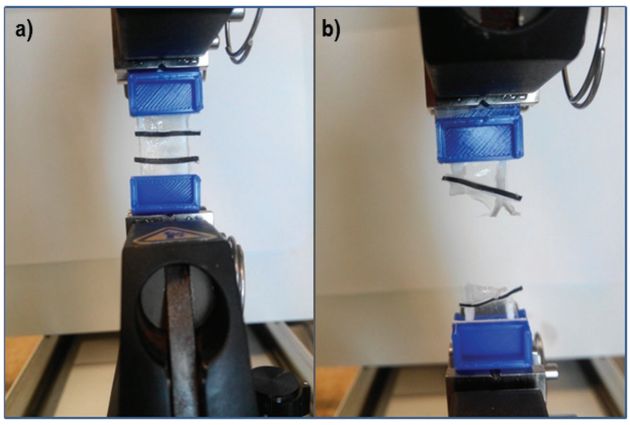

Figure 1. A sample glued between PLA grips and that fixed in the machine pneumatic system: a) at the beginning and b) at the end of traction test. Markers are visible on the sample surface.

The following parameters were acquired during the tensile test: (σ) stress (MPa), corresponding to the applied force (N) per unit sample area (mm^2), (ε) strain ($mm\ mm^{-1}$), defined as the sample deformation with respect to original length. Relying on the acquired parameters, the following were computed: (σ_{max}) stress at break (MPa), the maximum stress samples bear before breaking, (ε_{max}) strain at break (that can be expressed in $mm\ mm^{-1}$ or in %), the maximum elongation reached by the sample before breaking, (E) Young's modulus (MPa) that is a mechanical property of the material which describes the ratio between σ and ε, in the case of elastic behavior under uniaxial load.

2.4.12. Ex Vivo Preliminary Surgical Suture Test

A preliminary ex vivo test was performed on isolated excised porcine esophagus from 11-month-old adult porcines of slaughterhouse origin. The experimental goal was to test suture and patch resistance to an applied pressure.

Esophagus was intubated (to simulate the standard in vivo surgery procedure), and surgical incision of muscularis externa and mucosa was performed in selected position; mucosal and submucosal tissues were removed. Patch was inserted at the incised section, and sutured with a 4/0 hook needle using mid-term absorbable synthetic monofilament suture based on glyconate (HR26, ½ c, 26 mm Taper). Resistance and containment ability of ex vivo preparation was tested by perfusion of 20 mL PBS solution through a syringe; pressure was monitored to be within the 3–5 kPa range (flow through test). The test was performed on patch 2L.

Intact esophagus resistance to physiologic pressure applied by perfusion of 20 mL PBS solution in the same explained conditions was tested as control.

2.4.13. Statistical Analysis

All experiments were based on at least three independent samples, and experiments were repeated for three times. Results were reported as mean ± SD. Two-way analysis of variance was used to assess the statistical significance, and p-value of <0.05 was considered statistically significant.

3. Results and Discussion

The design of a patch suitable for esophageal reconstruction addressed to resorbable, biodegradable, biocompatible polymers, able to support cell migration and proliferation from surrounding tissues, and resistant to mechanical traction. The polymers selected in the present study are approved for medical use and known to be biodegradable and biocompatible. Lactide-*co*-caprolacton copolymers were selected in order to combine the glassy properties of polylactide (PLA) with the rubbery behavior of PCL, the better tensile strength of PLA with the higher toughness of PCL, and the slower degradation rate of PCL with the faster degradation rate of PLA, respectively, on the basis of the data in the literature.[21–23] These properties, together with polymer biodegradation time, depend on copolymer molecular weight.

The M_w values of the selected lactide-*co*-caprolacton copolymers were 181 492 Da for PLC 85:15 and 160 241 Da for PLC 70:30 since in vivo stability of the patch should be 28 d to allow tissue regeneration.

The anatomic esophagus structure addressed to design multilayered patches (see compositions listed in Table 1) where, in the case of bilayered patch, the external layer (the one in contact with esophagus lumen) should be richer in PLA than the inner layer in order to improve impermeability to fluids and prevent leaking of material from esophagus inside to outside, and the second layer, richer in PCL, increases patch mechanical resistance to traction. In the case of the three layered patch, an inner layer of hydrophilic chitosan was added with the aim to provide an environment furthermost favoring cell adhesion. In all these cases, the presence of two layers of partially hydrophilic lactide-*co*-caprolacton copolymers should provide suitable impermeability to fluids and suitable mechanical resistance to pressure rising during the ingestion process.

3.1. Morphologic Characterization

Patches were prepared by the temperature-induced precipitation method. Patch thickness was measured by SEM analysis and it ranged between 130 and 250 μm as a function of patch composition. As expected, two-layered and three-layered patches showed higher variability in thickness (see Table 1). Morphologic analysis performed by SEM, Figure 2, showed that the PLC 85:15 layer (patch 1L) was compacted with regular tiny pores (Figure 2a), PLC 70:30 layer (patch 2L) showed larger pores, as expected, due to the more diluted starting polymer solution composition (Figure 2b), while the chitosan layer (patch 3L) was highly porous with an ordered

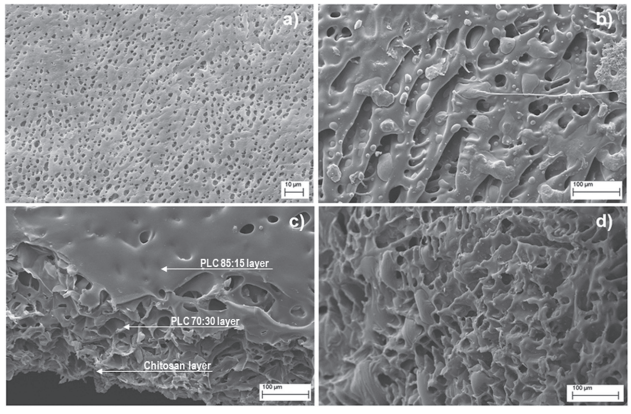

Figure 2. SEM micrographs a) one olayered patch 1L, b) inner layer of two layered patch 2L, c) section of three layered patch 3L, and d) inner chitosan layer of patch 3L.

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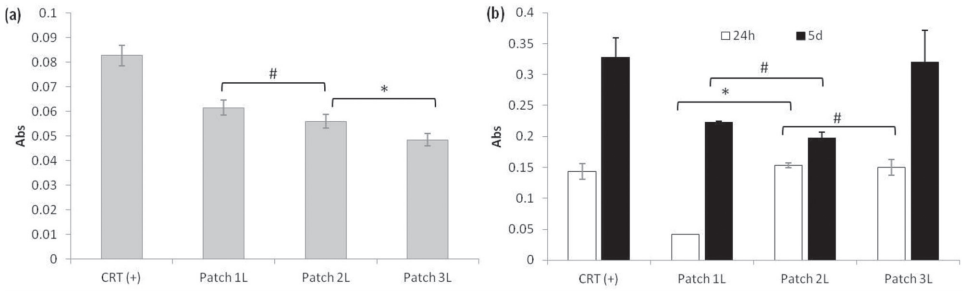

Figure 3. a) Cell attachment (5 h) and b) proliferation, assessed by MTT Assay of patches 3L, 2L, and 1L and cells w/o patch as positive control, CRT (+). Data are presented as mean ± SD ($n = 6$ per group, # indicates $p < 0.001$ and * indicates $p < 0.01$).

porous structure characteristic of the hydrophilic lyophilized component (Figure 2d). SEM analysis of patch section highlighted the 3D porous structure of patches, as shown in Figure 2c for patch 3L, also confirming a tiny pore structure in the PLC 85:15 layer, and a large pore structure in PLC 70:30.

3.2. In Vitro Evaluation of Cell Attachment and Proliferation

The results of biologic study on purpose differentiate between in vitro cell adhesion and proliferation. Cell attachment ability gives information on (i) patch cytocompatibility and (ii) how the patch structure is suitable to cell adhesion. Cell proliferation test results address to long-term suitability of the material composition.

Results of in vitro cell adhesion study are reported in Figure 3, and they are expressed in terms of absorbance. Cell adhesion, evaluated as percentage with respect to positive control (CRT+), varied from 58.6% for patch 3L to 67.65% and 74.3% for patches 2L and 1L, respectively. No significant differences in cell attachment ability were detected between patches 2L and 1L, highlighting that the PLC copolymers highly promoted cell adhesion. Addition of hydrophilic chitosan layer seems not to favor cell attachment, which was significantly lower for patch 3L as compared to patches 2L and 1L. CRT– did not show any absorbance, demonstrating that the polymers do not interfere with the carried out test.

Short-term cell proliferation (24 h, Figure 3b) results showed significantly higher values for 2L and 3L patches as compared to 1L patches, while medium-/long-term cell proliferation (5 d, Figure 3) results showed significant higher values for 3L patches (97.46%, cell viability with respect to CRT+) compared to 2L (60.30%, cell viability with respect to CRT+) and 1L patches (67.91%, cell viability with respect to CRT+). The results, obtained from in vitro proliferation study, lead to speculate that the composition and structure of 3L patches improve cell proliferation.

3.3. In Vitro Evaluation of Patches Degradation in Simulated In Vivo Physiologic and Pathologic Conditions

3.3.1. Water Uptake Determination

Results of water uptake are reported in Figure 4. Patches 1L absorb significantly higher amounts of water as compared to patches 2L, both in simulated physiologic (pH = 7.4) and pathologic (pH = 7.4 plus HCl intermittent addition) conditions. The behavior can be explained by the lower thickness of monolayered patch 1L (130 ± 4.5 μm) compared with patch 2L (200 ± 10.2 μm). Addition of a chitosan hydrophilic layer changes water uptake profile in such a way that patch 3L water uptake is

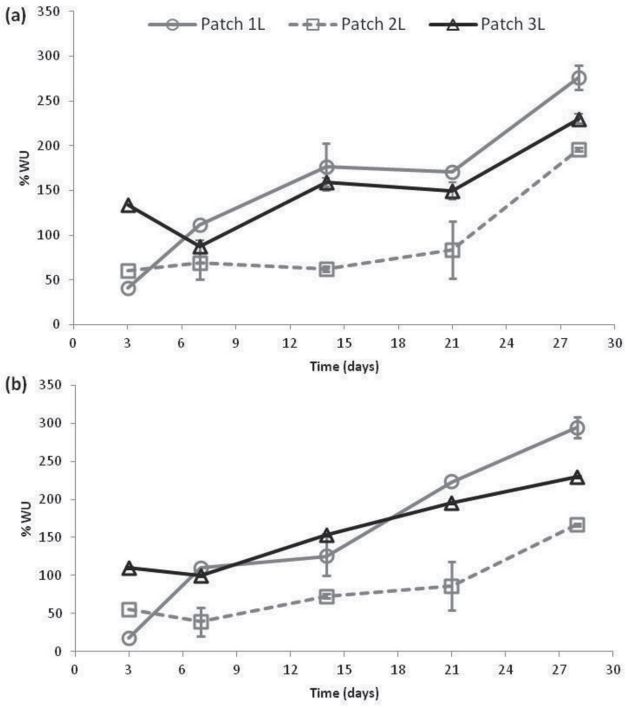

Figure 4. Water uptake of a) patches incubated in vitro simulated physiologic conditions; PBS, pH 7.4; b) patches incubated in vitro simulated pathologic conditions, PBS pH 7.4 supplemented with HCl 0.1 N.

the highest at day 3 (134.3% ± 6.7%), and it is superimposable to that of patches 1L from day 7 through day 21. This behavior can be explained by the presence of a chitosan hydrophilic layer improving patch hydration in the first incubation days. Indeed, the chitosan layer dissolves in about 48–72 h leaving a hydrated patch 3L, whose water uptake behavior is similar to that of patch 1L.

All samples tested in in vitro simulated esophageal reflux pathologic condition (Figure 4b) show the same water uptake trend of the corresponding samples incubated under simulated physiologic conditions (Figure 4a) with the exception of significantly higher water uptake values at day 21 for patches 3L and 1L. Water uptake percentages in 28 d range are between 196.0% and 276.0%.

3.3.2. Mass Loss Determination

Patch degradation, in terms of polymer mass remaining percentages along the in vitro degradation test at pH 7.4, shows that patches 1L and 2L have similar behavior keeping 98% of their mass during the first 7 d of incubation; mass remaining was reduced to 93%–94% after 28 d incubation. Patch 3L mass loss was faster and higher: mass remaining was 88% after 7 d incubation and further negligible mass reduction was measured between day 7 and day 21, reaching 87% of the mass remaining after 28 d of incubation (Figure 5a). The mass remaining profiles of patches 1L and 2L are always superimposable, while the one of patches 3L is superimposable only starting from day 21. The slight increased degradation shown by patches 3L in the first 14 d can be due to the presence of the chitosan hydrophilic layer that fastens water penetration into the patches; the result being consistent with water uptake results. Different behavior is verified in simulated pathologic conditions: degradation profiles of patches 1L and 2L are superimposable reaching 87% of mass remaining after 28 d (Figure 5b), patches 3L lose 20% of their mass in 28 d with degradation profile significantly different from those of patches 1L and 2L (Figure 5b).

The pH test along with the in vitro degradation study under pathologic conditions showed that addition of 500 μL 0.1 M HCl to 7 mL of PBS solution led to immediate pH lowering to 2.5 (data not reported). However, in 15 min the pH of the incubating PBS medium raised to 7.28, demonstrating its buffering ability and exposing patches to acidic pH only for short times. The behavior should be further in vivo investigated.

3.3.3. Polymer Molecular Weight Determination

Results of GPC analysis are reported in Figure 6, showing variation in M_w, M_n, and PI, upon incubation in PBS at pH 7.4 (physiologic conditions) and in PBS supplemented with 0.1 N HCL (pathologic conditions).

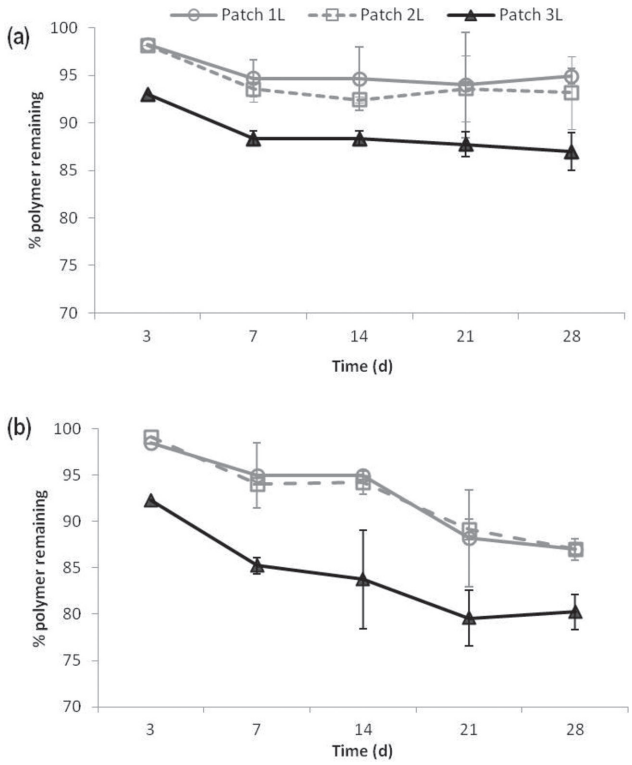

Figure 5. Evaluation of patch polymer mass remaining after incubation in a) in vitro simulated physiologic conditions, PBS pH 7.4; b) in vitro simulated pathologic conditions, PBS pH 7.4 supplemented with HCl 0.1 N.

In simulated physiologic conditions (Figure 6a), the polymer M_w and M_n values decrease with incubation time and the variation profiles of tested patches are superimposable after the first 7 d of incubation. However, the polymer M_w and M_n of patches 1L and 3L seem to increase in the first 7 and 14 d of incubation, respectively. This evidence can be due to loss of oligomers generated from polymer degradation, and their dissolution into incubation medium. This is more evident for patches 1L, in agreement with the faster patch hydration due to their single layer composition and lower thickness. Patches 2L, made of two layers of partially hydrophilic copolymers, show lesser extent of M_w decrease in the first 3 d of incubation (from 181 930 Da at time zero to 160 812 Da at third day), due to their greater thickness. At any rate, all the M_w values change in the first 3 d of incubation range within ±10%.

Eventually, M_n reduction percentages of patches 1L, 2L, and 3L at day 28 are 54.8%, 48.80%, and 69.31%, respectively, always greater than M_w reduction percentage values, namely 41.89%, 25.90%, and 26.10%, respectively. The behavior is confirmed by increasing PI, and it is congruent with an unzipping degradation mechanism of PLA and derivative polymers, as already suggested and discussed by the authors.[19]

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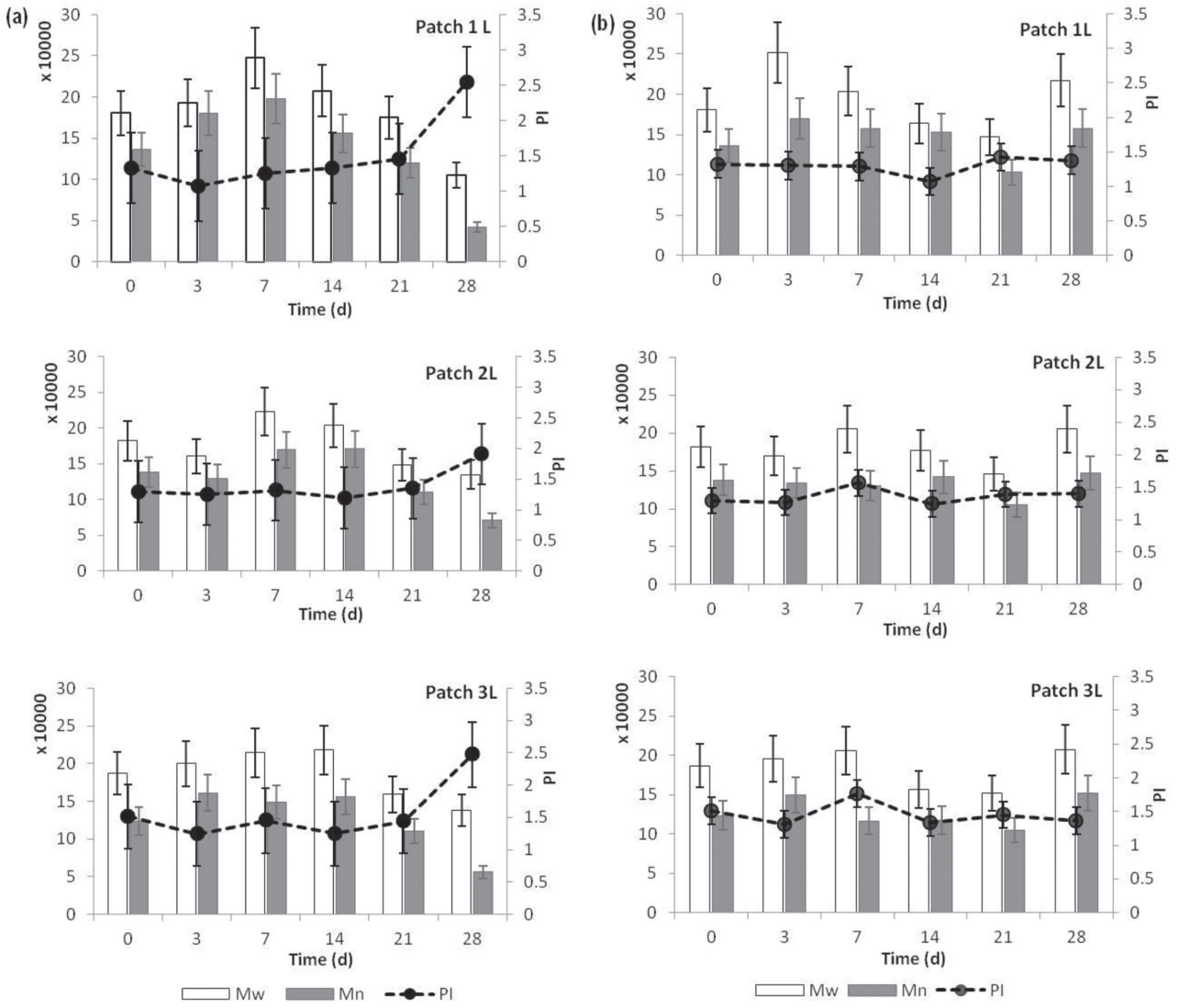

Figure 6. Variations of polymer molecular weight (M_w), molecular number (M_n), and polydispersity index (PI) upon patches in vitro degradation study in a) simulated physiologic conditions (PBS, 0.01 M, pH 7.4) and b) simulated esophageal reflux conditions (PBS 0.01 M supplemented with HCl 0.1 M, 37 °C).

Patches incubated in simulated pathologic conditions underwent periodic pH shifts between 7.4 and 2.5, and show significantly different polymer M_w and M_n variations (Figure 6b) with respect to the patches incubated under simulated physiologic conditions. The M_w values move from a slight increase at day 3 of incubation for patches 1L and at day 7 for patches 2L and 3L to a slight decrease in the following incubation times, in such a way that, at the end of the 28 d incubation, no significant M_w changes can be detected for all the polymer patches. The low polydispersity index values indicate that both the polymer degradation mechanisms, scission and unzipping, contribute in almost equal ratios.

Apparently, the M_w and M_n reductions after 28 d of incubation in simulated pathologic conditions are smaller and always not significant, if compared to the values obtained in PBS, pH 7.4; e.g., the M_w reduction percentages of patches 1L, 2L, and 3L under pH changed conditions are 0%, 12.85%, 0%, compared to 54.8%, 48.80%, and 69.31% at pH 7.4. However, the polymer mass loss values are significantly higher when the patches are treated under pH changing conditions. Therefore, comparing the results of mass loss with the M_w values via GPC analysis, it can be concluded that in acidic condition, polymer degradation products are more soluble and they are lost in the incubation medium.

3.3.4. Patch Hydrophilicity (Wettability)

The values of static contact angle are reported in Table 1. As known from the literature, about 0° contact angle values

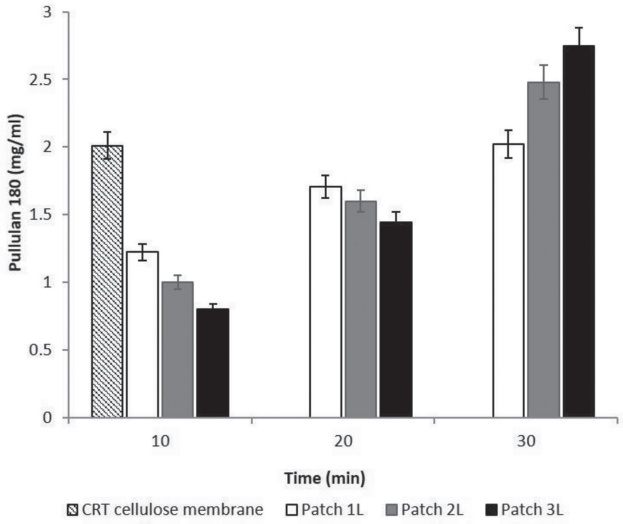

Figure 7. Results of patches' permeability evaluation toward pullulan 180 Da M_w; cellulose membrane represents the positive control.

correspond to material hydrophilicity, values between 0° and 90° correspond to partial material wettability, values higher than 90° but lower than 180° correspond to scarce wettability, and contact angle values of 180° correspond to hydrophobicity. As expected, the presence of chitosan layer (patches 3L) made the patches hydrophilic ($\theta = 0°$) while patches made of PLC are only partially wettable ($\theta = 41.63°$ and $\theta = 42.02°$). No significant difference, in terms of wettability, is highlighted among patches 2L and 1L.

3.3.5. Patch Porosity Evaluation

Results of patches porosity, as determined by the liquid displacement method, are reported in Table 1. The values are always about 80% and higher. No significant differences are attributable to patch composition.

3.3.6. In Vitro Permeability Study

Permeability study was aimed to evaluate patch permeability toward pullulan (180 Da) as a model of small nutrient molecules such as glucose. The results are reported in Figure 7 showing that pullulan gradually diffuses through patch matrices. The diffusion is completed in 30 min for all patches. The different permeability values highlighted for patches 1L, 2L, and 3L in the first 20 min can be attributed to the different composition and thickness of patches.

3.3.7. Mechanical Properties

The results of mechanical tests are reported in Table 2 and show patch composition significantly to affect mechanical performances. Results are expressed as a mean value within each group and a variation coefficient, computed as the ratio between the standard deviation and mean value. Patches 1L achieve the lowest elastic modulus, showing the highest maximum elongation, but the lowest maximum stress before breaking. Elastic modulus gives information on patch resistance and stiffness; thus, it is of utmost importance in a patch designed to mimik esophagus tissue. Elastic modulus is greater for patches 2L compared to 1L, consistently with the higher amount of polycaprolacton in patch 2L. Indeed, it is known from the literature that addition of PCL to PLA increases stiffness.[20] Chitosan reduces maximum elongation of patches since its elastic properties are limited by chain crosslinking performed to make the polymer stiff and to stabilize chitosan layer adhesion on synthetic ones. Patches 3L show maximum stress comparable to patches 1 L, but with significantly lower maximum elongation. It must be noticed that patches 2L and 3L show a high data variability; this could be due to the sample geometry limits, especially not in terms of homogeneous thickness.

Table 2. Mechanical properties of patches.

Patch code	σ_{max} [MPa]	ε_{max} [%]	E [MPa]
1L	0.129 ± 0.406	136.6 ± 0.4	0.395 ± 0.197
2L	0.928 ± 0.537	45.9 ± 54.2	5.089 ± 0.513
3L	0.153 ± 0.712	39.1 ± 69.1	0.832 ± 0.561

3.3.8. Ex Vivo Preliminary Surgical Suture Test

The ex vivo test gives preliminary information on (i) patch suitability to suture operation and (ii) resistance of the patch implanted into esophagus tissue. On the first purpose, commonly used surgical monofilament suture based on glyconate was selected on the basis of its properties, such as degradation profile suitable for soft tissues, filament attitude to pass thorough the patch, and tissue where the patch was fixed, excellent knot resistance and safety.

Figure 8 shows the ex vivo suture test procedure step by step. The test was successfully performed on patch 2L.

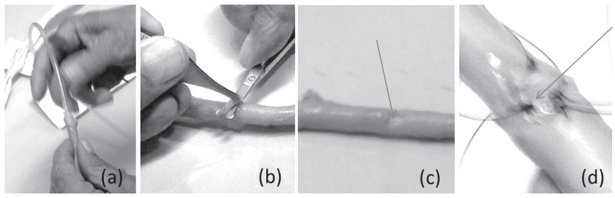

Figure 8. Ex vivo test of patch 2L on isolated pig esophagus: a) intubation, b) surgical incision of muscularis externa, and mucosa; c) insertion of patch 2L; and d) suture using midterm absorbable synthetic monofilament suture based on glyconate (HR 26, ½ c, 26 mm Taper).

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The patch and the suture resulted to be stable when the patch implanted esophagus underwent perfusion of with PBS solution. The test successfully simulated the step of fluids reinstatement usually performed 5 d after surgery.

4. Conclusions

The investigation allowed us to confirm multilayered patch fabrication feasibility. The temperature-induced precipitation method resulted to be a suitable preparation method in order to obtain stable multilayered patches made of polylactide-*co*-polycaprolacton copolymers with different composition, and also combining different types of polymers, such as a polysaccharide (chitosan) with polyesters (polylactide-*co*-polycaprolacton). The preparation method permits us to modulate the type of polymer layer composition and polymer layer structure (pore size) by simple steps such as selection of copolymer type and its M_w and selection of starting polymer solution concentration.

Preliminary in vitro investigation on functional patch properties demonstrated 1L and 2L patches are stable with about 7% mass loss after 28 d incubation in in vitro simulated physiologic conditions, and 12% mass loss in in vitro simulated pathologic acidic reflux. The presence of a chitosan layer (patches 3L) decreases patch stability, meanwhile improving cell proliferation.

Polymer degradation profile is accelerated in in vitro simulated pathologic conditions with the formation of soluble oligomers.

As long as the mechanical properties are concerned, taking into account the fact that esophagus physiologically withstands pressures of about 3–5 kPa, and its elongation is about 20%;[17,24] patches 2L and 3L comply with physiologic esophageal pressure. These results are confirmed by patch 2L positive preliminary results of the ex vivo suture test.

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