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A bilayered hybrid microfibrous PLGA–Acellular matrix scaffold for hollow organ tissue engineering

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ABSTRACT

Various synthetic and natural biomaterials have been used for regeneration of tissues and hollow organs. However, clinical outcome of reconstructive procedures remained challenging due to the lack of appropriate scaffold materials, supporting the needs of various cell types and providing a barrier function required in hollow organs. To address these problems, we have developed a bilayered hybrid scaffold comprising unique traits of polymeric microfibers and naturally derived acellular matrices and tested its potential for hollow organ regeneration in a rat bladder model. Hybrid scaffolds were fabricated by electrospinning of PLGA microfibers directly onto the abluminal surface of a bladder acellular matrix. Stability of this bilayered construct was established using modified spinning technique. The resulting 3-dimensional framework provided good support for growth, attachment and proliferation of primary bladder smooth muscle cells. Histological analysis *in vivo* at 4 and 8 weeks post implantation, revealed regeneration of bladder tissue structures consisting of urothelium, smooth muscle and collagen rich layers infiltrated with host cells and micro vessels. Furthermore, hybrid scaffolds maintained normal bladder capacity, whereas BAM recipients showed a significant distension of the bladder. These results demonstrate that this adaptable hybrid scaffold supports bladder regeneration and holds potential for engineering of bladder and other hollow organs.

1. Introduction

Hollow organs such as urinary bladder, urethra, ureter, oesophagus, intestine, uterus, vagina and blood vessels, generally share a similar functional anatomy consisting of luminal endothelium surrounded by smooth muscle cells [1]. Pathological changes in their functional anatomy caused by trauma, spinal cord injury, malignancy, infection, inflammation and congenital abnormalities may result in dysfunction and profoundly affect the patients' quality of life [2–4]. Many patients, including children, suffer from urinary bladder pathologies requiring reconstructive procedures [5,6]. Gastrointestinal segments are the gold standard for bladder reconstruction, however this procedure is associated with significant long-term complications including urinary tract infection, metabolic abnormalities, stone formation and malignancies [7,8]. Therefore, there is a strong clinical need for alternative biomaterials for these reconstructive procedures [9].

Tissue engineering (TE) approaches provide potential strategies to develop biological substitutes which can restore and maintain normal functional anatomy [10]. In TE, biomaterials play a central role by creating a pre-configured three-dimensional microenvironment that supports attachment, proliferation, migration and differentiation of transplanted or regenerating resident cells and promotes cell–cell interactions, extracellular matrix deposition and new tissue development [11,12]. Moreover, biomaterials should address the needs of various cell types involved in the regeneration process. The ideal biomaterial should possess desired properties concerning biodegradation and display a reliable biological performance without inducing a host immune response. Thus, the selection and design of ideal biomaterial mimicking the native architecture of the target tissue remains critical to generate functionally equivalent substitutes.

Two main classes of materials, i.e., acellular matrices [13,14] and biopolymers [15,16], have been used for the regeneration of hollow organs. Acellular matrices derived from donor tissue (e.g., bladder submucosa) offer excellent trophic support as they are naturally

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[2] Dr. Eberli and Dr. Gobet both serve as last authors. Justification: this work was perfomed by using the knowleage of 2 distinct labs (Dr. Eberli - Biomaterials, Dr. Gobet - Pediatric Urology). I hope you will be wiling to allow us the sharing of last authorship.

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enriched with a wide range of growth factors, thereby stimulating new tissue growth and providing attachment sequences that facilitate cell–material interactions and the maintenance of the functional phenotype [17,18]. In addition, they also prevent permeation of luminal contents into the abdominal cavity [19]. These matrices undergo biodegradation upon implantation and are easily remodelled by biological activity of transplanted or ingrowing cells [19]. However, these dense materials cannot harbour a high density of smooth muscle cells (SMCs) [10]. In contrast, synthetic polymer, i.e., polyglycolic acid (PGA), polylactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA) based fibrous scaffolds can be fabricated reproducibly with the desired and controllable mechanical and degradation properties, porosity as well as topographies providing guidance structures [20,21]. However, the biological competence of synthetic polymer based fibrous scaffolds is limited given their lack of both trophic support and natural barrier function of luminal endothelium.

Both of the above mentioned materials have desirable traits exclusive of each other and none of the scaffolds alone can mediate the functional regeneration of the defective hollow organ.

A functionally equivalent substitute for hollow organ tissue engineering should possess both tropic and topographical functions, while mediating barrier function. The development of such a pre-configured composite biomaterial construct providing the native architecture of the target organ might enhance the clinical outcome of tissue engineered reconstructive procedures. In this line of study, we have previously reported on a composite biomaterial fabricated by suturing a collagen matrix to PGA polymers which we tested in an *ex-situ* mice model. A major drawback of this scaffold is the potential leakage of urine through the holes created by threaded collagen stitches (1). The development of a stable composite material without the use of any external bonding materials may offer several advantages such as easy surgical handling and watertightness.

Therefore, we have developed a bilayered hybrid scaffold by electrospinning of polymer microfibers directly on a bladder acellular matrix and evaluated its biological performance *in vitro* and *in vivo* for the engineering of hollow organs using a rat bladder model. The stability of the bilayered scaffold was established by modifying the spinning technique and tested in aqueous buffer. Morphological features of the hybrid scaffold were characterized by scanning electron microscopy (SEM). SMCs, which are representing the main cell types of hollow organs, were seeded into microfibrous layers of hybrid scaffold and analysed *in vitro* for cell attachment and proliferation. Furthermore, the hybrid scaffolds were implanted into a rat bladder and the biological performance was evaluated at 4 and 8 weeks post-operatively by analysing anatomical and functional outcomes.

2. Materials and methods

2.1. Preparation of bladder acellular matrix

Urinary bladders from adult pigs were subjected to a multi-step detergent washing procedure based on an established protocol [1]. Briefly, the muscle layer from porcine bladder tissue was micro-dissected and removed leaving the lamina propria, also referred to as the bladder submucosa. All subsequent washes were performed on an elliptical shaker at 4 °C. The bladder submucosa was treated with distilled water for 2 days, changing the water twice a day to lyse the cells in the tissue. Subsequently, the tissue was treated with Trypsin 0.05% for 1 h. After blocking with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) overnight the tissues were treated with 1% Triton X100 and 0.1% ammonium hydroxide for 7 days, changing the detergent daily. Thereafter, the collagen matrix was rinsed with phosphate buffered saline (PBS) for two days. Representative samples of freshly processed matrices were analysed by haematoxylin eosin staining and DNA quantification by spectrophotometry to confirm the removal of all cellular constituents. The detergent wash was repeated if necessary to ensure complete extraction. The bladder acellular matrix (BAM) was stored in hydrated state at 4 °C.

2.2. PLGA-BAM composite scaffold production

The microfibrous scaffold was fabricated on the abluminal side of the BAM by electrospinning. An 8% PLGA (RG 85:15, Boehringer Ingelheim, Germany) polymer solution (wt/wt) in chloroform (Sigma–Aldrich, Switzerland) was loaded into 2 ml syringe (B. Braun Melsungen AG, Germany) fitted with a spinning head consisting of a blunt end stainless needle (1 mm inner diameter and 0.3 mm wall thickness, Angst & Pfister AG, Zurich, Switzerland). The polymer solution supply was adjusted to a flow rate of 1.6 ml/h using a programmable syringe pump (AL1000 Aladdin, World Precision Instruments, Germany) and horizontal spinning was performed with an accelerating voltage of 12 kV supplied by a high voltage power supply (Glassman High Voltage Inc., High Bridge, NJ, USA). The BAM was pre-fixed on a cylindrical collector (length: 100 mm, diameter:80 mm, wall thickness: 5 mm) by placing the luminal side of the BAM onto an aluminium foil-covered mandrel. The collector speed was set to 50 rpm and positioned at a distance of 20 cm from the tip of the needle. Fibers were electrospun directly onto the abluminal side of the BAM at room temperature (RT) and pressure. Three different spinning procedures were employed in order to define the optimal method to produce a stable hybrid scaffold. Those include continuous spinning of PLGA microfibers on dry BAM, continuous spinning of PLGA microfibers on wet BAM and layer-by-layer spinning of PLGA fibers on continuously rehydrated BAM. In contrast to the spinning on wet BAM, the rehydrated BAM was maintained hydrated throughout the spinning process by 20 min re-hydration with 100 mM PBS every hour during the layer-by-layer spinning procedure.

2.3. Stability of composite scaffold in aqueous buffer

The stable attachment of electrospun microfibers to the abluminal surface of the acellular matrix under aqueous conditions is an important feature of our hybrid scaffold allowing pre-implantation cell seeding and easy surgical handling. To test the attachment of electrospun fibers on the surface of acellular matrix and its stability, BAM-PLGA composite scaffolds were fabricated using a 5-(amino-acetamido) fluorescein (Invitrogen, Switzeralnd) (1 mg/ml) blended PLGA polymer solution. The 3 different techniques mentioned above were used for the production of electrospun scaffolds and full thickness scaffolds were resized into squares of 1 cm^2. Hybrid scaffold samples were incubated in PBS buffer at RT under mild shaking (50 rpm) and observed every 24 h over 7 days using an epifluorescence microscope (Zeiss Axiovert 200M, Germany) to verify whether the fluorescent fibrous PLGA layer was attached to the BAM, fully detached or if individual fibers remained attached on the BAM surface. Three parallel experiments were performed and 10 samples were taken for each condition in all the experiment. Hybrid scaffolds with partly or fully detached microfibrous layer were considered unstable. Scaffold stability was measured by using following method. Percent stability = Number of intact scaffolds x 100/Total number of scaffolds.

2.4. Scanning electron microscopy

Scaffold morphology, fiber diameter, interfiber space and scaffold thickness were analysed by SEM. To this effect, scaffolds were dried overnight in a vacuum chamber and sputter coated with gold using a Hummer V sputtering system (Technics Inc., Baltimore, MD) at 50 mA to obtain a 10 nm coating. Samples were analysed using a Zeiss SUPRA 50 VP (Zeiss, Cambridge, UK) SEM operated at an accelerating voltage of 6 kV at various magnifications between 100× and 20 k ×. ImageJ 1.31v software (National Institutes of Health, Bethesda, USA) was used for the quantitative analysis of the fibrous scaffolds and randomly selected fibers ($n = 20$) from different locations were chosen for size measurement.

2.5. Porosity measurement

Electrospun PLGA microfibrous scaffolds were dried in a vacuum oven and resized into squares of 1 cm^2. The apparent density of electrospun PLGA polymer scaffolds were determined gravimetrically using the weight measurements of the precisely cut scaffolds of defined area and thickness. The scaffold dimensions were measured from SEM micrographs of the electrospun PLGA scaffolds. Apparent density and porosity were calculated using the following equations as described previously [22,23].

$$\mathrm{PLGA}_{\text{apparent density}}\left[\mathrm{g\cdot cm^{-3}}\right] = \frac{10\cdot \mathrm{PLGA}_{\mathrm{mass}}[\mathrm{mg}]}{\mathrm{PLGA}_{\mathrm{thickness}}[\mu\mathrm{m}] \times \mathrm{PLGA}_{\mathrm{area}}\left[\mathrm{cm^2}\right]}$$

$$\mathrm{PLGA}_{\mathrm{porosity}}[\%] = \frac{\mathrm{PLGA}_{\text{bulk denisty}}\left[\mathrm{g\cdot cm^{-3}}\right] - \mathrm{PLGA}_{\text{apparent density}}\left[\mathrm{g\cdot cm^{-3}}\right]}{\mathrm{PLGA}_{\text{bulk denisty}}\left[\mathrm{g\cdot cm^{-3}}\right]} \times 100$$

note: Bulk density of the PLGA is 1.15 g/cm^3 [23]

2.6. Smooth muscle cell isolation, proliferation and seeding

Primary SMCs were harvested from rat bladders. After removal of the urothelium by mechanical scraping the muscle layer was cut in small pieces of 1 × 1 mm