Нанофибриллярный каркас устойчив к действию желчи и мочи: эксперимент на свиньях

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Функция каркаса на основе биоматериалов состоит в том, чтобы заменить анатомические и функциональные потери, а затем восстановить и поддерживать нормальную функцию поврежденной или пораженной ткани.
Материал и методы. В наших экспериментах мы использовали каркас PHEA-PLA+PCL у 2 самок свиней и оценивали его устойчивость к желчи и моче.
Результаты. Обе свиньи выжили после хирургических вмешательств. По данным электронной микроскопии, через 1 мес волокна не изменились по форме и размерам. В ходе проведения микроскопической оценки внутри каркаса были выявлены клетки и факторы экстрацеллюлярного матрикса.
Заключение. Плоские и трубчатые каркасы были колонизированы как клеточными, так и внеклеточными матричными элементами. Исследование, проведенное на свинье, показало, что этот тип материала может противостоять действию желчи и мочи. Этот материал может быть предложен в качестве заменителя ткани.

Ключевые слова:
повреждение желчных протоков, повреждение мочевого пузыря, восстановление желчного пузыря, 3D-матрица, свиньи, PHEA-PLA+PCL

Финансирование. Исследование не имело спонсорской поддержки.
Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.

Для цитирования: Фаззотта С., Бушеми С., Палумбо В.Д., Дамиано Дж., Гераччи Дж., Личиарди М., Палумбо Ф.С., Фиорика К., Кудиа Б.М., Дженова П., Ло Монте А.И. Нанофибриллярный каркас устойчив к действию желчи и мочи: эксперимент на свиньях // Клиническая и экспериментальная хирургия. Журнал имени академика Б.В. Петровского. 2020. Т. 8, № 4. С. 29–34. DOI: https://doi.org/10.33029/2308-1198-2020-8-4-29-34
Статья поступила в редакцию 01.11.2020. **Принята в печать** 18.11.2020.

Nanofibrillar scaffold resists to bile and urine action: experiences in pigs

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Biomaterial-based-scaffolds' functions are to replace anatomical and functional features loss of an injured tissue. They can replace native tissue after their reabsorption.
Material and methods. In our experimental procedures we utilized the PHEA-PLA+PCL scaffold in 2 female pigs to assess its resistance to bile and urine.
Results. Both pigs survived to surgical procedures. After a month fibres appeared unchanged in term of form and dimension at electronic microscopy. Cells and ECM factors were founded inside the scaffold in a microscopical evaluation.
Conclusion. Planar and tubular scaffolds were colonized by cells and extracellular matrix elements. The study conducted on pig suggested that this type of material can resist to the action of bile and urine. This material could be proposed as a tissue substitute.

Keywords:
bile duct injury, bladder injury, gallbladder repair, 3D matrix, porcine models, PHEA-PLA+PCL

Funding. The study had no sponsor support.
Conflict of interests. The authors declare that they have no conflict of interests.

For citation: Fazzotta S., Buscemi S., Palumbo V.D., Damiano G., Geraci G., Licciardi M., Palumbo F.S., Fiorica C., Cudia B.M., Genova P., Lo Monte A.I. Nanofibrillar scaffold resists to bile and urine action: experiences in pigs. Clinical and Experimental Surgery. Petrovsky Journal. 2020; 8 (4): 29–34. DOI: https://doi.org/10.33029/2308-1198-2020-8-4-29-34
Received 01.11.2020. **Accepted** 18.11.2020.

Tissue engineering (TE) is an interdisciplinary filed of science that aims to meet the medical needs by recreating tissues and organs or improving their original biological functions. It's based on cell biology, materials science and engineering. Biomaterial-based scaffolds have the task to replace anatomical and functional features, lost in injured or diseased tissues, and to restore and maintain normal functions [1, 2]. Scaffolds can be obtained by natural or synthetic materials. In particular, polycaprolactone (PCL) is FDA-approved linear polyester, biodegradable, relatively cheap. Its properties makes it fit for different biomedical application, such as medical implants, medical devices, wound dressing materials, drug delivery systems, and tissue engineering scaffolds (bone, cardiovascular, and liver substitutes) [3–5]. Highly porous networks for cellular support miming the natural extra-cellular matrix (ECM) can be produced from PCL through different techniques like the electrospinning. This technique allows to produce fibrillar structures characterized by fiber diameters ranging from nanometers to microns [6]. Electrospun nanofibrous scaffolds show a high surface/volume ratio, tunable porosity, and flexibility in order to suit over a wide variety of sizes and shapes [7–9]. Moreover, the biomaterial composition can be modulated in features and functionality. In particular, in order to improve chemical-physical properties and optimize cell growth, the scaffold can be prepared starting by blends of PCL with a variety of natural polymers (chitosan, silk fibroin, collagen, elastin) or synthetic polymers (PLLA, PLGA, polystyrene) [7, 10]. For example, Fadaie and colleagues prepared a bionanocomposite electrospun scaffold of PCL/chitosan with improved mechanical properties, wettability and cell compatibility [11]. In addition, this blend scaffold, employed as vascular engineered vascular graft, showed ECM deposition (especially elastin and collagen) and, above all, endothelialisation [12]. PCL/starch nanofibrillar mat, prepared by elettrospinnig, was employed in haemostatic applications, due to its ability to promote blood clotting formation in 156 s [13]. Different PCL/PLA blends have been assessed to optimize properties of electrospun scaffold in term of morphology, in vitro degradation, mechanical behaviour and cell compatibility [14].

Taking into account the advantages to use hybrid PCL scaffolds, this study aimed to assess the resistance to bile and urine of an electrospun blend of PCL, α,β-poly(N-2-hydroxyethyl-$_D$-$_L$)-aspartamide (PHEA) and polylactic acid (PLA) (named PHEA-PLA). Tubular PCL/PHEA-PLA has been already employed to create arteriovenous fistulas (AFV) in pigs [15, 16]. In this case, the material showed great elasticity, probably thanks to regularity and dimensional uniformity of fibres, and good mechanical features. A tubular PCL/PHEA-PLA scaffold was also tested to create a biliary-digestive anastomosis, on rabbits. Three months after implant, the fibrillar structure did not show any sign of lithic digestion by bile and a new epithelial tissue appeared on scaffold surface, suggesting potential reparative features of the material [17]. The aim of this work is to evaluate the biological stability of planar and tubular PCL/PHEA-PLA matrices in different biological conditions (bile and urine).

Material and methods

in our experimental procedures we utilized the PHEA-PLA+PCL scaffold in two female pigs to assess its resistance to bile and urine.

α,β-Poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified according to the reported procedures [18]. Spectroscopic data (FT-IR and ^{1}H-NMR) were in agreement with previous results [19]. PHEA weight-average molecular weight was determined by size exclusion chromatography and was equal to 38 kDa (M_W/M_N = 1.78).

In the first step, PLA N-hydroxysuccinimide (NHS) derivative was prepared and purified [20–22]. In the second step, PHEA and PLA–NHS were solubilised using an opportune amount of DEA as catalyst. The reaction between the hydroxyl groups of PHEA and the activated carboxylic groups of PLA-NHS was carried out for 24 hours [20]. After work-up process, obtained copolymer was characterized by ^{1}H NMR, and the value of molar derivatisation degree in PLA was in agreement with previous results. Proton nuclear magnetic resonance (^{1}H-NMR) was performed using a Brucker AC-300 instrument of 300 MHz.

For electrospinning process, a programmable syringe pump (Aitecs PLUS SEP-21S) and a high volt-

age power supply (Spellman CZE 1000 R) were used. Electrospinning process was carried out horizontally with an accelerating voltage of 20–25 kV and a constant polymeric solution rate of 1 mL/h obtained through a programmable syringe pump. Scaffold with different shapes were obtained by changing the rotative collector of the electrospinning apparatus. In particular, we used a 2 cm^2 planar scaffold and a tubular scaffold with 6 mm diameter and 4 cm length. In both scaffolds the thickness was 0,6 mm. All scaffolds obtained were washed several time with bidistilled water, then dried under vacuum and sterilized.

All animal experimental procedures were in compliance with the Animal Research: Reporting of In Vivo Experiments guidelines. The study designed was initially submitted for approval to the Animal Welfare Body (O.P.B.A.) of the "A.Mirri" Institute (according to Italian Legislative Decree No. 26 of 4 March 2014 transposing Directive 2010/63/EU on the protection of animals used for scientific purposes) that expressed a favourable opinion.

We used two porcine models, female of 20 kg and 4 months of age. During a procedure under general anaesthesia (premedication: Zolazepam + Tiletamine 6.3 mg/Kg + Xylazine 2.3 mg/Kg – induction: Propofol 0.5 mg/Kg – Maintenance: Isoflurane + Pancuronium 0.07 mg/Kg) [23]. In all animals a central venous catheter was placed to be used also for post-operative blood tests [24]. With the animal in a prone position and the 4 limbs secured to the operating table, the region of interest was shaved and the operating field was disinfected with povidone iodine 10%. After the surgical procedure, all the pigs received post-operative antibiotic treatment with oxytetracycline (20 mg/Kg a day for 3 days). In post-operative period, the animals were monitored clinically and blood samples were drawn daily for the first seven days and then once a week to control the inflammation and cholestasis parameters. A follow-up ultrasound was performed at post-op day 7. The sacrifice of the animals was scheduled at one month, except in the case of a worsening of clinical conditions requiring a change of the scheduled deadlines.

Experimental test 1 (t1): resistance test *in vivo* to bile

We assessed the ability of our scaffold to replace a portion of the gallbladder wall. We already tested the biocompatibility of the scaffold and its resistance against bile in other surgical procedures in rat (17) and rabbit (15). In this surgical procedure we want to simulate a bigger damage as close as possible to real surgical injuries and to value how the scaffold works. After a median longitudinal incision we accessed the hepato-duodenal region and isolated the gallbladder. Once the gallbladder was identified and isolated, we clamped the gallbladder fundus and made an incision of about 2 cm^2. We sutured a planar scaffold made of PHEA-PLA+PCL on the gallbladder by means of interrupted 6-0 Prolene stitches.

Experimental test 2 (t2): resistance test *in vivo* to urine

We assessed the ability of our scaffold to resist against pig's urine. After a median longitudinal incision we accessed the pelvic region and isolated the bladder. We performed an approx. 5 cm incision on the bottom of the bladder. Inside the bladder, about 1 cm from the section margin, we have sutured the scaffold to the inner wall of the bladder with continuous suture in non-absorbable material 5-0. After that we closed the surgical bladder incision.

After the explant of gallbladder and bladder, we evaluated scaffold morphology and cells adhesion by electron microscopy (SEM) using a scanning electron microscope (ESEM Quanta FEI) on sample cross section. After removal, all samples were washed with phosphate buffer and were treated with a 4% (v/v) formaldehyde solution. Samples were dehydrated with ethanol (30%, 50%, 70%, 90% and pure ethanol), then were treated with hexamethyldisilazane and freeze-dried. Histopathological analysis on tissue samples taken from areas of contact with gallbladder mat were performed by the coloration with hematoxylin-eosin. In particular, 5 μm thick sections were cut and were analysed using an optical digital microscope.

Results

In post-operative period, both the animals were monitored clinically and blood samples were drawn daily for the first seven days and then once a week to control the inflammation, cholestasis parameters in the first pig and kidney function in the second one. According to our schedule, a follow-up ultrasound was performed at post-op day 7. There wasn't leakage or peritonitis signs in both pigs. After a month we performed cholecystectomy in the first pig and a partial bladder resection in the second one.

In the first case (t1), a microscopic histological examination of the grafted section was done to assess if there were any response of the host and the degree of tissue regeneration. After fixation and reduction, the samples were processed as done routinely for paraffin embedding. Serial sections (5 μm) of each sample were stained with hematoxylin-eosin (H&E). An immunohistochemical exam was done to search for specific endothelial markers by using Ig anti-CD31. In both cases the samples were embedded in formalin, treated with 30% sucrose, included in OCT (antibodies) and stored at -80 °C. Part of the samples were cut into slices with

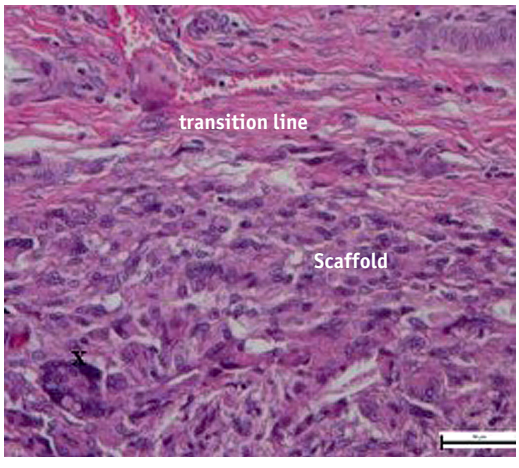

Fig. 1. Hystological microscopy on tissue sample after 30 days of implantation (20x)

a thickness of 8 mm in a cryostat for subsequent immunofluorescence and analysis under confocal microscopy.

Histological examination of tissue samples recovered from areas of contact with gallbladder mat showed a mucosal tissue on the scaffold. In addition, a correct stratification of epithelial cells was evident. The boundary line between electrospun matrix and mucosal tissue formed was visible (fig. 1). Under this transition zone, there was a monocyte infiltration with the presence of giant cells and macrophages.

Micrograph obtained revealed an interpenetration of cells and extracellular matrix components (fig. 2A). In addition, fibres appeared unchanged in term of form and dimension and, above all, the absence of fusion among each other was demonstrated (fig. 2B).

In the second case (t2) a tubular PCL/PHEA-PLA scaffold was sutured into pig's bladder. After 30 days the scaffold was explanted and SEM analysis was conducted. Micrograph acquired showed the optimal cell colonisation of electrospun matrix. The native structure of scaffold was maintained over time and the suture thread was detectable after explant (fig. 3). In addition, the accumulation of different type of insoluble inorganic salt (such as phosphate, oxalated or uronic salts) appeared evident.

All the micrographs obtained confirmed that materials maintained *in vivo* unaltered its fibrillary structures after the contact with bile and urine.

Discussion

Biocompatible PHEA is already used to prepare drug carriers systems and as a starting materials for different biomedical and pharmaceutical applications [22, 25–27]. PHEA-PLA derivative was extensively employed to design biocompatible scaffolds thank its easy chemical processability and optimal biocompatibility toward different cell lines [20, 22, 28]. In particular, nanofibrillar scaffolds starting by PHEA-PLA can be obtained through electrospinning technique how reported in a previous work [29]. This polymeric derivative of PHEA can be mixed with PCL to prepare different type of electrospun matrixes, following a procedure reported elsewhere [15]. Taking into account the potential versatility of these scaffolds, the aim of this work was to study the biological stability of blended PCL/PHEA-PLA matrixes *in vivo* animal models.

The scaffold used was characterized by a large surface area to volume ratio, superior mechanical performance like stiffness and tensile strength compared with any other known form of the material [30]. Micrographs obtained in the two experimental trails confirmed that materials maintained unaltered its fibrillary structures after the contact with extracellular environment. Electrospun matrix seemed to mime the native ECM structure promoting cells adhesion and the infiltration of extracellular matrix components. These features were confirmed by histological analysis.

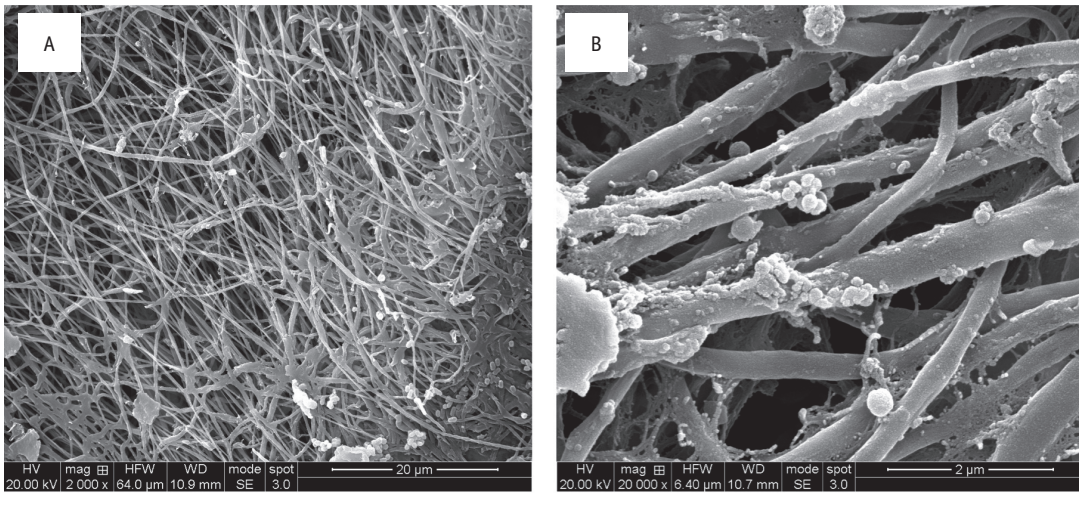

Fig. 2. SEM micrograph of PCL/PHEA-PLA scaffold after 30 days of implantation in pig gallbladder. Scale bar corresponds to 20 µm for A and to 2 µm for C panel

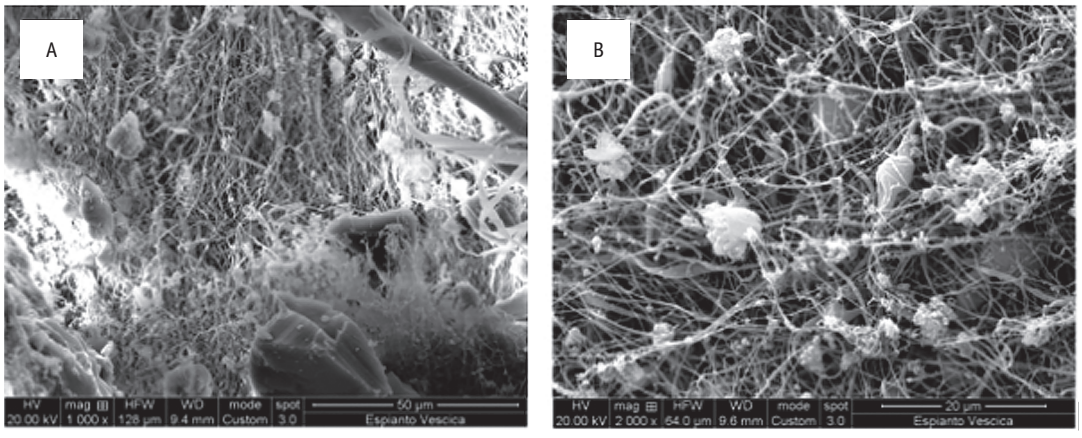

Fig. 3. SEM micrograph of PCL/PHEA-PLA tubular scaffold after 30 days of implantation in pig bladder. Scale bar corresponds to 500 µm for A and to 20 µm for B panel

The opportune cell infiltration and proliferation observed allowed to assess the biocompatibility of material used. However, an inflammatory response of modest degree was observed, which has a key role in the long-term resorption of the material leading potentially to the migration of stem cells.

The excessive chronic inflammatory response can be a limit of this study because we observed the scaffold ongoing only 30 days. This limit would be exceeded by testing the material for a longer time frame. In addition, this type of scaffold is easily functionalized with anti-inflammatory factors, growth factors, antibiotics and anti-coagulants that can be a key-factor in the future use of this device.

Conclusion

Electrospun PCL/PHEA-PLA matrix showed a high biological versatility. Fibers maintained a uniform size and shape over time. Data obtained allowed to suppose that the three-dimensional architecture features promote the interaction between cells and the regeneration of tissue. As a result, both mat and tubular scaffold were colonized by cells and extracellular matrix elements.

In conclusion, these results confirmed the ability of PCL/PHEA-PLA scaffold to promote tissue regeneration thanks to the integration with native component of tissues. Further studies will be necessary to evaluate the long-term effects of the scaffold.

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