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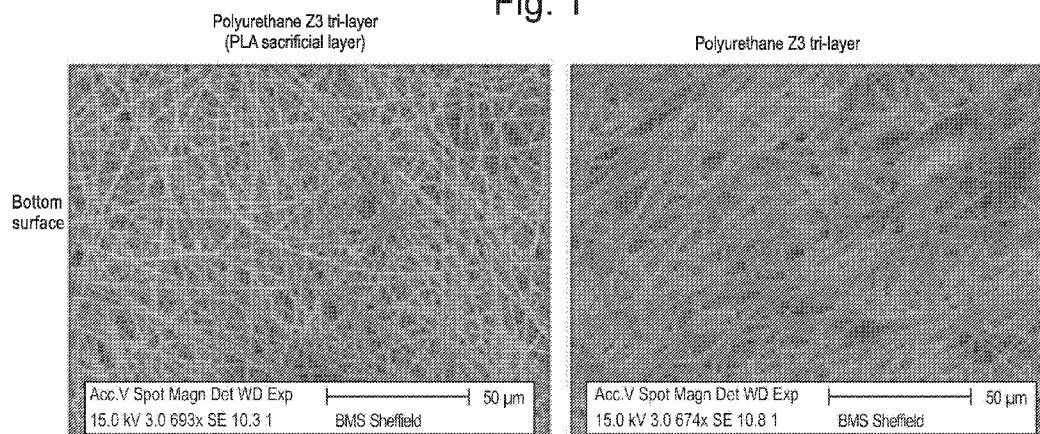
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Fig. 1



(57) Abstract: The present invention relates to scaffolds for the treatment of hernia and methods of making such scaffolds.

Scaffold

The present invention relates to scaffolds for the treatment of hernia and methods of making such scaffolds.

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BACKGROUND

Polypropylene (PP) mesh has been used for the last 50 years being the gold standard material to treat hernia. While the successful rates for curing the symptoms are high, complications have been associated with these meshes, such as contraction and pain. There has been a learning process during that time about the critical characteristics of the mesh which influence the integration and performance of the mesh after being implanted into the native tissues (Zhu LM, Schuster P, Klinge U1. Mesh implants: An overview of crucial mesh parameters. World J Gastrointest Surg. 2015 Oct 27;7(10):226-36). New generations of mesh materials have been developed with special attention paid to the pore characteristics, particularly the collapse of pores under strain, the amount of mesh material and their mechanical properties.

PP mesh is a non-degradable synthetic knitted material with a macroporous structure creating macrofilaments. In 1997, Amid (Classification of biomaterials and their related complications in abdominal wall hernia surgery. Hernia. 1997;1:15–21) identified mesh porosity as the decisive factor for risk of infection, reduced by having pores larger than 75 μm . However, the porosity is a major determinant for the tissue reaction and risk of scar entrapment (Klinge U, Klosterhalfen B. Modified classification of surgical meshes for hernia repair based on the analyses of 1,000 explanted meshes. Hernia. 2012;16:251–258). Alternatively, the “effectiveness porosity” depends on the raw material selected. It has been showed that PP meshes with larger pores exhibit less inflammatory infiltrate, connective tissue, fistula formation, calcification and bridging (i.e., the pores are filled by scar tissue) (Klosterhalfen B, Klinge U. Retrieval study at 623 human mesh explants made of polypropylene--impact of mesh class and indication for mesh removal on tissue reaction. J Biomed Mater Res B Appl Biomater. 2013;101:1393–1399; Greca FH, Souza-Filho ZA, Giovanini A, Rubin MR, Kuenzer RF, Reese FB, Araujo LM. The influence of porosity on the integration histology of two polypropylene meshes for the treatment of abdominal wall defects in dogs. Hernia. 2008;12:45–49). Granuloma normally forms around individual fibres as a normal foreign body reaction, but bridging describes the process whereby individual granulomas become confluent with each other and encapsulate the entire mesh, which leads to scar plate and reduced flexibility increasing contraction of the mesh (Jerabek J, Novotny T, Vesely K, Cagas

J, Jedlicka V, Vlcek P, Capov I. Evaluation of three purely polypropylene meshes of different pore sizes in an onlay position in a New Zealand white rabbit model. *Hernia*. 2014;18:855–864). Following this rationality new lightweight meshes have been developed with larger pore sizes introducing less foreign material to the body. Although lighter meshes can compromise the mechanical properties, such as the tensile and burst strength; these meshes induce less-pronounced foreign body reaction and decreased inflammatory response, which results in better tissue incorporation, increased prosthesis compliance and decreased patient discomfort and pain (Novitsky YW, Cristiano JA, Harrell AG, Newcomb W, Norton JH, Kercher KW, Heniford BT. Immunohistochemical analysis of host reaction to heavyweight-, reduced-weight, and expanded polytetrafluoroethylene (ePTFE)-based meshes after short- and long-term intraabdominal implantations. *Surg Endosc*. 2008;22:1070–1076). However, Weyhe et al (Weyhe D, Belyaev O, Müller C, Meurer K, Bauer KH, Papapostolou G, Uhl W. Improving outcomes in hernia repair by the use of light meshes--a comparison of different implant constructions based on a critical appraisal of the literature. *World J Surg*. 2007;31:234–244) considered the textile mesh construction, characterized in terms of pore size and filament structure, as a more important determinant of foreign body reaction after implantation than absolute material reduction.

It has been shown that the tensile strength of heavy-weight PP meshes is far too high and these meshes could restrict the abdominal distention (Klosterhalfen B, Junge K, Klinge U. The lightweight and large porous mesh concept for hernia repair. *Expert Rev Med Devices*. 2005;2:103–117) leading to poor functional results with pain. This suggest that softer and stretchier materials may be required; however, grate elongation rates at low loads could lead to recurrence of hernia (Deeken CR, Abdo MS, Frisella MM, Matthews BD. Physicomechanical evaluation of polypropylene, polyester, and polytetrafluoroethylene meshes for inguinal hernia repair. *J Am Coll Surg*. 2011;212:68–79). Eliason et al (Effect of repetitive loading on the mechanical properties of synthetic hernia repair materials. *J Am Coll Surg*. 2011;213:430–435) demonstrated significantly reduced tensile strength and significantly increased permanent elongation after exposure to 1000 cycles of repetitive loading sequences that simulated changes in the intrabdominal pressure. Finally, mechanical properties of several commercial meshes have shown big differences in maximum tensile strength (11.1 ± 6.4 to 100.9 ± 9.4 N/cm), stiffness (0.3 ± 0.1 to 4.6 ± 0.5 N/mm), and elongation at break ($150\% \pm 6\%$ to $340\% \pm 20\%$ based on the load direction (Pott PP, Schwarz ML, Gundling R, Nowak K, Hohenberger P, Roessner ED. Mechanical properties of mesh materials used for hernia repair and soft tissue augmentation. *PLoS One*. 2012;7:e46978).

Polyurethane (PU) materials demonstrate greater elasticity and biocompatibility than PP when used in abdominal hernia repair (Badylak *et al.* 2008). The current inventors also demonstrated that in a 90 days implantation animal model single layer PU and PLA materials maintain mechanical integrity, and do not show a degree of sustained inflammation when compared to 2 commercially available surgical mesh devices. Good tissue integration was observed with both materials (Roman *et al.* 2016). Previously, the inventors have investigated single layer polyurethane meshes as repair material slings for the treatment of stress urinary incontinence (Hillary *et al.* 2016). They investigated a single layer of polyurethane, poly-L-lactic acid (PLA) and combinations as scaffolds, as PLA is known to show good cell attachment and matrix production *in vitro*. PU scaffolds showed good dynamic strain properties, however they showed reduced cellular interaction properties and cell penetration when compared to PLA scaffolds. PLA scaffolds, however, lacked the mechanical properties to be suitable for load bearing. Scaffolds of PU containing PLA were weaker and stiffer than PU or PP, but were significantly better than PU scaffolds alone at supporting cell attachment and growth. However, the properties were still sub-optimal.

Therefore, there remains a need to find scaffolds with suitable properties to act as alternative surgical treatments for hernia repair. The scaffolds previously explored lack the properties to successfully mimic the natural fascia.

BRIEF SUMMARY OF THE DISCLOSURE

The present invention has surprisingly provided a scaffold suitable for the treatment of hernia which has sought to provide the following: viscoelastic properties similar to native tissue fascia, resistance to delamination and penetration of cells through the mesh for successful integration in the patient.

In a first aspect, the present invention provides a scaffold for the treatment of a hernia, wherein the scaffold comprises at least two layers of polyurethane: at least one layer in which the polyurethane fibres are randomly orientated and at least one layer in which the fibres are aligned.

Suitably, the scaffold may comprise at least two layers in which the polyurethane fibres are randomly orientated and wherein the at least two layers are separated by at least one layer in which the fibres are aligned.

Suitably, the scaffold may comprise at least two layers in which the polyurethane fibres are aligned and wherein the alignment of the at least two layers are in different orientations. For example, the alignment of fibres in the second layer may be substantially perpendicular to the alignment of fibres in the first layer. Suitably, the scaffold may comprise at least two layers in which the polyurethane fibres are randomly orientated and are separated by at least one layer in which the fibres are aligned.

Suitably, the polyurethane may have a hardness of between 80A to 90A.

Suitably, the polyurethane may be polycarbonate urethane.

Suitably, the scaffold may have an ultimate tensile strength of between 0.7 and 3 MPa.

Suitably, the scaffold may have a strain at ultimate tensile strength of between 250% and 300%.

Suitably, the layer(s) in which the polyurethane fibres are randomly orientated may comprise pores from the outermost surface of a depth of at least 16µm suitable for penetration by patient's cells from the adjacent layers of skeletal muscle or connective tissue dependent on the site of implantation of the scaffold into the patient's abdominal wall. This material can be implanted on fascia or adjacent to muscle which is the preferred site of implantation for abdominal hernia repair. For the development of the mesh human adipose derived mesenchymal stem cells were used to assess cell penetration *in vitro* and materials were subsequently implanted in sheep abdominal wall to assess cell penetration *in vivo*.

Suitably, the mean pore size in at least one layer in which the polyurethane fibres are randomly orientated may be at least 10µm.

Suitably, at least 20% of the pores on the outer surface of the at least one layer in which the polyurethane fibres are randomly orientated are greater than 18µm.

Suitably, at least 5% of the pores on the outer surface of the at least one layer in which the polyurethane fibres are randomly orientated are greater than 20µm.

The scaffold is a suitable shape for hernia applications (e.g. circular, oval, rectangular or square) and has the appropriate dimension. Suitably, the scaffold may have a width of at least

4 cm and a length of at least 10cm. Suitably, the scaffold may have a depth of about 100 to 500µm.

In another aspect, the present invention provides a method of preparing a scaffold for treatment of a hernia, the method comprising:

- a. Electrospinning a sacrificial layer of poly-lactic acid (PLA) onto a rotating surface;
- b. Electrospinning a layer of polyurethane in which the fibres are spun in random orientations;
- c. Electrospinning a layer polyurethane in which the fibres are spun in an aligned orientation;
- d. Removing the sacrificial layer of PLA to produce the scaffold.

Suitably, the method may further comprise one or more steps between steps c and d selected from:

- e. Electrospinning a further layer polyurethane in which the fibres are spun in aligned longitudinal orientation, wherein the alignment of fibres is in a different orientation to the alignment in step c; and
- f. Electrospinning a further layer in which the fibres are spun in random orientations.

In one embodiment of the method of the invention, the method may comprise the use of at least two separate syringe pumps, one delivering random fibres and one delivering aligned fibres.

Suitably, in the method:

- i. step c may start before step b finishes such that there is an overlap between the layers; and/or
- ii. step e or f may start before step c finishes such that there is an overlap between the layers.

Suitably, the sacrificial layer may be:

- i. applied to a surface rotating from about 200 to 400 rpm;
- ii. produced with a needle to surface distance of from about 15cm to 20cm;
- iii. produced by delivering polymer solutions at a rate of from about 30 µl/min to 40 µl/min per syringe with an accelerating voltage of from about 15kV to 19kV DC.

Suitably, step b may be conducted after any excess solvent from step a. has evaporated.

Suitability, step b may be:

- i. applied to a surface rotating from about 200 to 400 rpm;
- 5 ii. produced with a needle to surface distance of from about 20cm to 25cm;
- iii. produced by delivering polymer solutions at a rate of from about 30 μ l/min to 40 μ l/min per syringe with an accelerating voltage of from about 15kV to 25kV DC.

Suitability, step c may be:

- 10 i. applied to a surface rotating from about 500 to 700 rpm;
- ii. produced with a needle to surface distance of from about 5cm to 10cm;
- iii. produced by delivering polymer solutions at a rate of from about 30 μ l/min to 40 μ l/min per syringe with an accelerating voltage of from about 20kV to 25kV DC.

- 15 In another embodiment of the method of the invention, the method may comprise the use of at least one syringe pumps for the delivering of both random fibres and aligned fibres.

Suitably, in the method: step c may comprise a step-by-step increase and then decrease of the rotating collector speed to avoid delamination of the layers; and/or

- 20 ii. if applicable, step e may comprise a step-by-step increase and then decrease of the rotating collector speed before starting step e and/or f to avoid delamination of the layers.

Suitably, the step a may be:

- 25 i. applied to a surface rotating from about 50 to 100 rpm;
- ii. produced with a needle to surface distance of from about 20cm to 30cm;
- iii. produced by delivering polymer solutions at a rate of from about 30 μ l/min to 100 μ l/min per syringe with an accelerating voltage of from about 13kV to 22kV DC.

- 30 Suitably, step b may be conducted after any excess solvent from step a. has evaporated.

Suitability, step b may be:

- i. applied to a surface rotating from about 50 to 150 rpm;
- ii. produced with a needle to surface distance of from about 20cm to 30cm;
- 35 iii. produced by delivering polymer solutions at a rate of from about 50 μ l/min to 150 μ l/min per syringe with an accelerating voltage of from about 13kV to 22kV DC.

Suitability, step c may be:

- i. applied to a surface rotating from about 250 to 350rpm for about 15 to 20 minutes, followed by a surface rotating from about 550 to 650rpm for about 15 to 20 minutes, followed by a surface rotating from about 1150 to 1250rpm for about 15 to 20 minutes, followed by a surface rotating from about 550 to 650rpm for about 15 to 20 minutes, and followed by a surface rotating from about 250 to 350rpm for about 15 to 20 minutes;
- ii. produced with a needle to surface distance of from about 20cm to 30cm;
- iii. produced by delivering polymer solutions at a rate of from about 50 μ l/min to 150 μ l/min per syringe with an accelerating voltage of from about 13kV to 22kV DC.

Suitably, the method may provide a scaffold of the invention.

In a further aspect, the present invention provides a scaffold produced by a method of the invention.

In another aspect, the present invention provides a scaffold of the invention for use as a medicament.

In a further aspect, the present invention provides a scaffold of the invention for use in the treatment of a hernia.

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of them mean "including but not limited to", and they are not intended to (and do not) exclude other moieties, additives, components, integers or steps.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

Various aspects of the invention are described in further detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the invention are further described hereinafter with reference to the accompanying drawings, in which:

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Figure 1 shows scanning electron microscopy images of the bottom surface of the polyurethane scaffolds. The left hand picture shows the bottom surface of the polyurethane Z3 tri-layer produced using a PLA sacrificial layer. Clearly defined polymer fibres and pores between the fibres are visualised. The right hand picture shows a polyurethane tri-layer produced in a similar manner but without a PLA sacrificial layer. This shows a 'warped' polymer fibre morphology on the bottom surface of the polyurethane tri-layer scaffold without the presence of a sacrificial layer of PLA.

Figure 2 shows the fibre diameter and pore size of polyurethane scaffolds. This shows that while the fibre diameter of the bottom surface of the polyurethane scaffold with a sacrificial layer is smaller than that of the plain polyurethane tri-layer, the pore size is greater for that of PU Z3/PLA.

Figure 3 shows the ability of adipose derived stem cells (in white) to penetrate between the polymer fibres at different depths from the scaffold surface. This shows that cells are better able to penetrate both the top (-TOP) and bottom (-BOTTOM) surfaces of the polyurethane scaffold with a sacrificial layer (PU Z3/PLA) than the plain polyurethane tri-layer scaffold (PU Z3). When a sacrificial layer of PLA is used both the top layer and bottom layer adipose derived stem cells penetrate to at least 16µm whereas when adipose derived stem cells do not penetrate the bottom layer of a tri-layer prepared without the use of a sacrificial layer.

Figure 4 shows steps 1 – 3 of a schematic of the preparation of a polyurethane (PU) scaffold. In step 1, a sacrificial layer of PLA is electrospun, in step 2 a random layer of polyurethane is electrospun, in step 3 there is an overlap wherein the layer of randomly electrospun polyurethane and a layer of polyurethane in which the fibres are aligned are both spun simultaneously.

Figure 5 shows steps 4 – 6 of a schematic of the preparation of a polyurethane (PU) scaffold. In step 4, the electrospinning of the first layer of randomly electrospun polyurethane has ceased and only the second layer aligned is electrospun, in step 5 there is an overlap wherein the second layer of aligned electrospun polyurethane and a third layer of randomly electrospun

polyurethane fibres are both spun simultaneously, in step 6 only the third layer of randomly electrospun polyurethane fibres is spun.

Figure 6 shows mechanical testing data. The stress strain curves obtained are shown for these in Figure 6A. Figure 6B shows the Young's modulus for the materials tested at the end of the first cycle and at the end of the second cycle. Figure 7C shows PU and PP samples tested for a uniaxial tensile test after 7 days cultured into an EBERS bioreactor under dynamic distention (Hillary et al. 2016). Sample 1 and sample 2 are scaffolds in accordance with the present invention, "one layer PU" refers to an electrospun scaffold of only one layer of polyurethane and "polypropylene" refers to the PP mesh which is currently used to treat SUI.

Figure 7 summarises the values obtained for Young's modulus (A), ultimate tensile strength (B) and strain at ultimate tensile strength (C) for all of the materials following a tensile lab test.

Figure 8 shows, using scanning electron microscopy (SEM), random PU fibres which have been spun directly onto the collector using the same solution described for the tri-layers. The bottom (A) surface shows some merging of the fibres almost certainly due to solvent evaporation. The upper surface (B) shows an open porous network.

Figure 9 shows sample 1. Figure 9A shows the first layer which has been deliberately spun onto PLA fibres. This shows some residual fibres of PLA which are much thicker, around $2\mu\text{m}$, as can be seen from Figure 9B, whereas the PU fibres are around $1\mu\text{m}$ diameter. Also shown is the third layer at low (Figure 9C) and high (Figure 9D) magnification. This shows an open network of fibres. Figure 9E shows a cross section of the scaffold and has been labelled to show the areas that represent the random, the aligned and then the random fibres of this tri-layer from the SEM. The thicknesses of the three layers were calculated to be $80\mu\text{m}$, $30\mu\text{m}$ and $80\mu\text{m}$ respectively.

Figure 10 shows Sample 2. Figure 10A shows the first layer which has been deliberately spun onto PLA. This shows some residual fibres of PLA which are much thicker around $2\mu\text{m}$ as can be seen from Figure 10B, whereas the PU fibres are around $0.5\mu\text{m}$ diameter. Also shown is the third layer at low (Figure 10C) and high (Figure 11D) magnification. This shows an open network of fibres. Figure 10E shows a cross section of the material and has been labelled to show the areas that represent the random, the aligned and then the random fibres of this tri-layer from the SEM. The thicknesses of the three layers were calculated to be $50\mu\text{m}$, $20\mu\text{m}$ and $50\mu\text{m}$ respectively.

Figure 11 shows the mesh implantation and fixation abdominal walls.

Figure 12 shows H+E staining of abdominal wall implants at 60 and 180 days. 100X magnification. Scale bar = 0.2mm.

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Figure 13 shows Masson-Goldner trichrome staining of abdominal implants at 60 and 180 days. 100X magnification. Scale bar = 0.2mm.

Figure 14 shows immunohistochemistry staining of abdominal wall implants at 60 days for 6 different antibodies. 400X magnification, scale bar = 0.1 for CD45, HLA-DR, CD163 and CD34. 100X magnification, scale bar = 0.2 for smooth muscle actin (SMA) and PGP9.5.

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Figure 15 shows immunohistochemistry staining of abdominal wall implants at 180 days for 6 different antibodies. 400X magnification, scale bar = 0.1 for CD45, HLA-DR, CD163 and CD34. 100X magnification, scale bar = 0.2 for smooth muscle actin (SMA) and PGP9.5.

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Figure 16 shows graphs and a table of the semi-quantitative scoring of the H&E data. A score of 0 - 3 was used for all where 0 = none and 3 = extensive.

Figure 17 shows graphs and table of the semi-quantitative scoring of the trichrome data. A score of 0 - 4 was used for all where 0 = none and 4 = great abundance.

20

Figure 18 shows graphs and a table of the semi-quantitative scoring of the IHC data (A score of 0 - 4 was used for all where 0 = none and 4 = great abundance).

25

Figure 19 shows the M2/M1 ratios for 60 and 180 days of abdomen implants. These were calculated for each group using the values from the blind scoring of the immunostaining, where the M1 response is the HLA-DR staining and the M2 response the D163 staining.

30 **DETAILED DESCRIPTION**

In a first aspect, the present invention provides a scaffold for the treatment of a hernia, wherein the scaffold comprises at least two layers of polyurethane: at least one layer in which the polyurethane fibres are randomly orientated and at least one layer in which the fibres are aligned.

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The term "scaffold", as used herein, refers to any material that allows attachment of cells, preferably attachment of cells involved in wound healing. "Attachment", "attach" or "attaches" as used herein, refers to cells that adhere directly or indirectly to a substrate as well as to cells that adhere to other cells.

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Suitably, the scaffold may comprise at least two layers in which the polyurethane fibres are randomly orientated and wherein the at least two layers are separated by at least one layer in which the fibres are aligned.

- 10 Suitably, the scaffold may comprise at least two layers in which the polyurethane fibres are aligned and wherein the alignment of the at least two layers are in different orientations. For example, the alignment of fibres in the second layer may at any angle with respect of the alignment of fibres in the first later of between 20° to 160°, or between 50° to 130°, or between 70° to 110°, or between 80° to 100° or about 90°. Advantageously, having the two or more
15 layer of aligned fibres may provide optimal strength and elasticity to the scaffold for hernia repair.

The term "orientated" is used herein to refer to the arrangement of the polyurethane fibres relative to ones another within each polyurethane layer. Orientated may be used herein
20 interchangeably with "spun" for embodiments where the scaffold is produced by electrospinning, though the scaffold of the invention may be produced by any suitable means including bioprinting.

A hernia occurs when an organ or fatty tissue squeezes through a weak spot in a surrounding
25 muscle or connective tissue called fascia. The most common types of hernia are inguinal (inner groin), incisional (resulting from an incision), femoral (outer groin), umbilical (belly button), and hiatal (upper stomach).

In an inguinal hernia, the intestine or the bladder protrudes through the abdominal wall or into
30 the inguinal canal in the groin. In an incisional hernia, the intestine pushes through the abdominal wall at the site of previous abdominal surgery. A femoral hernia occurs when the intestine enters the canal carrying the femoral artery into the upper thigh.

Hollinsky *et al.* (Fabrication of large pores in electrospun nanofibrous scaffolds for cellular
35 infiltration: a review. Tissue Eng Part B Rev. 2012 Apr;18(2):77-87) measured the tensile load of the abdominal wall being 10 MPa horizontally, and 4.5 MPa vertically. Williams *et al.* (Force

measurement in the abdominal wall. Biomed Eng. 1975;10:181–183) estimated the maximum force applied to the abdominal wall after hernia repair surgery as 22 N/mm in the cranial/caudal direction and 32 N/mm in the lateral direction. The natural elasticity of the abdominal wall at 32 N/cm is approximately 38%, with higher resilience in the horizontal direction than the longitudinal direction. A load of 16N/cm is accepted as the maximum load in the groin because of the more sphere-like anatomy of the groin (Klinge U, Klosterhalfen B, Conze J, Limberg W, Obolenski B, Ottinger AP, Schumpelick V. Modified mesh for hernia repair that is adapted to the physiology of the abdominal wall. Eur J Surg. 1998 Dec;164(12):951-60). Alternatively, the scaffold will ultimately be implanted with in the rectus fascia to give support to the abdominal wall. Therefore, we have used the mechanical properties of this rectus fascia as a reference to match with the scaffold. Chapin K *et al.* (Chapin K, Khalifa A, Mbimba T, McClellan P, Anderson J, Novitsky Y, Hijaz A, Akkus O. In vivo biocompatibility and time-dependent changes in mechanical properties of woven collagen meshes: A comparison to xenograft and synthetic mid-urethral sling materials. J Biomed Mater Res B Appl Biomater. 2018 Jun 13. doi: 10.1002/jbm.b.34138. [Epub ahead of print]) measured the ultimate tensile strength of rectus fascia to be 1.1 (± 0.4) MPa and a Young's modulus of 4.4 (± 2.7) MPa. Rubod C *et al.* (Rubod C, Brieu M, Cosson M, Rivaux G, Clay JC, de Landsheere L, Gabriel B. Biomechanical properties of human pelvic organs. Urology. 2012 Apr;79(4):968.e17-22) found similar ultimate tensile strength of the fascia from the rectum and a strain at this maximum strength of 30%.

Polyurethane is a polymer composed of organic units joined by carbamate (urethane) links. Polyurethanes may be classified as reaction polymers. Polyurethanes may be produced by reacting an isocyanate containing two or more isocyanate groups per molecule ($R-(N=C=O)_n$ [16]) with a polyol containing on average two or more hydroxyl groups per molecule ($R'-(OH)_n$ [16]) in the presence of a catalyst or by activation with ultraviolet light. They can be formulated to be long changed with low crosslinking to give stretchy, flexible polymers, or with short chains and high cross linking, to give hard polymers. They can also be formulated to be foams.

Suitably, the polyurethane used may have a hardness of between 80A to 90A. The Shore-hardness is a characteristic value for material properties of Elastomers and plastics. It is specified in the DIN 53505 and DIN EN ISO 868 standards. The Shore-hardness-tester (or „Durometer“) consists of a spring-loaded indenter, which elastic indentation is inversely related to the Shore-hardness of the material. The scale is from 0 to 100. A high figure means a high hardness. Shore A hardness applies to soft elastomers.

Suitably, the polyurethane may be any medical grade polyurethane. Suitably, the polyurethane may be derived from any medical grade polyether and/or polycarbonate material. Suitably, the polyurethane may be Z3. Polyurethane Z3 is a commercially available medical grade polyurethane (such as from Biomer Technologies (Cheshire)). Advantageously, when polyurethane Z3 is used the scaffold of the present invention may advantageously provide a scaffold which has viscoelastic properties similar to natural fascia. Other polyurethanes having viscoelastic properties similar to polyurethane Z3 may be used in accordance with the present invention. "Viscoelastic properties" may be measured by any one of the following: Young's modulus (e.g. in MPa); ultimate tensile strength (e.g. in MPa) or as a percentage strain at ultimate tensile strength. Other polyurethanes having similar viscoelastic properties to Z3 are known to a person of ordinary skill in the art including, for example: polyether and polycarbonate based medical grade material from DSM Biomedical Inc. (Chemelot Gate 2 (loge Campus), Urmonderbaan 22, 6167 RD GELEEN, The Netherlands), Lubrizol LifeSciences (Chaussée De Wavre, 1945, Brussels, B-1160, Belgium) and AdvanSource Biomaterials (229 Andover St, Wilmington, MA 01887, United States of America). All these polyether and polycarbonate based medical grade materials are designed for biomedical applications with a similar hardness grade to Z3.

Suitably, the scaffold may have an ultimate tensile strength of between 0.7 and 1.5 MPa.

Suitably, the scaffold may have a strain at ultimate tensile strength of 30% at least.

Suitably the scaffold may have a strain at an ultimate tensile strength which is similar to native fascia.

Suitably, the scaffold may have a Young's modulus of less than 7.1 MPa, such as in the range of 1.7 to 7.1 MPa. Suitably, the scaffold may have a Young's modulus similar to that of native fascia.

Suitably, the at least one layer in which the polyurethane fibres are randomly orientated comprise pores from the outermost surface of a depth of at least 16µm suitable for penetration by human adipose derived mesenchymal stem cells. It is desirable for the scaffold to allow for penetration by human adipose derived mesenchymal stem cells to aid the initial healing phase following implantation of the scaffold and to reduce the inflammatory response to the scaffold.

Suitably, the layer(s) in which the polyurethane fibres are randomly orientated may comprise pores suitable for penetration by human adipose derived mesenchymal stem cells (used as test cell, as described in Figure 3 above), wherein the pores from the outermost surface have

a depth of at least 16µm or at least 20µm or at least 30µm or at least 40µm or at least 50µm or at least 60µm or at least 70µm or at least 80µm or at least 90µm or at least 100µm.

5 A skilled person would readily understand that the maximum depth for penetration is dependent of the depth of the random layers of the scaffold. Suitably, the layer(s) in which the polyurethane fibres are randomly orientated may comprise pores suitable for penetration by human adipose derived mesenchymal stem cells throughout the depth of the random layer.

10 *In vitro* cell penetration was improved by the use of a PLLA sacrificial layer as demonstrated *in vitro* (Figure 3) and this material implanted *in vivo* in sheep showed penetration throughout the random layers of the scaffold, which were about 100 microns each with a central layer of 50 microns of compact aligned fibres.

15 Suitably, the depth each of the at least one layer(s) in which the polyurethane fibres are randomly orientated may be in the range of about 50µm to 150 µm. Suitably, each of the at least one layer(s) comprise pores suitable for penetration by human adipose derived mesenchymal stem cells of at least 30% of the depth of the layer or at least 50% or at least 70% or at least 80% or at least 90%.

20 Suitably, at least 20% or at least 30% or at least 40% or at least 50% of the pores on the outer surface of the first and/or third layer may be greater than 20µm in diameter. Suitably, about 55% of the pores on the outer surface of the first and/or third layer may be greater than 20µm in diameter.

25 The scaffold has dimensions suitable for the intended purpose of hernia repair. However, a skilled person would readily understand that any dimensions which render the scaffold suitable for the intended purpose could be used and this would be dependent on the type and size of hernia. For example, oval, rectangular and square shaped supports are often used in hernia repair and such scaffold have dimensions such as 5cm by 10cm, 10 cm by 20 cm, 15 cm by 20 cm, 20 cm by 25 cm (e.g. for oval or rectangular supports) or 10 cm by 10 cm or 15 cm by 30 15 cm for square shaped supports. Accordingly, the scaffold may have a width of at least 4 cm and a length of at least 10cm.

35 Suitably, the scaffold may have a depth of about 100µm to 500µm, or about 200µm to 300µm, or about 250µm. A skilled person would readily understand that a balance is to be achieved by having enough of a depth that the desired mechanical properties of the scaffold are achieved whilst balancing with the desire to not implant excessive foreign material.

Suitably, the scaffold may be of a density of about 60 to 100 g/m².

Suitably, the scaffold may be prepared in the form of a tape, sheet or reel.

In another aspect, the present invention provides a method of preparing a scaffold for treatment of a hernia, the method comprising:

- 5 a. Electrospinning a sacrificial layer of poly-lactic acid (PLA) onto a rotating surface;
- b. Electrospinning a layer of polyurethane in which the fibres are spun in random orientations;
- c. Electrospinning a layer polyurethane in which the fibres are spun in an aligned
10 orientation;
- d. Removing the sacrificial layer of PLA to produce the scaffold.

The terms "electrospinning" or "electrospun," as used herein to refer to any method where materials are streamed, sprayed, sputtered, dripped, or otherwise transported in the presence
15 of an electric field. The electrospun material can be deposited from the direction of a charged container towards a grounded target, or from a grounded container in the direction of a charged target. In particular, the term "electrospinning" means a process in which fibres are formed from a charged solution comprising at least one natural biological material, at least one synthetic polymer material, or a combination thereof by streaming the electrically charged
20 solution through an opening or orifice towards a grounded target.

As used herein, the terms "solution" and "fluid" refers to a liquid that is capable of being charged and which comprises at least one natural material, at least one synthetic polymer, or a combination thereof. The polymer may be a co-polymer. The term "co-polymer" as used
25 herein is intended to encompass co-polymers, ter- polymers, and higher order multiple polymer compositions formed by block, graft or random combination of polymeric components.

Poly-L-lactic acid is a biodegradable aliphatic polyester, typically formed from natural sources such as corn starch. In production, two main monomers are typically utilised; lactic acid, and
30 the cyclic di-ester, lactide. The most common route to PLA is the ring-opening polymerization of lactide with various metal catalysts (typically tin octoate) in solution, in the melt, or as a suspension. Alternative, lactic acid monomers can be directly condensed together. Polylactic acid is chiral, and several distinct forms can exist; poly-L-lactide (PLLA) is the product resulting from polymerization of L,L-lactide (also known as L-lactide). Polymerization of a racemic
35 mixture of L- and D-lactides usually leads to the synthesis of poly-DL-lactide (PDLLA), which

is amorphous. The ring-opening polymerization of lactide with various metal catalysts in solution typically leads to a racemic mix of isomers. Use of stereospecific catalysts can lead to heterotactic PLA which has been found to show crystallinity, largely controlled by the ratio of D to L enantiomers used, and to a lesser extent on the type of catalyst used.

5

Suitably, the method may further comprise one or more steps between steps c and d selected from:

- e. Electrospinning a further layer polyurethane in which the fibres are spun in aligned longitudinal orientation, wherein the alignment of fibres is in a different orientation to the alignment in step; and
- 10 f. Electrospinning a further layer in which the fibres are spun in random orientations.

In one embodiment, the method may comprise the use of at least one syringe pumps to deliver both random fibres and aligned fibres.

The number of needles to be utilised may depend in part of the size of the scaffold to be produced.

In one embodiment, step c. may comprise a step-by-step increase and then decrease of the rotating collector speed after step b to avoid delamination of the layers.

20 Suitably, step e., if applicable, may comprise a step-by-step increase and then decrease of the rotating collector speed before starting step e and/or step f to avoid delamination of the layers.

Whilst, in the methods of the present invention, it is preferable that there is a step-by-step increase and then decrease of the rotating collector speed at steps c. and e., these steps do not overlap so that two layers are clearly distinguishable in the cross-section of the scaffold. Hence, no two layers are fully intermixed. Likewise, for embodiments having three or four layers, the current method for step c and e will increase resistance to delamination whilst each layer is clearly distinguishable in the cross-section of the scaffold.

Suitably, the sacrificial layer may be applied to a surface rotating from about 50 to 100 rpm. 30 Suitably, the sacrificial layer may be applied to a surface rotating at about 60 rpm.

Suitably, the sacrificial layer may be produced with a needle to surface distance of from about 20cm to 30cm. Suitably, the sacrificial layer may be produced with a needle to surface distance of about 25cm.

Suitably, the sacrificial layer may be produced by delivering polymer solutions at a rate of from about 30 $\mu\text{l}/\text{min}$ to 100 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 13kV to 22kV DC.

5 Suitably, step b may be conducted after any excess solvent from step a. has evaporated. For example, step b. may be conducted at least 5 minutes or at least 10 minutes or at least 15 minutes or at least 20 minutes or at least 25 minutes after step a. finishes. Suitably, step b. may be conducted about 30 minutes after step a. finishes.

10 Suitably, the layers in which the polyurethane fibres are randomly aligned may be applied to a surface rotating from about 50 rpm to 150 rpm. Suitably, the such layer(s) may be applied to a surface rotating at about 100 rpm.

Suitably, the layers in which the polyurethane fibres are randomly aligned may be produced with a needle to surface distance of from about 20cm to 30cm. Suitably, the first and/or third layers may be produced with a needle to surface distance of about 25cm.

15 Suitably, the layers in which the polyurethane fibres are aligned (e.g. longitudinally or perpendicular thereto) may be produced with a needle to surface distance of from about 20cm to 30cm. Suitably such layer(s) may be produced with a needle to surface distance of about 25cm.

20 Suitably, the layers in which the polyurethane fibres are randomly aligned may be produced by delivering polymer solutions at a rate of from about 50 $\mu\text{l}/\text{min}$ per syringe to 150 $\mu\text{l}/\text{min}$ per syringe ; with an accelerating voltage of from about 13kV DC to 22kV DC. Suitably, the first and/or third layer may be produced by delivering polymer solutions at a rate of from about 130 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 17kV DC.

25 Suitably, the second layer may be applied to a surface rotating from about 250 rpm to 1200 rpm. Suitably, the second layer may be applied to a surface rotating from about 250 to 350rpm for about 15 to 20 minutes, followed by a surface rotating from about 550 to 650rpm for about 15 to 20 minutes, followed by a surface rotating from about 1150 to 1250rpm for about 15 to 20 minutes, followed by a surface rotating from about 550 to 650rpm for about 15 to 20 minutes, and followed by a surface rotating from about 250 to 350rpm for about 15 to 20 minutes.

30 Suitably, the second layer may be produced by delivering polymer solutions at a rate of from about 50 $\mu\text{l}/\text{min}$ per syringe to 150 $\mu\text{l}/\text{min}$ per syringe; with a voltage of from about 13kV DC to 22kV DC. Suitably, the second layer may be produced by delivering polymer solutions at a rate of from about 130 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 17kV DC.

Suitably, the humidity during the electrospinning of the sacrificial layer may be about 30%.

Suitably, the amount of polymer used in each layer can be modified according to desired use and/or to yield a desired thickness or ratio between the layers. For example, it may be desirable for the scaffold to have a cross-section which is about 2:1:2 (first layer to second layer to third layer).

A more detailed method of producing a scaffold of the present invention follows. This method provides preferred ranges of conditions for the method.

The poly-L-lactic acid which may be used as a sacrificial layer in the method of the invention may be used in any suitable concentration. Suitably, a concentration of PLA at about 10% wt/v to about 15% wt/v may be used. Suitably, the concentration may be about 12% wt/v.

The poly-L-lactic acid may be dissolved in any appropriate solvent or combination of solvents. Suitably, the solvent may be dichloromethane (DCM).

The polyurethane used to electrospin the first, second and/or third layer may be used in any suitable concentration. Suitably, a concentration of polyurethane at about 8% wt/v to about 25% wt/v may be used. Suitably, the concentration may be about 20% wt/v.

The polyurethane may be dissolved in any appropriate solvent or combination of solvents. Suitably, one example of an applicable solvent is 60:40 to 80:20 Dimethylformamide (DMF):Tetrahydrofuran (THF). Suitably, the solvent may be 75:25 Dimethylformamide (DMF):Tetrahydrofuran (THF).

Alternative method

In another embodiment, the method may comprise the use of at least two separate syringe pumps, one delivering random fibres and one delivering aligned fibres. The method may comprise a plurality of syringes for delivering the random fibres and/or a plurality of syringes for delivering aligned fibres.

The number of needles to be utilised may depend in part of the size of the scaffold to be produced.

Suitably, step c. may start before step b. finishes such that there is an overlap between the layers. Advantageously, by starting step c. prior to finishing step b., the scaffold may have increased resistance to delamination between layers.

Suitably, step e. may start before step c. finishes and/or step f. may start before step e. finishes such that there is an overlap between the layers.

Whilst in the methods of the present invention it is preferable that there is some overlap between steps b. and c.. these steps do not fully overlap so that two layers are clearly distinguishable in the cross-section of the scaffold. Hence, no two layers are fully intermixed. Figures 5 and 6 show a preferred method of the invention. Likewise, for embodiments having
 5 three or four layers it is preferable that there is some overlap for increased resistance to delamination whilst each layer is clearly distinguishable in the cross-section of the scaffold.

Suitably, the sacrificial layer may be applied to a surface rotating from about 200 to 400 rpm or from about 250 to 350 rpm. Suitably, the sacrificial layer may be applied to a surface rotating at about 300 rpm.

10 Suitably, the sacrificial layer may be produced with a needle to surface distance of from about 15cm to 20cm or from about 16cm to 18cm. Suitably, the sacrificial layer may be produced with a needle to surface distance of about 17cm.

Suitably, the sacrificial layer may be produced by delivering polymer solutions at a rate of from about 30 μ l/min to 40 μ l/min per syringe with an accelerating voltage of from about 15kV to
 15 19kV DC.

Suitably, step b may be conducted after any excess solvent from step a. has evaporated. For example, step b. may be conducted at least 5 minutes or at least 10 minutes or at least 15 minutes or at least 20 minutes or at least 25 minutes after step a. finishes. Suitably, step b. may be conducted about 30 minutes after step a. finishes.

20 Suitably, the layers in which the polyurethane fibres are randomly aligned may be applied to a surface rotating from about 200 rpm to 400 rpm or from about 250 rpm to 350 rpm. Suitably, the such layer(s) may be applied to a surface rotating at about 300 rpm.

Suitably, the layers in which the polyurethane fibres are randomly aligned may be produced with a needle to surface distance of from about 20cm to 25cm or from about 21cm to 24cm.

25 Suitably, the first and/or third layers may be produced with a needle to surface distance of about 23cm.

Suitably, the layers in which the polyurethane fibres are aligned (e.g. longitudinally or perpendicular thereto) may be produced with a needle to surface distance of from about 5cm to 10cm or from about 5cm to 7cm. Suitably such layer(s) may be produced with a needle to
 30 surface distance of about 5cm.

Suitably, the layers in which the polyurethane fibres are randomly aligned may be produced by delivering polymer solutions at a rate of from about 30 μ l/min per syringe to 40 μ l/min per syringe or from about 35 μ l/min per syringe to 40 μ l/min per syringe; with an accelerating

voltage of from about 15kV DC to 25kV DC or from about 19kV DC to 21kV DC. Suitably, the first and/or third layer may be produced by delivering polymer solutions at a rate of from about 40µl/min per syringe with an accelerating voltage of from about 20kV DC.

5 Suitably, the second layer may be applied to a surface rotating from about 500 rpm to 700 rpm or about 550 rpm to 650 rpm. Suitably, the second layer may be applied to a surface rotating from about 600 rpm.

Suitably, the second layer may be produced by delivering polymer solutions at a rate of from about 30µl/min per syringe to 40µl/min per syringe or from about 35µl/min per syringe to 40µl/min per syringe; with a voltage of from about 20kV DC to 25kV DC or from about 21kV
10 DC to 23kV DC. Suitably, the first and/or third layer may be produced by delivering polymer solutions at a rate of from about 40µl/min per syringe with an accelerating voltage of from about 23kV DC.

Suitably, the humidity during the electrospinning of the sacrificial layer may be about 30%.

Suitably, the amount of polymer used in each layer can be modified according to desired use
15 and/or to yield a desired thickness or ratio between the layers. For example, it may be desirable for the scaffold to have a cross-section which is about 2:1:2 (first layer to second layer to third layer).

A more detailed method of producing a scaffold of the present invention follows. This method provides preferred ranges of conditions for the method.

20 The poly-L-lactic acid which may be used as a sacrificial layer in the method of the invention may be used in any suitable concentration. Suitably, a concentration of PLA at about 10% wt/v to about 15% wt/v may be used. Suitably, the concentration may be about 10% wt/v.

The poly-L-lactic acid may be dissolved in any appropriate solvent or combination of solvents. Suitably, the solvent may be dichloromethane (DCM).

25 The polyurethane used to electrospin the first, second and/or third layer may be used in any suitable concentration. Suitably, a concentration of polyurethane at about 10% wt/v to about 15% wt/v may be used. Suitably, the concentration may be about 10% wt/v.

The polyurethane may be dissolved in any appropriate solvent or combination of solvents. Suitably, one example of an applicable solvent is 60:40 to 70:30 Dimethylformamide
30 (DMF):Tetrahydrofuran (THF). Suitably, the solvent may be 70:30 Dimethylformamide (DMF):Tetrahydrofuran (THF).

A sacrificial PLA layer of random fibres may be produced in accordance with step a., by delivering a suitable amount of the polymer solution comprising PLA towards a rotating mandrel. Suitably, about 5ml to 10ml may be used. Suitably, about 10 ml may be used.

The polymer solution may be divided between an appropriate number of syringes. For example, where 10ml of polymer solutions comprising PLA is used, the solution may be divided between 2 or more or 3 or more syringes. Suitably, the solution may be equally divided between 4 syringes. The syringes may be placed into a syringe pump (such as GenieTMPlus, Kent Scientific, USA).

PLA fibres were produced by delivering polymer solutions at an appropriate rate (e.g., from 30 μ l/min to 40 μ l/min) per syringe with an appropriate accelerating voltage (e.g. from 15kV to 19kV) DC from a high voltage supply (e.g. Genvolt, UK). The electrospun material may be collected on a covered (e.g. aluminium foil covered) earthed rotating surface (e.g. mandrel) of an appropriate size for the scaffold being produced.

A rotating speed of from 200 rpm to 400 rpm may be used. The needle to collector distance may be 15cm to 20cm at 21°C and ~30% humidity. Though a person of ordinary skill in the art could readily adapt these parameters appropriately when different temperatures or humidity levels are used.

Suitably, a break between step a. and step b. may be desired to allow any excess solvent to evaporate. The polyurethane (hereinafter PU) tri-layers may then be directly electrospun onto the surface of the PLA fibres produced in step a.

PU scaffolds may be created using a similar set up as for the sacrificial layer. For example, by delivering a suitable amount of the polymer solution comprising PU towards a rotating surface covered with PLA fibres. Suitably, about 40ml to 80ml of the polymer solution comprising PU may be used. Suitably, about 60 ml may be used.

The polymer solution may be divided between an appropriate number of syringes. For example, where 60ml of polymer solutions comprising PU is used, the solution may be divided between 2 or more or 3 or more syringes. Suitably, the solution may be equally divided between 4 syringes. The syringes may be placed into a syringe pump (such as GenieTMPlus, Kent Scientific, USA).

In the method of the present invention each PU polymer solution is electrospun such that the scaffold comprises at least two layers at least one random and at least one aligned layer. Suitably, the method may be used to form a trilayer scaffold in random-aligned-random orientations or aligned-aligned-random orientation. Suitably, the method may be used to form a four layer scaffold in random-aligned-aligned-random orientations.

Random fibres may be produced by delivering polymer solutions at a rate from 30 µl/min to 40 µl/min per syringe with an accelerating voltage of about 15kV to 25kV DC from a high voltage supply onto a rotating surface rotating at from about 200rpm to about 400rpm.

5 Suitably, the needle to collector distance may be from about 20cm to 25cm at 21°C and ~30% humidity. However, a person of ordinary skill in the art could readily adapt these parameters appropriately when different temperatures or humidity levels are used.

Aligned fibres may be produced using a voltage from about 20kV to 25kV, and a rotating surface of about 500 rpm to 700 rpm, such as 600 rpm. Suitably, the needle to collector may be from about 5cm to 10cm, preferably about 5cm.

10 Interwoven random-aligned fibre morphologies may be produced using two separate syringe pumps. Suitably, each random layer may be produced using e.g. about 16mls of polymer and e.g. about 8ml may be used for the aligned layer, with e.g. a 4ml overlap between separate layers. For example, this could be achieved using at least one syringe pump delivering random fibres, and at least one syringe pump delivering aligned fibres. With this set up, it is possible
15 to obtain 40:20:40 for proportions of the polymer amount of each layer. It is a matter of routine to adjust the mounts of polymer used in each layer to obtain a different desired ratio between the layers.

Suitably, the sacrificial layer may readily be removed from the scaffold without the need for further processing techniques.

20 The properties of the electrospun materials can be adjusted in accordance with the needs and specifications of the cells to be suspended and grown within them. The porosity, for instance, can be varied in accordance with the method of making the electrospun materials matrix.

There are many factors involved in the electrospinning process which may affect scaffolds fibre diameter and pore size. The key variables are solution viscosity, surface tension, and
25 viscoelasticity of the spinning solution. These are directly related to the concentration of, and molecular weight of the polymer, as well as the solvent used. The dielectric properties of the solution also play a key role (Kowalczyk *et al.*, 2008).

Another source of variation in electrospinning, which is perhaps not well documented, is that once the polymer is in solution it can change or degrade on storage and the same
30 concentration of polymer does not always yield a solution with the same viscosity. Thus the molecular weight of the polymer will decrease rapidly over time (particularly the 50/50 PLGA). Therefore, fresh polymer is preferably used for spinning.

In a further aspect, the present invention relates to a scaffold produced by the method of the present invention. Suitably, the scaffold may have the properties of a scaffold as disclosed herein.

5 In another aspect, the present invention provides a scaffold of the present invention or produced by a method of the invention for use as a medicament.

In further aspect, the present invention provides a scaffold of the present invention or produced by a method of the invention for use in the treatment of a hernia

10 In another aspect, the present invention provides a method of preparing a scaffold for treatment of a hernia wherein the method comprises using additive manufacturing methodologies (e.g. three-dimensional printing). Advances in three-dimensional printing has led to the printing of biocompatible materials. Such 3D bioprinting can be used to create complex 3D materials. Bioprinting of 3D materials are known to a person of ordinary skill in the art. For example, bioprinting of 3D materials have been described by using layer-by-layer
15 methodology in which the settings can be readily adapted by a person of ordinary skill in the art to produce fibres with different orientations in each layer without restrictions of structural complexity and spatial heterogeneities. The method is thus able to mimic the natural structure of the target tissue in a precise and controlled placement (Hong *et al.* 2018).

20 As used herein, “urinary incontinence” (abbreviated UI) refers to any involuntary leakage of
As used herein, “mesenchymal stem cells” refers to multipotent stem cells that can differentiate into a variety of cell types, including: osteoblasts (bone cells), chondrocytes (cartilage cells), and adipocytes (fat cells).

Other Applications

25 Throughout the patent application the scaffold, methods for preparing the scaffold and uses thereof are suitable for the treatment of hernia. Hence, the dimensions of the scaffold ,, strength and out parameters are optimised accordingly.

However, the mechanical properties and biocompatibility profile of the scaffold disclosed herein would be suitable for use in other applications. A person of ordinary skill in the art is
30 readily aware that the scaffold may be designed to be an appropriate shape, size and strength of other surgical applications, such as cosmetic surgery applications.

For example, a scaffold of the invention (such as a trilayer scaffold) could be used as the external material that covers cardiovascular stents or it could be used as reconstructive

material for other soft tissues, such as breast reconstruction after a skin-sparing or nipple-sparing mastectomy.

The material is softer and stretchier than other materials used for covering cardiovascular stents or than ultralight meshes used for breast reconstruction, and it mimics the mechanical properties of soft tissues providing the right forces and frictions when in contact with them. Also, the biocompatibility data (e.g. illustrated with a trilayer scaffold) support the potential to allow cell infiltration and neo-vascularization for a good long-term integration within native tissues. Hence, scaffolds as defined herein may have utility in such applications.

Within the cosmetic sector, this material could have potential for the reconstruction of any soft tissue. Nevertheless, the mechanical properties for each application may need to be adjusted by increasing or decreasing the thickness of the middle layer with aligned fibres, as well as, by adjusting the whole thickness of the final product to introduce more or less supportive material.

There have been some improvements on the surgical introduction of these meshes in the past. For incisional hernia repair, onlay mesh repairs were replaced with sublay repairs where the mesh is placed underneath a thick muscle tissue (retro-rectus) in a well vascularized wound bed and away from the skin. Onlay mesh repairs required a large area of the mesh to stay in very close proximity to skin increasing the chances of mesh colonization and infection (Binnebösel, M. et al. Impact of mesh positioning on foreign body reaction and collagenous ingrowth in a rabbit model of open incisional hernia repair. *Hernia* 14, 71–77 (2010)).

A person of ordinary skill in the art would readily understand that the dimensions of the scaffold could readily be optimised for the desired use. For example, the scaffold of the invention can be readily made in sizes which are typically used in the desired application. For example, for breast surgery, the support may be a length of at least 15 cm or at least 20 cm or at least 25 cm and a width of at least 15 cm or at least 20 cm or at least 25 cm. Suitably, the scaffold may be substantially circular in shape and may have a central substantially circular hole of between 5 to 6 cm.

Hence, in one aspect, the present invention provides a scaffold suitable for a cosmetic surgical procedure, wherein the scaffold comprises at least two layers of polyurethane: at least one layer in which the polyurethane fibres are randomly orientated and at least one layer in which the fibres are aligned. For example, the scaffold may comprise at least two layers in which the polyurethane fibres are randomly orientated and wherein the at least two layers are separated by at least one layer in which the fibres are aligned, or the scaffold may comprise at least two layers in which the polyurethane fibres are aligned and wherein the alignment of the at least two layers are in different orientations.

The present invention further provides methods of making a scaffold suitable for a cosmetic surgical procedure and uses thereof.

The reader's attention is directed to all papers and documents which are filed concurrently with or previous to this specification in connection with this application and which are open to public inspection with this specification, and the contents of all such papers and documents are incorporated herein by reference.

All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

Each feature disclosed in this specification (including any accompanying claims, abstract and drawings), may be replaced by alternative features serving the same, equivalent, or similar purpose, unless expressly stated otherwise. Thus, unless expressly stated otherwise, each feature disclosed is one example only of a generic series of equivalent or similar features.

The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

EXAMPLES

Production of a polyurethane tri-layer using a sacrificial poly-L-lactic acid layer, analysis of cell penetration

Methods

Polymers

Poly-L-lactic acid (PLA) was purchased from Goodfellow (Cambridge, UK), polyurethane Z3A1 (Z3) was purchased from Biomer technologies (Cheshire, UK). PLA at 10% (wt/v) was dissolved in dichloromethane (DCM), Z3 was dissolved in 70:30 DMF:THF at 10% (wt/v).

Electrospinning

Polyurethane tri-layer scaffolds were created by loading solutions of PU Z3 into 5ml syringes fitted with blunt tipped 21G needles, placed into a syringe pump (Genie™Plus, Kent Scientific, USA). Tri-layers consisted of random-aligned-random orientations. Random fibres were

produced by delivering polymer solutions at a rate of 40µl/min per syringe with an accelerating voltage of 20kV DC from a high voltage supply (Genvolt, UK) and collected on an aluminium foil covered earthed mandrel (80mm diameter, 160mm length) rotating at 300rpm, with a needle to collector distance of 20cm at 21°C and ~30% humidity. Aligned fibres were produced using a voltage of 23kV, a mandrel rotation speed of 600rpm and a needle to collector distance of 5cm.

Interwoven random-aligned-random fibre morphologies were produced using two separate syringe pumps. Each layer was produced using 20mls of polymer, using a 5ml overlap between separate layers (one syringe pump delivering random fibres, while the other pump delivered aligned fibres).

The sacrificial PLA layer of random fibres was produced by delivering 10mls of the polymer solution towards the rotating mandrel using the same steps for random fibre production above. A 30 minute break period between separate polymer delivery (PLA and PU Z3) was performed to allow any excess solvent to evaporate. PU Z3 tri-layers were then directly electrospun onto the surface of the PLA fibres.

The PLA layer was readily removed from the PU Z3 tri-layer, without the need for further processing techniques. There was no gross delamination of the individual layers of the PU Z3 tri-layer. Scaffolds were allowed to dry for 12 hours at room temperature, prior to packaging in vacuum packs and were stored at -20°C.

Adipose-Derived Mesenchymal Stem Cell (ADSC) Culture

ADSC were isolated from human subcutaneous fat, donated by patients giving informed consent under a research tissue bank license (number 08/H1308/39) under the Human Tissue Authority, isolated and cultured. Cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum (FCS) (Advanced Protein Products, Brierley Hill, UK), 2mM glutamine, 0.625µg/mL amphotericin B, 100IU/mL penicillin and 100µg/mL streptomycin (Gibco Invitrogen, Paisley, UK).

Sample Preparation and Culture of Cells on Scaffolds

Scaffolds were cut to 1.5cm x 1.5cm and sterilized in 70% ethanol for 20 minutes followed by 3 washes in PBS. 500,000 passage 6 ADSC were seeded per scaffold into the centre of steel rings (internal diameter 1cm) placed onto each scaffold, creating a defined area for cell attachment. Rings were removed after 12 hours and samples cultured for 2 weeks at 37°C, 5% CO₂. DMEM was changed three times per week.

Scanning electron microscopy assessment of scaffolds

For imaging of cells on scaffolds these same fixed samples were processed and gold sputter coated (Edwards sputter coater S150B, Crawley, England). Samples were imaged using a
5 Phillips XL-20 scanning electron microscope (Cambridge, UK). Fibre diameter and pore size of each scaffold was assessed.

Assessment of cell penetration

For imaging of live cells within scaffolds, a fluorescent dye was used to label the cells and
10 second harmonic generation was used to image the scaffolds.

500,000 ADSC were seeded on each of the 5 sterilised scaffolds as previously described and incubated with media (DMEM) changed three times per week. Cell-scaffolds were cultured for 3 weeks, following which, 0.5mls of serum free DMEM with 10 μ M Celltracker™ red CMTPIX
15 (Invitrogen, Oregon USA) was added per well and incubated for one hour. Cells were imaged live, using a Zeiss LSM 510 Meta upright laser-scanning confocal microscope (Carl Zeiss Micro Imaging, Germany) using a 40x 1.3 NA oil immersion objective attached to a tuneable (700– 1060 nm) Chameleon Ti:sapphire multiphoton laser (Coherent, CA, USA) for second harmonic generation (SHG) signal. Red cell tracker signal was created by illuminating
20 constructs at 543nm with 30% transmission and detected between 565nm and 615nm. For SHG signal, constructs were illuminated at 840nm and signals were detected between 415nm and 426nm. Images (512 x 512), with a pixel dwell time of 6.39 μ s were captured at a range of depths by moving the focal plane down from the surface of the scaffold, where there was the greatest number of cells present and without any polymer fibres visible, at 1 μ m intervals
25 until no further cells were visible and polymer fibres dominated the field of view.

Results*Scanning electron microscopy assessment of scaffolds*

Figure 1 demonstrates the electron microscopy images of the two produced scaffolds. The
30 bottom surface of the tri-layer that was electrospun onto the surface of the sacrificial PLA layer demonstrates a confluent surface, while the bottom layer of the basic tri-layer shows a warped appearance. Figure 2 demonstrate the fibre diameter and pore size of PU scaffolds. Figure 2 demonstrates that the pore size of the bottom surface of the tri-layer that is electrospun onto the sacrificial PLA layer is much greater. This shows that while the fibre diameter of the bottom
35 surface of the polyurethane scaffold with a sacrificial layer is smaller than that of the plain PU tri-layer, the pore size is much greater for that of PU Z3/PLA.

Assessment of cell penetration

Figure 3 shows the ability of adipose derived stem cells (in white) to penetrate between the polymer fibres at different depths from the scaffold surface, as measured using confocal microscopy. The bottom layer of the tri-layer that is electrospun onto the sacrificial PLA layer demonstrates that cells are present at greater depths than either the bottom surface of the tri-layer without the sacrificial PLA layer or the top surface of either scaffold. For the top surface of both the sacrificial and non-sacrificial tri-layer scaffolds, cells represented only a thin superficial layer, while a control that consisted of only a single random fibre orientation of Z3 fibres demonstrates a much greater cell penetrative ability. This shows cell tracker red stained cells at a variety of scaffold depths, as measured using confocal microscopy. The bottom layer of the tri-layer that is electrospun onto the sacrificial PLA layer demonstrates that cells are present at greater depths than either the bottom surface of the tri-layer without the sacrificial PLA layer or the top surface of either scaffold. For the top surface of both the sacrificial and non-sacrificial tri-layer scaffolds, cells represented only a thin superficial layer.

Conclusions

The production of a tri-layer of random-aligned-random orientated fibres represents a fascia-like structure. These scaffolds can be reliably produced using basic electrospinning equipment. However, these techniques can frequently lead to the production of a 'warped' bottom fibre surface that results from the electrospinning of polymer directly onto a flat surface. The use of a sacrificial layer consisting of randomly orientated degradable polymer fibres that can be readily separated from the final product is a novel method for overcoming this commonly encountered problem. The use of the sacrificial layer results in improved porosity of the bottom surface of the scaffold, and the inventors demonstrate that this is associated with an improved ability of cells to penetrate the scaffold fibres and therefore this technique can potentially have an impact on the initial healing phase of a repair material following implantation.

Mechanical testing of scaffolds

Preparation of scaffolds

Figures 4 and 5 show a schematic of the preparation of the polyurethane (PU) scaffolds. Three layers are spun - the first layer to produce random fibres, the second layer to produce aligned fibres and then the third layer to produce random fibres.

In a modification of this technique the inventors introduced an initial sacrificial layer of poly-L-lactic acid (PLA). These fibres are spun first to provide a template then the tri-layer spun onto them and then post spinning the PLA layer is gently peeled off the PU layers.

The motivation for spinning onto a random scaffold of PLA fibres was to avoid the adverse effects of residual solvent on PU fibres which tended to cause fibre merging and small voids between the fibres which would not support cell entry into the scaffolds. The PLA acts as a sacrificial template layer.

Polymers

PLA was purchased from Goodfellow (Cambridge, UK), PU Z3A1 was purchased from Biomer technologies (Cheshire, UK). PLA at 10% (wt/v) was dissolved in dichloromethane (DCM), PU was dissolved in 70:30 Dimethylformamide (DMF):Tetrahydrofuran (THF) at 10% (wt/v).

Electrospinning

A sacrificial PLA layer of random fibres was produced by delivering 10mls of the polymer solution towards the rotating mandrel. 2ml was loaded into each of 4 syringes (5ml syringes) fitted with blunt tipped 21G needles, placed into a syringe pump (GenieTMPlus, Kent Scientific, USA). PLA fibres were produced by delivering polymer solutions at a rate of 40µl/min (from 30 µl/min to 40 µl/min) per syringe with an accelerating voltage of 17kV (from 15kV to 19kV) DC from a high voltage supply (Genvolt, UK) and collected on an aluminium foil covered earthed mandrel (80mm diameter, 160mm length) rotating at 300rpm, with a needle to collector distance of 17cm at 21°C and ~30% humidity.

A 30 minute break period between separate polymer delivery (PLA and PU) was performed to allow any excess solvent to evaporate. PU tri-layers were then directly electrospun onto the surface of the PLA fibres.

PU tri-layer scaffolds were created by loading 20 ml solution of PU into 5ml syringes (5 ml each) fitted with blunt tipped 21G needles, placed into a syringe pump (GenieTMPlus, Kent Scientific, USA). Tri-layers consisted of random-aligned-random orientations. Random fibres were produced by delivering polymer solutions at a rate of 40µl/min per syringe with an accelerating voltage of 20kV DC from a high voltage supply (Genvolt, UK) and collected on an aluminium foil covered earthed mandrel (80mm diameter, 160mm length) rotating at 300rpm, with a needle to collector distance of 20cm at 21°C and ~30% humidity. Aligned fibres were produced using a voltage of 23kV, a mandrel rotation speed of 600rpm and a needle to collector distance of 5cm.

Interwoven random-aligned-random fibre morphologies were produced using two separate syringe pumps. Each random layer was produced using 16mls of polymer and 8mL for the

aligned layer, using a 4ml overlap between separate layers (one syringe pump delivering random fibres, while the other pump delivered aligned fibres). 40:20:40 proportions of the polymer amount of for each layer was obtained. However, the middle layer looks thinner (more than half than the others) because fibres are aligned occupying less space.

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The sacrificial PLA layer of random fibres was produced by delivering 10mls of the polymer solution towards the rotating mandrel using the same steps for random fibre production above. A 30 minute break period between separate polymer delivery (PLA and PU) was performed to allow any excess solvent to evaporate. PU tri-layers were then directly electrospun onto the surface of the PLA fibres.

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The PLA layer was readily removed from the PU tri-layer, without the need for further processing techniques. There was no gross delamination of the individual layers of the PU tri-layer.

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Mechanical testing of the materials

Three materials were tested – a commercial available PP used for stress urinary incontinence (used here as a reference), a random scaffold of PU and a tri-layer scaffold of PU consisting of random, aligned and random fibres. These was produced twice by two operators – Sample 1 and Sample 2. The materials were subjected to cyclic strain up to 5 cycles applying 25% of distention.

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The stress strain curves obtained are shown for these in Figure 6A. A plastic deformation at the second cycle is shown in Table 1 and the thickness of the materials in Table 2. Figure 6B shows the Young's modulus for the materials tested at the end of the first cycle and at the end of the second cycle.

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Table 1: Plastic deformation (%) at the second cycle

Sample	Plastic deformation (%) at the second cycle
Sample 1	0.47± 0.22
Sample 2	0.24±0.14
One layer pU	0
PP	1.14±0.54

Table 2: Thickness of the material (mm) measured with a digital micrometre

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Sample	Thickness of the material (mm) measured with a digital micrometre
Sample 1	0.5
Sample 2	0.3
One layer pU	0.4
PP	0.5

The dotted lines in Figure 6B show the Young's modulus reported for human healthy abdominal tissues from Chapin et al., (Chapin *et al.*, 2018). All materials showed a reduction (softening) following cyclic strain. The three PU materials have a Young's modulus that is around the lower end of the normal range, reducing slightly further after cyclical strain. In contrast the PP starts off much stiffer than the native tissues and it gets even stiffer after cyclic strain applied.

Figure 6C shows PU and PP samples tested for a uniaxial tensile test after 7 days cultured into an EBERS bioreactor under dynamic distention (Hillary *et al.*, 2016). While PU, in this case, one layer (random) scaffolds maintained its stretchability, PP mechanically failed by snapping during the test.

Figure 7 summarises the values obtained for Young's modulus (Figure 7A), ultimate tensile strength (Figure 7B) and strain at ultimate tensile strength (Figure 7C) for all of the materials following a tensile lab test. Again, the dotted lines indicate the reference values for normal patient's tissues. This shows that with respect to Young's modulus, PP is much stiffer while the PU materials are within the values for the range of native tissues. With respect to ultimate tensile strength PP is much stronger than the native tissues. PU materials are within the values for the range of native tissues. Finally, the strain at ultimate tensile strength, the PU materials can extend more than the native tissues, the PP material is just above the strain of normal tissue. PP is a very stiff and strong material for the abdominal environment as compared to human native tissues from that area. One layer random PU seems to be slightly stiffer and stronger than the tri-layer scaffolds.

Mechanical testing of the scaffold materials before and after dynamic conditions

Strips of all scaffolds materials, including PP as a reference material, were measured, cut and clamped to a tensiometer (BOSE Electroforce test instruments, Minnesota, USA) with a 22 N load cell. Mechanical properties were measured using a ramp test at a rate of 0.1 mm/s or a cyclic test at rate of 1mm/s up to 25% of displacement (from its original length) at 5 cycles.

Strips from all materials measuring 3cm x 1cm were cut and clamped in an EBERS bioreactor. Chambers were filled with DMEM and all samples were under cyclic uniaxial distension was set at 25% elongation, 0.1mm/s rate and 18 cycles per minute over 7 days at 37°C, 5% CO₂.

5 Thereafter, samples were tested for both ramp and cyclic tensile uniaxial tests as described above.

Stress vs strain plots were shown as strength (y axis, MPa) by % of displacement (x axis, %) for example in Figure 6C. The linear gradient of each plot was taken as the Young's modulus (MPa) which is used to measure stiffness of the material. Values for Young's modulus and ultimate tensile strength are represented as compared to values derived for healthy abdominal tissues (Chapin *et al.*, 2018), for example in Figure 7.

The appearance of the tri-layer materials

15 Figure 8 shows, using scanning electron microscopy (SEM), random PU fibres which have been spun directly onto the collector using the same solution described for the tri-layers. The bottom surface shows some merging of the fibres almost certainly due to solvent evaporation. The upper surface shows an open porous network. Due to problems with solvent evaporation fibres of the bottom layer (the one in contact with foil on the collector) melt together giving very small pores. This makes it difficult for cell entry. This may explain the highest mechanical properties of this material compared with the tri-layers. A sacrificial layer of PLA when producing the tri-layer avoids this problem.

Figures 9 (sample 1) and Figure 10 (sample 2) show tri-layer material made on two occasions. Figure 9 shows sample 1. Figure 9A shows the lower surface which has been deliberately spun onto PLA fibres. This shows some residual fibres of PLA which are much thicker, around 2µm, as can be seen from Figure 9B, whereas the PU fibres are around 1µm diameter. Figure 9 shows the upper surface at low (Figure 9C) and high (Figure 9D) magnification. This shows an open network of fibres. Figure 9E shows a cross section of the material and has been labelled to show the areas that represent the random, the aligned and then the random fibres of this tri-layer from the SEM. The thicknesses of the three layers were calculated to be 80, 30 and 80 respectively.

Figure 10 shows sample 2. Figure 10A shows the lower surface which has been deliberately spun onto PLA. This shows some residual fibres of PLA which are much thicker around 2µm as can be seen from Figure 10B, whereas the PU fibres are around 0.5µm diameter. Figure 10 shows the upper surface at low (Figure 10C) and high (Figure 10D) magnification. This

shows an open network of fibres. Figure 10E shows a cross section of the material and has been labelled to show the areas that represent the random, the aligned and then the random fibres of this tri-layer from the SEM. The thicknesses of the three layers were calculated to be 50µm, 20µm and 50µm respectively. These values do not correspond with the values measured with the digital micrometre for analysing the mechanical testing data. The sample is cut bending its edges and placed with an angle for the SEM what makes a non-accurate measurement.

Scanning electron microscopy assessment of scaffolds

For imaging of scaffolds these same fixed samples were processed and gold sputter coated (Edwards sputter coater S150B, Crawley, England). Samples were imaged using a Phillips XL-20 scanning electron microscope (Cambridge, UK). Fibre diameter and pore size of each scaffold was assessed.

Sheep vaginal and abdominal wall biomechanical properties after implantation of electrospun meshes

Methodology

The study was for preclinical evaluation (biomechanics, histomorphology, local complications) of newly produced electrospun meshes (PU).

Implants were prepared in two sizes:

50x50mm abdominal implant

Groups and time points (Table 3):

Table 3: Groups and time points

Time points (days)	60	180
PU	6	6

Surgical procedure

All animals underwent abdominal wall implantation with single type of the implant under sterile conditions under general anaesthesia.

Experimental surgery - abdominal implantation

Following anaesthesia, sheep were placed in a back lithotomy position. Lower abdominal wall was shaved and disinfected. Longitudinal 5 cm long paramedian skin incisions were made 3cm lateral to the midline and 3cm caudally to the umbilicus. Following lateral dissection, a 40x5mm full thickness defect (abdominal fascia, muscles and peritoneum) were made through the abdominal wall parallel to the midline. Incision was primarily repaired with continuous running 1/0 polydioxanone suture (PDS II 1) and overlaid by the 50x50mm implant. Implant was fixed tension-free with interrupted 3/0 PP sutures in the corners and with additional sutures along the borders (in the middle of the side and half of this distance) (Figure 11). The subcutis and skin was closed with a running 2/0 poliglecaprone (Monocryl) suture.

Postoperative care and analgesia

Postoperative analgesia consisted of Meloxicam (0,5mg/kg) and Buprenorphine 0,3 mg/mL and Chlorocresol 1,35 mg/mL (Vetergesic, Ecuphar, Belgium) 1 mL/day i.m. injection up to the third day after surgery.

Animals were clinically observed for one week. Surgical sites were regularly observed to noted early postoperative complications. Sheep were euthanized at 60 or 180 days.

Mesh explantation

Euthansia

Sheep were euthanized at 60 and 180 days by i.v. pentobarbital (Release, Belgium) IV after sedation with Xylazine HCl (Xyl -M®; VMD; Arendonk ; Belgium) 1 mL/50 kg IM injection.

Abdominal wall explantation

Abdominal explants were retrieved “en-block” following skin removal. Before obtaining the specimen picture with a ruler on side were taken. The implant with surrounding and underlying tissue and muscles (further referred as to the abdominal explant) was resected.

1. Ball burst test specimens - circular specimen (diameter 30mm)
2. Histology - rectangular specimen – rectangular specimen (5x10mm)
3. Contractility – 1x rectangular specimen – caudal and cranial (5x10mm)
4. OCT – rectangular specimen (5x10mm)
5. Snap frozen

Macroscopical evaluation

During dissection evaluation of the presence of herniation, erosions, fluid collections or infections was undertaken and pictures will be taken. The presence and severity of adhesion formation was documented (also by a picture). This involves documentation of the area (%) of the implant surface that was covered by adhesions. The density of adhesions was graded on a scale of 0–III, where 0 represents no adhesions, I adhesions that can be easily separated, II mild adhesions that are more difficult to separate and III dense adhesions, which can only be surgically separated (Toosie et al., 2000).

The longitudinal and transversal dimensions were measured similarly along/perpendicular to cranio-caudal body axis (analogue calliper). The later were used to calculate area reduction of the implant.

The surface of the implant was measured before implantation and at sacrifice (width and length at 3 levels). Proportional shrinkage will be defined as % shrink = (surface of the implant at implantation minus surface of the implant at sacrifice)/surface at implantation X 100%.

Assessment of the explant

Tissue collection and histological analyses

1. 1.0x0.5 cm tissue pieces fixed in 10% neutral buffered formalin overnight, washed in PBS for 2 hours and stored in Ethanol 70% before embedding

2. Specimens were embedded in paraffin and cut into 6 µm slices in a longitudinal fashion so that each slice contained the implant, interface and surrounding native tissue.

3. Sections were stained with

a. Haematoxylin & Eosin - basic inflammatory reaction (FBGC, PMN according to Badytax)

b. Trichrome - morphometry

c. Alpha-smooth muscle actin (α-SMA) - smooth muscle content (1:200 dilution)

d. CD34 vascularisation (1:2000 dilution)

e. CD 45 – lymphocytes (1:200 dilution)

f. HLA-DR, CD163 Macrophages subtypes (M1 and M2 respectively) (1:100 and 1:300 dilution, respectively)

g. PGP9.5 Innervation staining (1:1000 dilution)

Histologic assessment

H&E stains

Sections for H&E staining were deparaffinized with xylene for 2 min and then re-hydrated in 2 changes in IMS from 100% absolute alcohol (1 min) to 70% alcohol (30 sec). After this, samples were washed for 1 min in distilled water, and were stained in Harris haematoxylin for 1 min and 30 sec. After another wash in running tap water for 4 min samples were stained in eosin for 5 min. Then, samples were dehydrated in 70% alcohol (IMS) by dunking the samples and then in 100% alcohol for 30 sec. Finally, they were cleaned in xylene and mount with a coverslip using a DPX mounting medium.

H&E stains were performed to quantify the presence of foreign body giant cells (FBGC), polymorphonuclear (PMN) and vessels (vascularity). Five randomly chosen non- overlapping fields per slide scored at a magnification of $\times 400$ and averaged. Fields randomly selected at the interface between the implant and surrounding tissue. An ordinal scale was used similar to that described by Badylak, where scores are made as follows: none of the cells/vessels per high-power field (score 0), 1–5 (score 1), 6–10 (score 2) and >10 (score 3).

Trichrome stains

Sections for trichrome staining were deparaffinized and re-hydrated as above for the H&E method. Once washed in distilled water sections are incubated in Weigert's haematoxylin for 5 min and washed in running tap water for another 5 min. Then samples were washed with 1% acetic acid for 30 sec and incubated with Azophloxine solution (Reagent 1, Masson-Goldner staining kit) for 10 min. After another wash with 1% acetic acid for 30 sec samples were incubated with Tungstophosphoric acid orange G solution (Reagent 2, Masson-Goldner staining kit) for 1 min. After another wash with 1% acetic acid for 30 sec samples were incubated with Light green SF solution (Reagent 3, Masson-Goldner staining kit) for 2 min. After a final washed with 1% acetic acid for 30 sec samples were dehydrated by increased alcohol incubations from 70% IMS for 30 sec, to 96% IMS form 30 sec, to 3 washes with 100% IMS of 30 sec, 30 sec and 2 min respectively. Finally samples were washed with xylene doing 2 incubations of 2 min each. Samples were then mount with a coverslip using a DPX mounting medium.

Trichrome stains extracellular connective tissue (mainly unspecified collagen) blue. Five non-overlapping images 400x magnification were obtained and semi-quantitatively evaluated using a blind scoring done by 3 researchers for percentage of area occupied by collagen at interface mesh-surrounding tissue.

Immunohistochemistry

Sections for immunohistochemistry (IHC) were deparaffinized by 2 changes of xylene, 2 min each, and then, re-hydrated with 2 changes in 100% absolute alcohol (IMS), 2 min each, and 10 min in 95% alcohol. After this, samples were washed briefly in distilled water, and 2 washes more with Tween 20-PBS were performed of 2 min each. Sections were incubated for 10 min with hydrogen peroxide (Mouse and Rabbit Specific HRP/DAB Detection IHC Kit) to quench endogenous peroxidase activity. After two washes in Tween 20-PBS, 2 min each, an antigen retrieval step was performed to break the protein cross-links and therefore to unmask the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies, with 0.05% trypsin (v/w) and 0.1% Calcium Chloride (v/w) in distilled water, by 20 min incubation at 37°C. After 10 min at room temperature to cool down samples, sections were washed twice in Tween 20-PBS, 2 min each, and incubated with protein blocking serum (Mouse and Rabbit Specific HRP/DAB Detection IHC Kit) for 10 min to avoid non-specific staining. After this, samples were incubated for 2 hours with primary antibodies diluted in 1% bovine albumin serum, as above. Then sections were washed 3 times in Tween 20-PBS, 2 min each, and incubated for 10 min with a biotinylated secondary antibody (Mouse and Rabbit Specific HRP/DAB Detection IHC Kit). After 3 more washes with Tween 20-PBS and samples were incubated with streptavidin (Mouse and Rabbit Specific HRP/DAB Detection IHC Kit) for another 10 min. After 3 more washes in Tween 20-PBS, 2 min each, samples were incubated with a DAB chromogen (Mouse and Rabbit Specific HRP/DAB Detection IHC Kit) for another 10 min for brown staining being developed. Samples were then washed 2 more times with Tween 20-PBS, 2 min each. Samples were counterstained with Harris haematoxylin for 3 seconds and excess staining was eliminated by several washes in distilled water. Finally, samples were dehydrated again for 10 min in 95% alcohol, followed by 2 changes in 100% alcohol of 2 min each. Samples were cleaned by 2 changes of xylene, 2 min each, and were mounted with a coverslip using DPX mounting medium. Controls consisted of samples incubated without primary and secondary antibodies, or incubated only with secondary antibodies.

Semi-quantitative assessment of the extent of immunostaining was performed on a blinded observer basis using a qualitative grading scale; absent=0, mild presence=1, large presence=2, abundance=3, great abundance=4. Five representative images from 2 representative samples at each time point were assessed by three blinded researchers (n=30). Example photographs depicting 0, 1, 2, 3 and 4 were provided for reference and the median value from these scores was used. The M2/M1 ratio was also calculated for each group using the values from the blind scoring of the immunostaining.

Statistics

Differences in the different staining were tested for statistical significance with a GraphPad Prism 8 software considering both factors together (group and implantation site) using a two-way ANOVA test and doing multiple comparisons between individual groups using a Sidak's test.

5

Results

Figure 12 (H+E staining) and Figure 13 (Masson-Goldner trichrome staining) show integration of PP meshes and tri-layer PU scaffolds within abdominal at 60 and 180 days. Whilst a strong host cell response around the macro-filaments of the PP meshes can be seen, there is a large cell infiltration within the tri-layer PU scaffolds with new blood vessels formed within the synthetic material. At 180 days it can still be seen that the 3 layer structure of the PU scaffolds with the aligned layer of fibres in the middle act as a barrier which cells cannot penetrate. There is a lot of new tissue formation around and between the microfilaments of the PP meshes similar to the new tissue formed at both sides of the tri-layer PU scaffolds.

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Figure 16 (semi-quantitative scoring of the H&E data, of Figure 12) shows a similar number of foreign body giant cells (FBGC) between PP meshes and tri-layer PU scaffolds, which was null for the control and sham. The number of polymorphonuclear cells (PMN) was higher for PP compared to all the other groups. This may indicate a higher risk of infection when using PP meshes. Similar vascularity was measured between materials which was higher than controls at both time points.

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Figure 17 (semi-quantitative scoring of the Masson-Goldner trichrome data of Figure 13) shows similar values of trichrome staining for all groups in implant sites, suggesting similar collagen/connective tissue form surrounding the materials.

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Figure 18 (semi-quantitative scoring of the IHC data of Figures 12 – 15) shows a similar vascularization between materials, as demonstrated by the CD34 staining, with higher values than the control group only visible within the abdomen at 180 days. Similar to trichrome staining, there was a similar smooth muscle actin (SMA) staining for all groups, which was again only lower for the control group at 180 days within the abdomen. There was a similar nerve (PGP9.5) staining between all groups. In conclusion, both materials stimulate regeneration of a functional new connective tissue similar to the tissue formed by the normal wound healing represented by the sham group, with neo-vascularization, new collagenous matrix and new innervation respectively.

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In terms of host/inflammatory response, Figure 18 shows that while the lymphocyte (CD45) staining was much higher for both materials than the control and sham groups at 180 days within the abdomen. Same results were found for the M1 response (HLA-DR staining), with a higher value at 180 days for both materials than the control and sham groups within the abdomen. Again, the M2 response (CD163 staining) was higher for both materials at 180 days within the abdomen compared to control and sham groups. The results suggest, as expected, a higher host response against the materials. Nevertheless, looking at the M2/M1 ratio (Figure 23), it is positive and similar for both materials at 180 days within the abdomen, being similar to the sham group and being negative for the PP meshes more related to rejection with a chronic inflammatory response. The positive ratio for the tri-layer PU scaffolds suggest better integration with reconstructive remodelling.

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CLAIMS

1. A scaffold for the treatment of a hernia, wherein the scaffold comprises at least two layers of polyurethane: at least one layer in which the polyurethane fibres are randomly orientated and at least one layer in which the fibres are aligned.
2. The scaffold in accordance with claim 1, wherein the scaffold comprises at least two layers in which the polyurethane fibres are randomly orientated and wherein the at least two layers are separated by at least one layer in which the fibres are aligned.
3. The scaffold in accordance with claim 1, wherein the scaffold comprises at least two layers in which the polyurethane fibres are aligned and wherein the alignment of the at least two layers are in different orientations.
4. The scaffold in accordance with claim 3, wherein the scaffold comprises at least two layers in which the polyurethane fibres are randomly orientated and are separated by at least one layer in which the fibres are aligned.
5. The scaffold in accordance with any one of claims 1 to 4, wherein the polyurethane has a hardness of between 80A to 90A.
6. The scaffold in accordance with any one of claims 1 to 4, in which the polyurethane is polycarbonate urethane.
7. The scaffold in accordance with claim 1 or claim 2, wherein the scaffold has an ultimate tensile strength of between 0.7 and 3 MPa.
8. The scaffold in accordance with any one of claims 1 to 3, wherein the scaffold has a strain at ultimate tensile strength of 250% and 300%.
9. The scaffold in accordance with any one of the preceding claims, wherein the layer(s) in which the polyurethane fibres are randomly orientated comprise pores from the outermost surface of a depth of at least 16 μ m.

10. The scaffold in accordance with any one of the preceding claims, wherein the mean pore size in at least one layer in which the polyurethane fibres are randomly orientated is at least 10 μ m.
11. The scaffold in accordance with any one of the preceding claims, wherein at least 20% of the pores on the outer surface of the at least one layer in which the polyurethane fibres are randomly orientated are greater than 18 μ m.
12. The scaffold in accordance with any one of the preceding claims, wherein at least 5% of the pores on the outer surface of the at least one layer in which the polyurethane fibres are randomly orientated are greater than 20 μ m.
13. The scaffold in accordance with any one of the preceding claims, wherein the scaffold has a width of at least 4 cm and a length of at least 10cm.
14. A method of preparing a scaffold for treatment of a hernia, the method comprising:
 - a. Electrospinning a sacrificial layer of poly-lactic acid (PLA) onto a rotating surface;
 - b. Electrospinning a layer of polyurethane in which the fibres are spun in random orientations;
 - c. Electrospinning a layer polyurethane in which the fibres are spun in an aligned orientation;
 - d. Removing the sacrificial layer of PLA to produce the scaffold.
15. The method of claim 15 further comprising one or more steps between steps c and d selected from:
 - e. Electrospinning a further layer polyurethane in which the fibres are spun in aligned longitudinal orientation, wherein the alignment of fibres is in a different orientation to the alignment in step; and
 - f. Electrospinning a further layer in which the fibres are spun in random orientations.

16. The method according to claim 14 or claim 15, wherein the method comprises the use of at least one syringe pump, wherein either one syringe pump is used for delivering both random fibres and aligned fibres or separate syringe pumps are used to deliver random fibres and aligned fibres.
17. The method according to any one of claims 14 to 16, wherein:
- step c comprises a step-by-step increase and then decrease of the rotating collector speed; and/or
 - wherein step e comprises a step-by-step increase and then decrease of the rotating collector speed.
18. The method according to any one of claims 14 to 17, wherein the sacrificial layer is:
- applied to a surface rotating from about 50 to 100 rpm;
 - produced with a needle to surface distance of from about 20cm to 30cm;
 - produced by delivering polymer solutions at a rate of from about 30 $\mu\text{l}/\text{min}$ to 100 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 13kV to 22kV DC.
19. The method according to any one of claims 14 to 18, wherein step b is conducted after any excess solvent from step a. have evaporated.
20. The method according to any one of claims 14 to 19, wherein the polyurethane layers of random fibres are:
- applied to a surface rotating from about 50 to 150 rpm;
 - produced with a needle to surface distance of from about 20cm to 30cm;
 - produced by delivering polymer solutions at a rate of from about 50 $\mu\text{l}/\text{min}$ to 150 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 13kV to 22kV DC.
21. The method according to any one of claims 14 to 20, wherein the polyurethane layers of aligned fibres are:
- applied to a surface rotating from about 250 to 350rpm for about 15 to 20 minutes, followed by a surface rotating from about 550 to 650rpm for about 15 to 20 minutes, followed by a surface rotating from about 1150 to 1250rpm for about 15 to

20 minutes, followed by a surface rotating from about 550 to 650rpm for about 15 to 20 minutes, and followed by a surface rotating from about 250 to 350rpm for about 15 to 20 minutes;

- ii. produced with a needle to surface distance of from about 20cm to 30cm;
- iv. produced by delivering polymer solutions at a rate of from about 50 $\mu\text{l}/\text{min}$ to 150 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 13kV to 22kV DC.

22. The method according to any one of claims 14 to 16, wherein:

- i. step c starts before step b finishes such that there is an overlap between the layers; and/or
- ii. wherein step d starts before step c finishes such that there is an overlap between the layers.

23. The method according to any one of claims 14 to 16 and 22, wherein the sacrificial layer is:

- i. applied to a surface rotating from about 200 to 400 rpm;
- ii. produced with a needle to surface distance of from about 15cm to 20cm;
- iii. produced by delivering polymer solutions at a rate of from about 30 $\mu\text{l}/\text{min}$ to 40 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 15kV to 19kV DC.

24. The method according to any one of claims 14 to 16 and 22 to 23, wherein the polyurethane layers of random fibres are:

- i. applied to a surface rotating from about 200 to 400 rpm;
- ii. produced with a needle to surface distance of from about 20cm to 25cm;
- iii. produced by delivering polymer solutions at a rate of from about 30 $\mu\text{l}/\text{min}$ to 40 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 15kV to 25kV DC.

25. The method according to any one of claims 14 to 16 and 22 to 24, wherein the polyurethane layers of aligned fibres are:

- i. applied to a surface rotating from about 500 to 700 rpm;
- ii. produced with a needle to surface distance of from about 5cm to 10cm;

- iv. produced by delivering polymer solutions at a rate of from about 30 $\mu\text{l}/\text{min}$ to 40 $\mu\text{l}/\text{min}$ per syringe with an accelerating voltage of from about 20kV to 25kV DC.

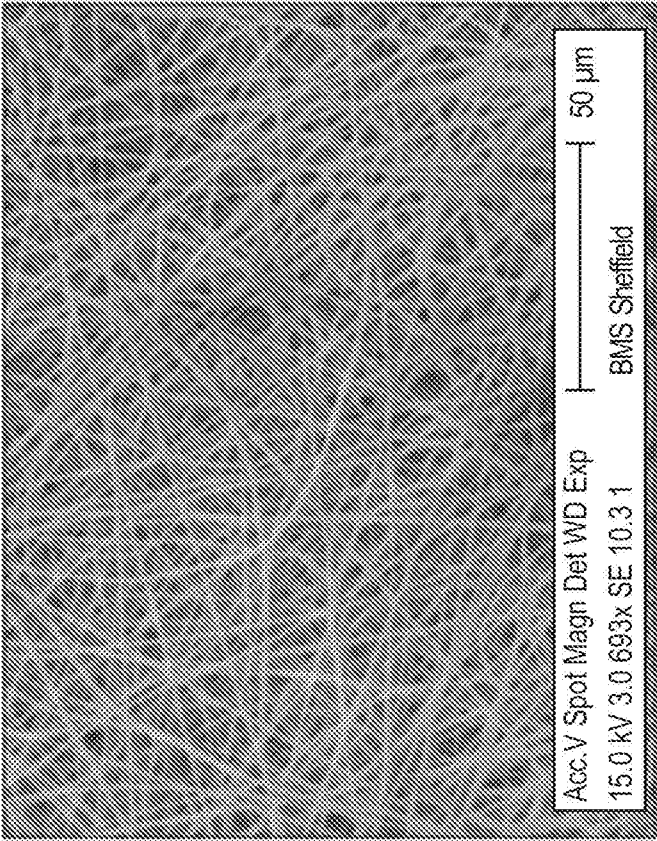
26. The method according to any one of claims 14 to 18, wherein step b is conducted after any excess solvent from step a. have evaporated

27. A scaffold produced by the methods of any one of claims 14 to 26.

28. The scaffold of any one of claims 1 to 13 or claim 27 for use as a medicament.

29. The scaffold of any one of claims 1 to 13 or claim 27 for use in the treatment of a hernia.

Polyurethane Z3 tri-layer
(PLA sacrificial layer)



Bottom
surface

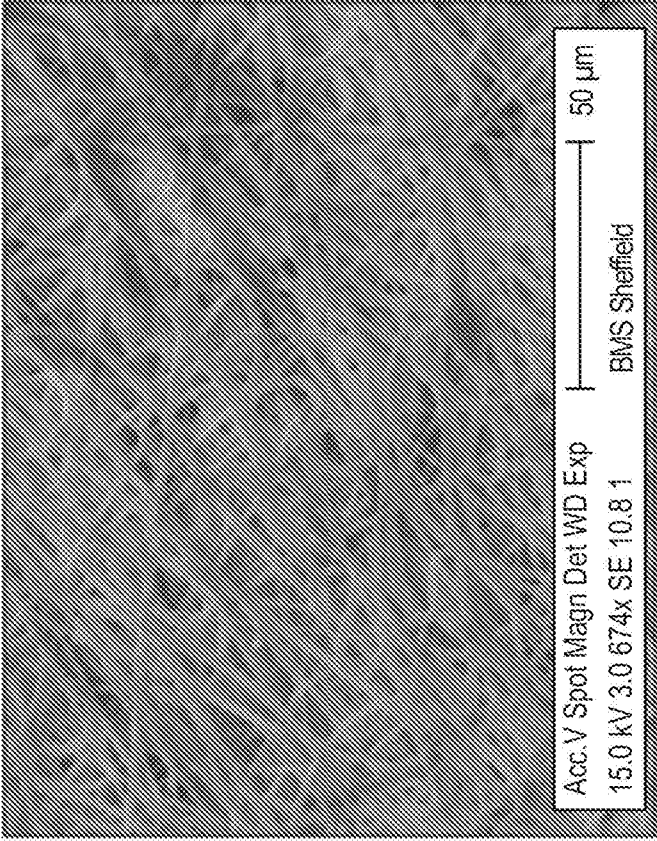


Fig. 1

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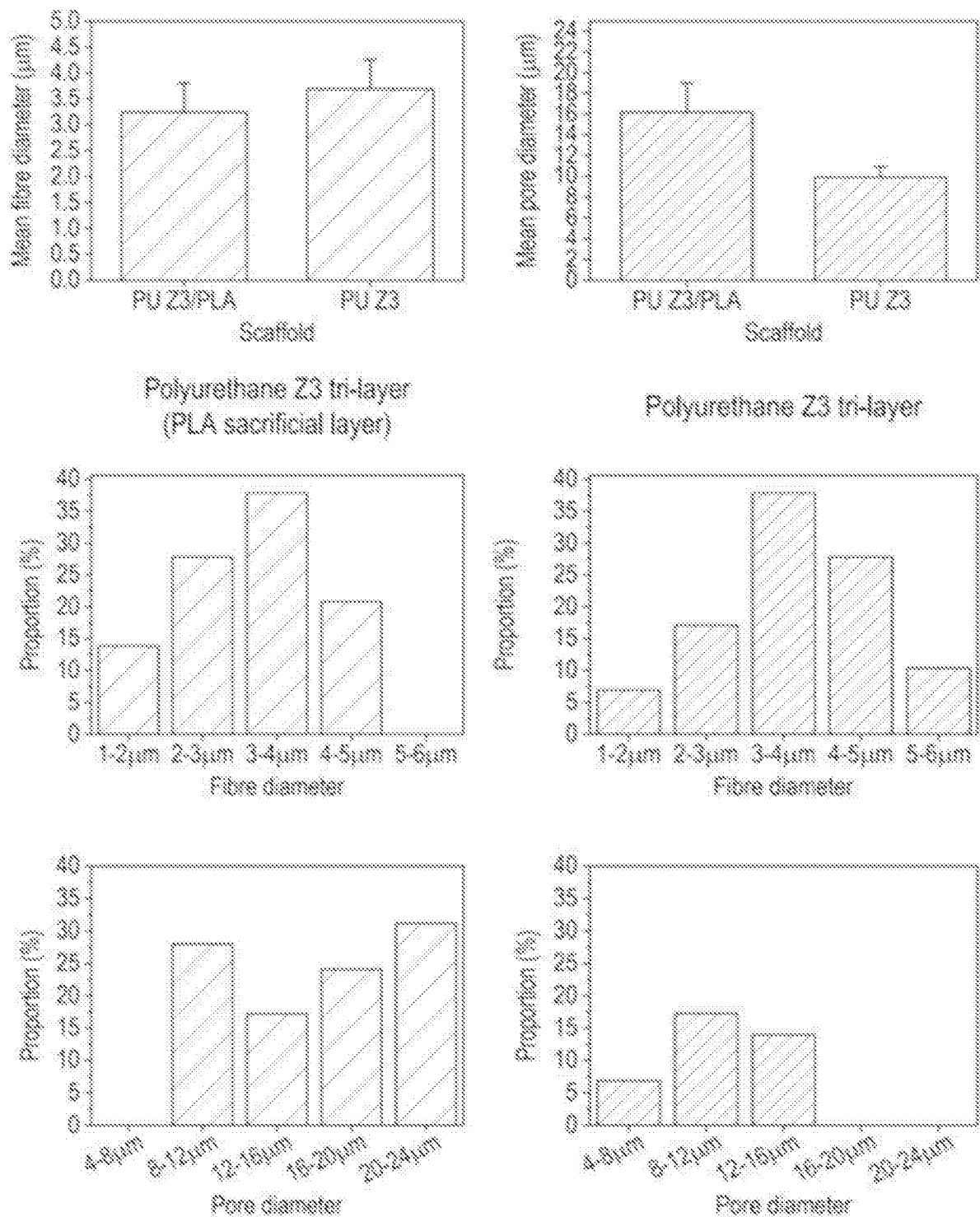


Fig. 2

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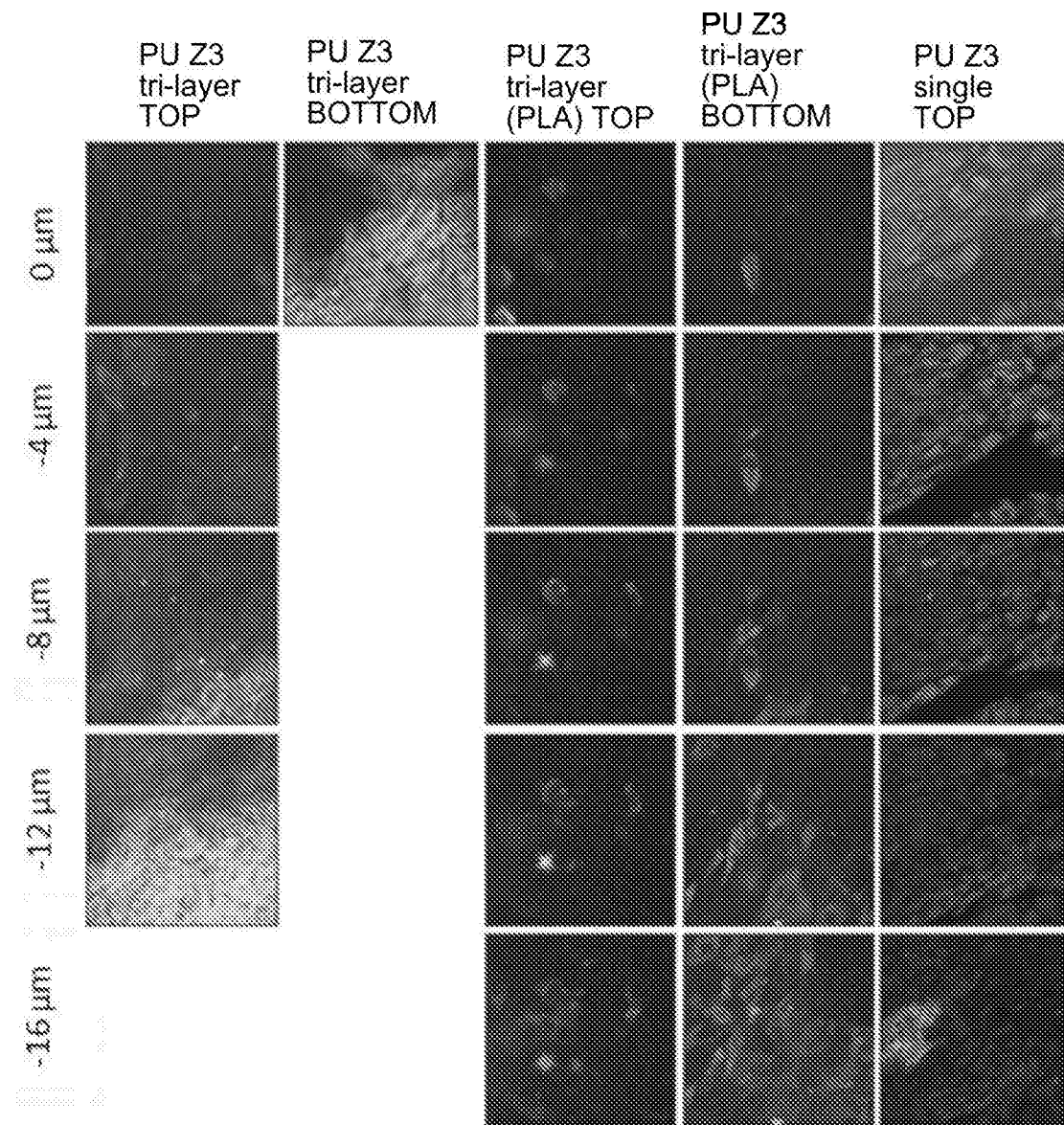
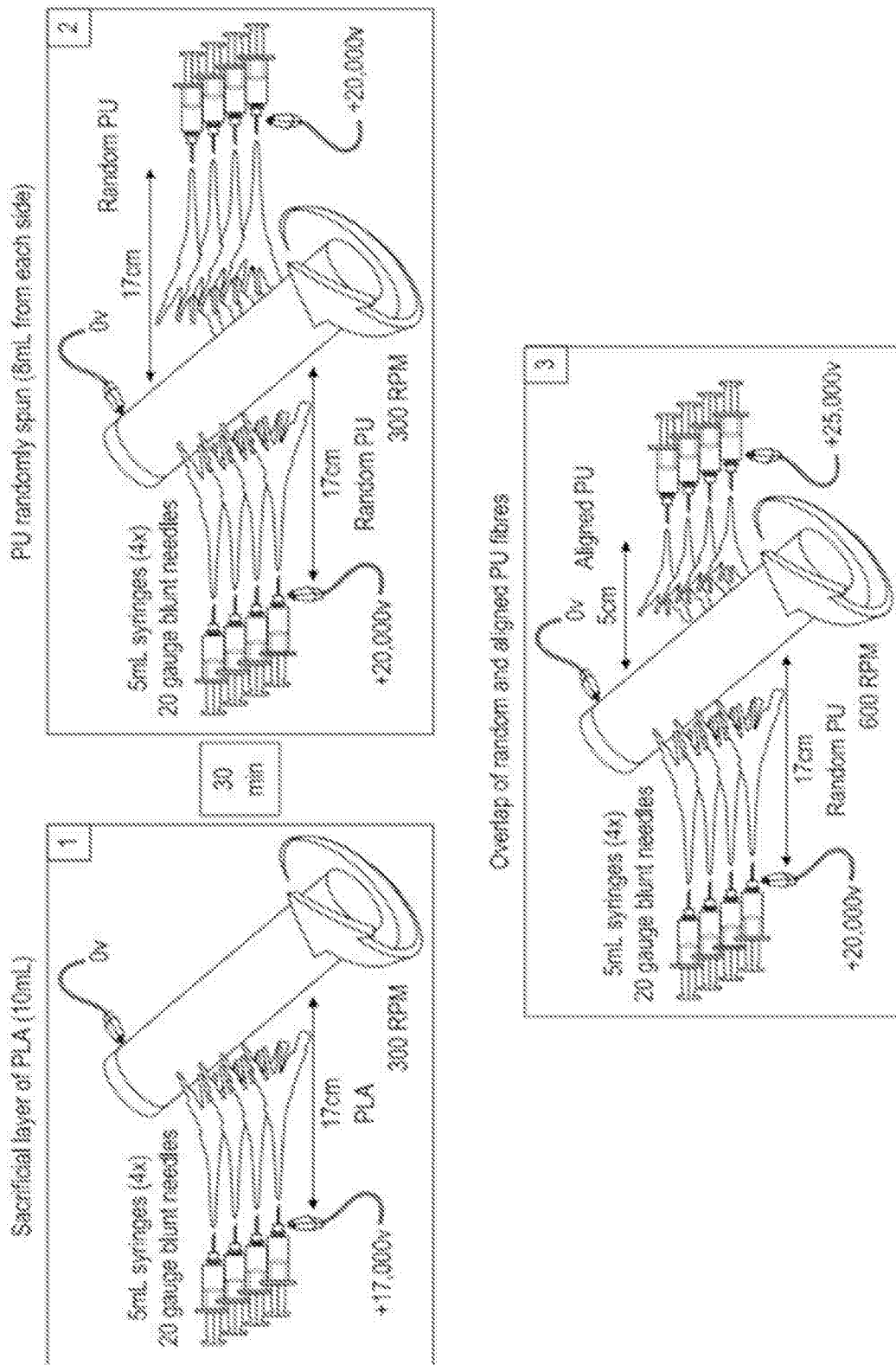


Fig. 3



4.9.

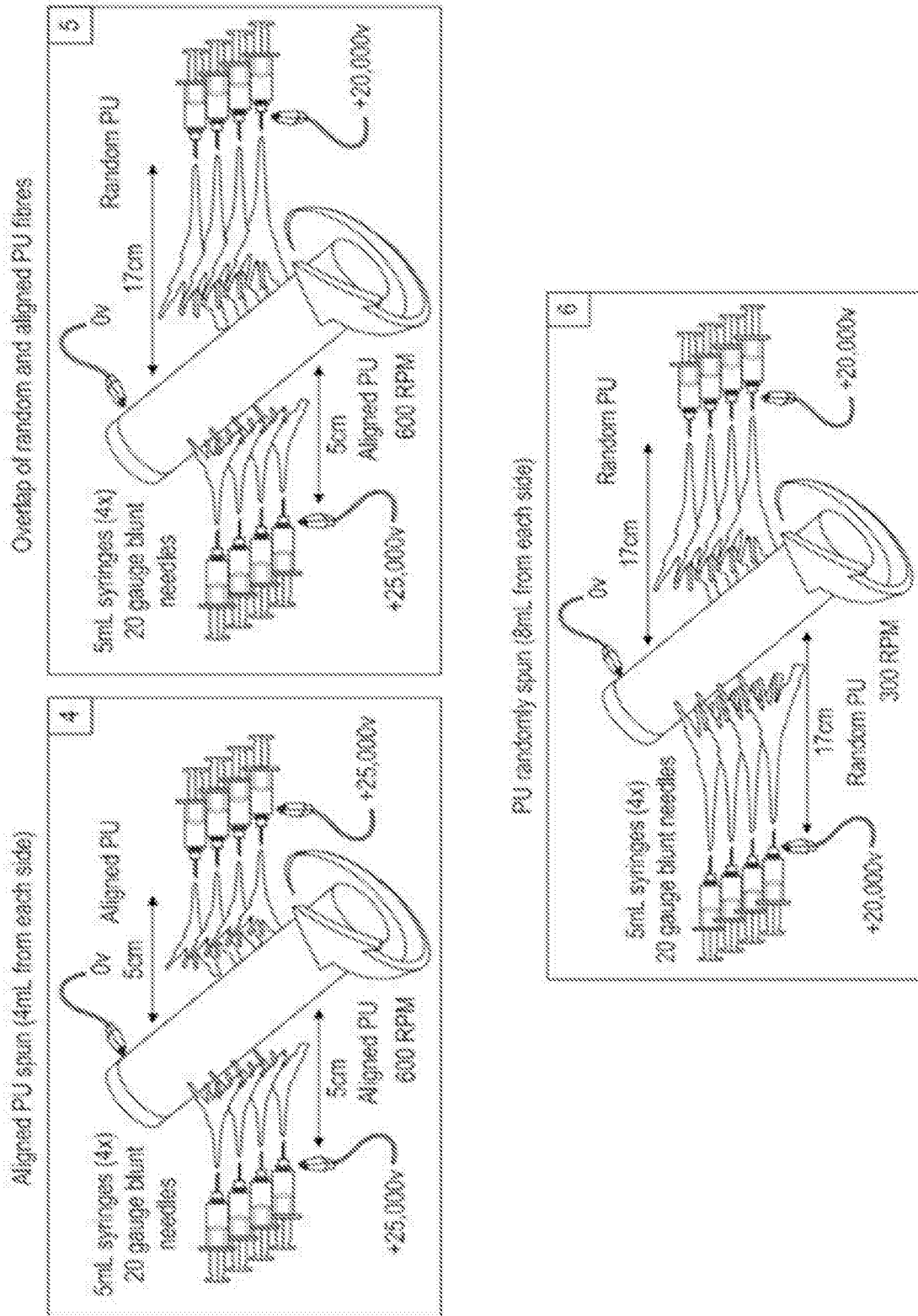


Fig. 5

2 trilayer samples (1 and 2) were tested and compared to previously produced one layer (random) PU scaffolds and commercial polypropylene meshes, as reference material

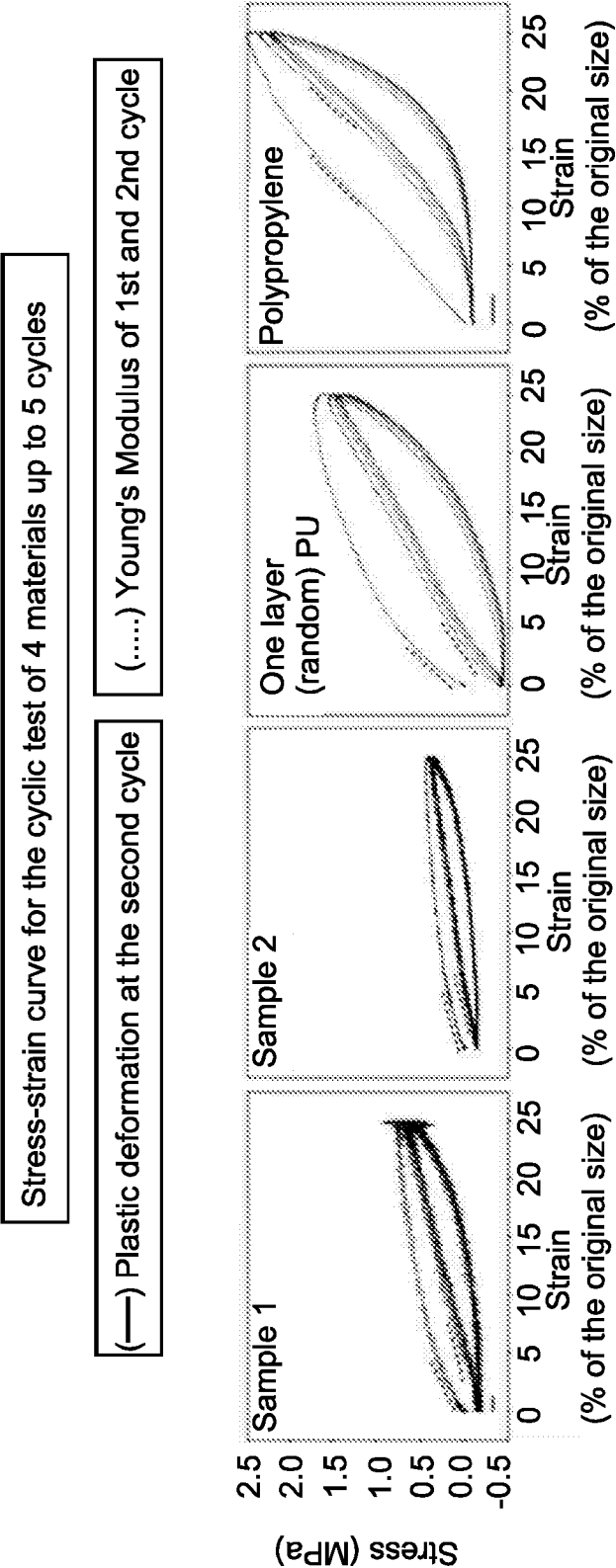


Fig. 6A

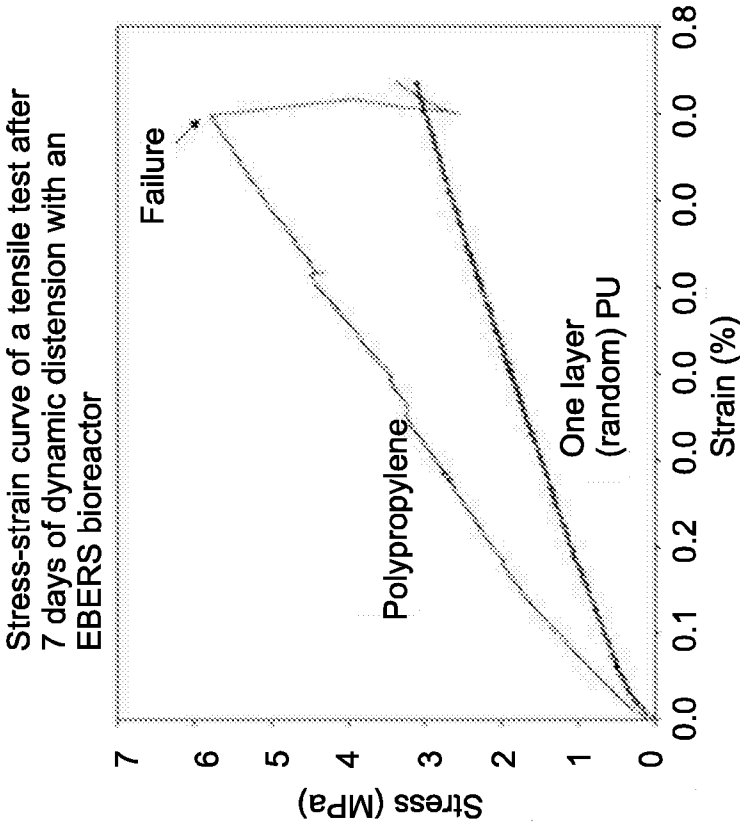


Fig. 6C

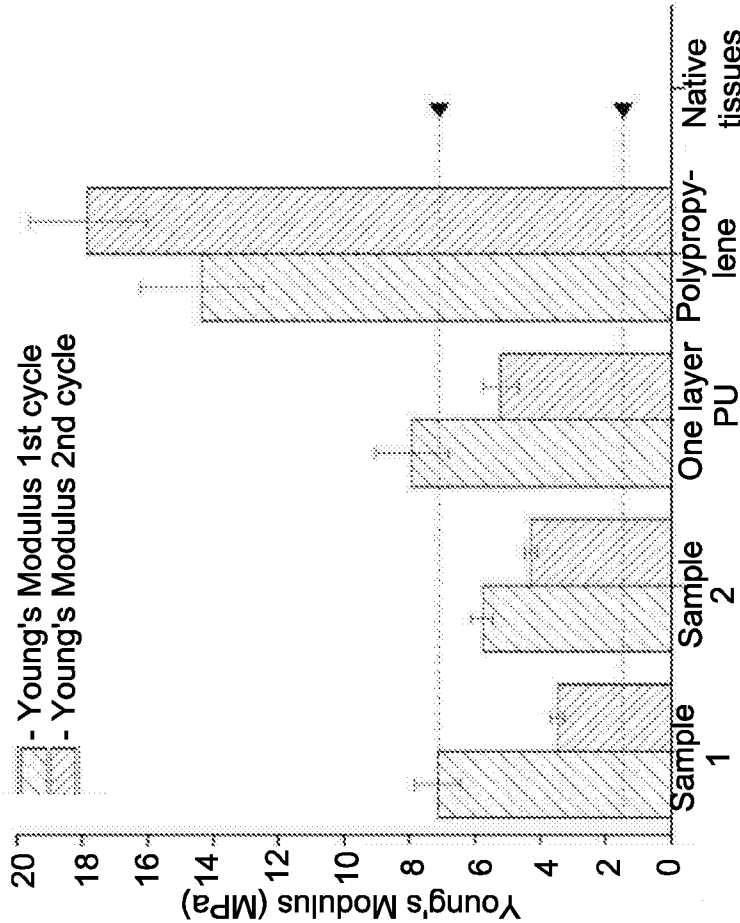
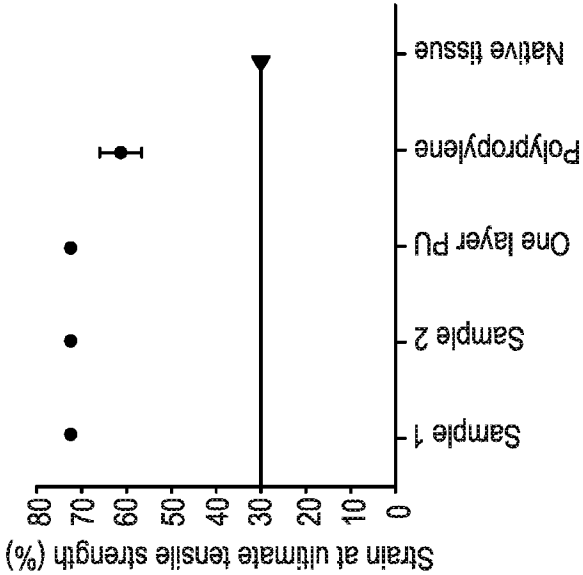
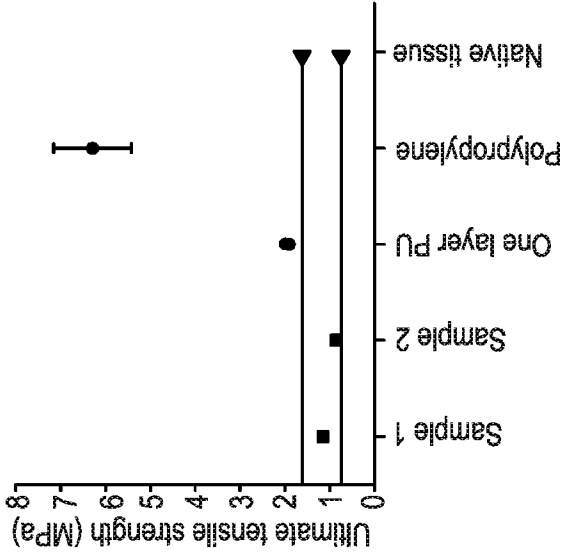
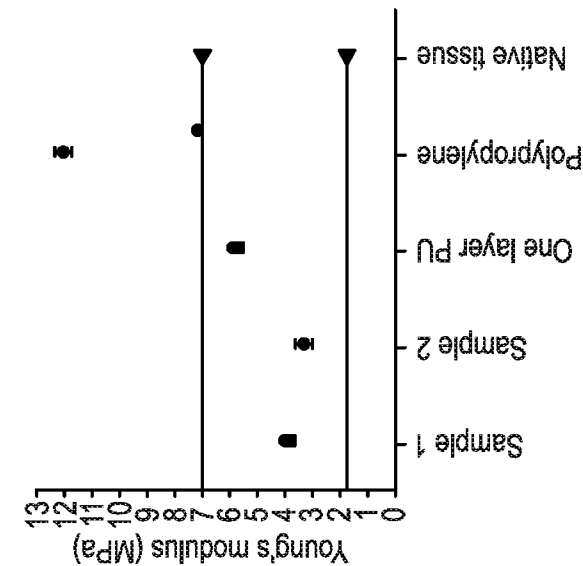


Fig. 6B



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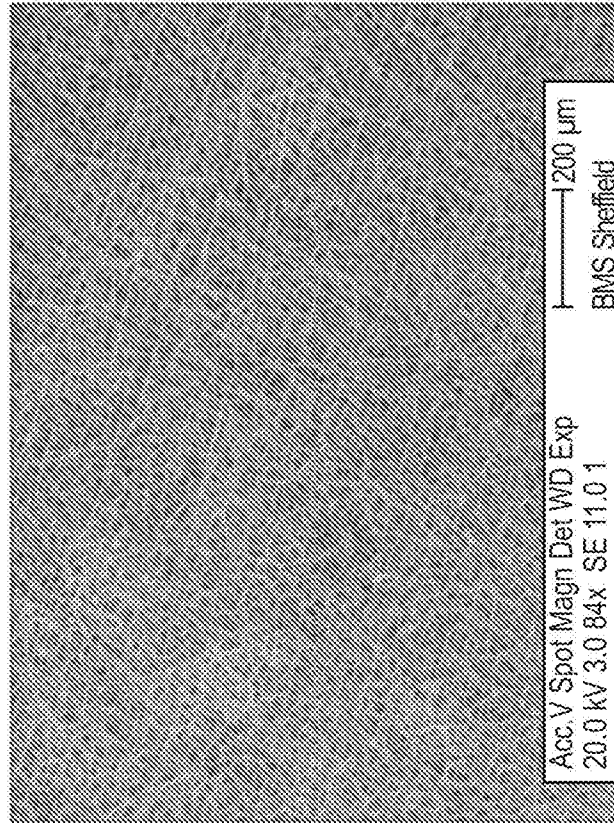


Fig. 8B

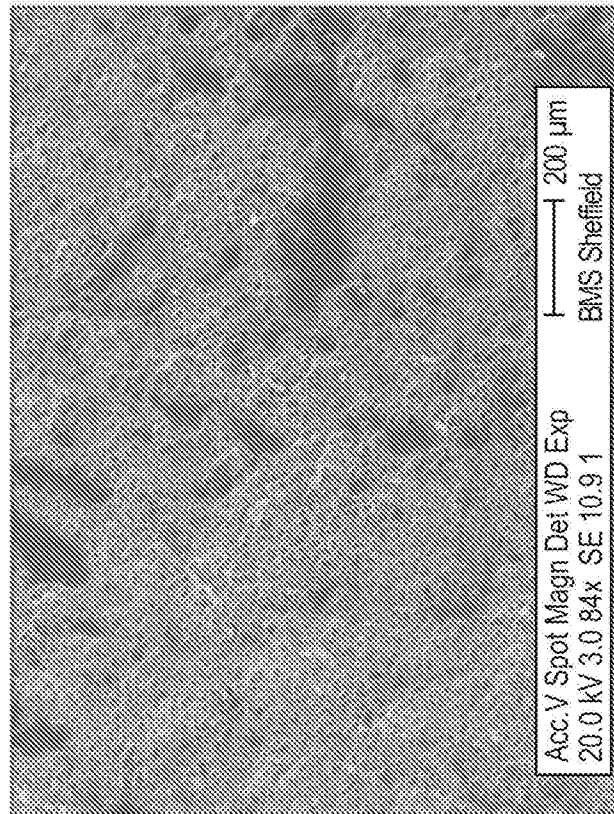
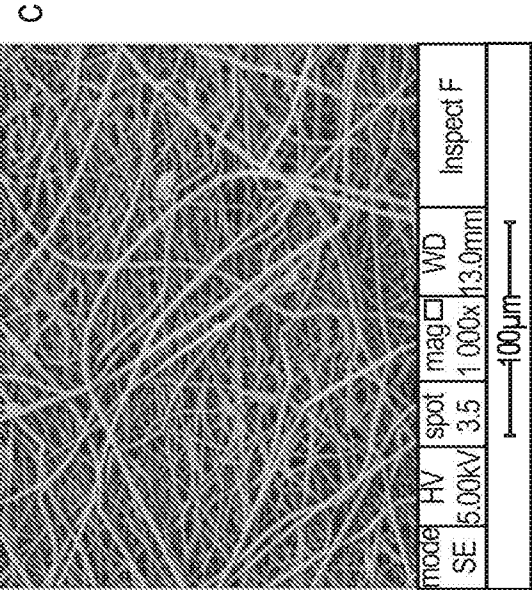
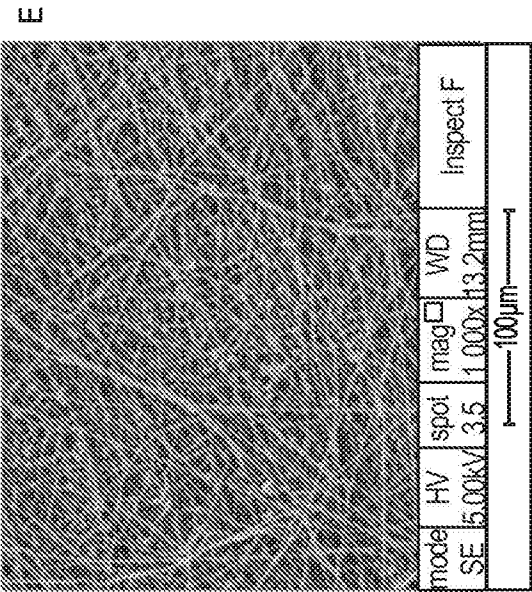
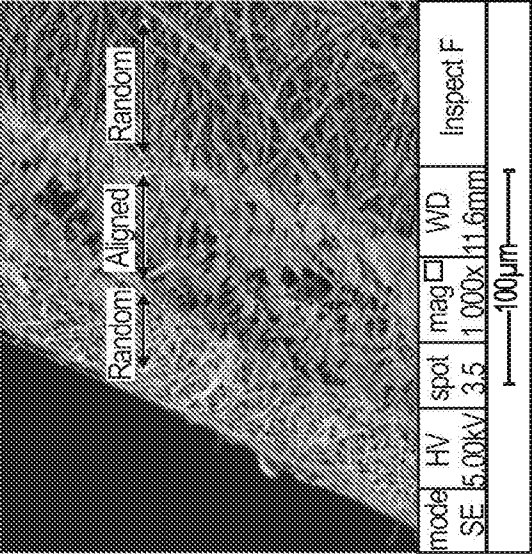
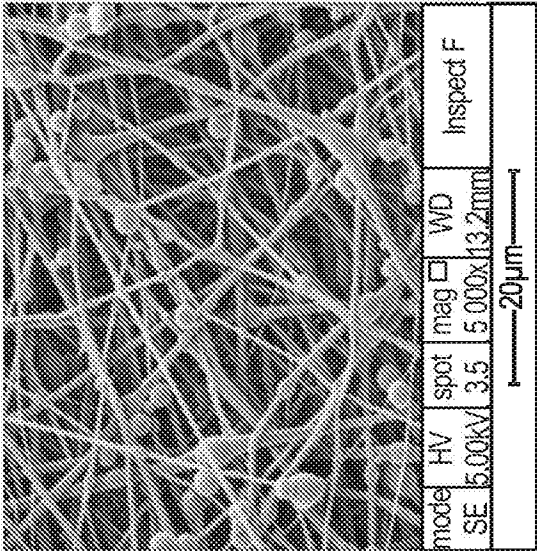


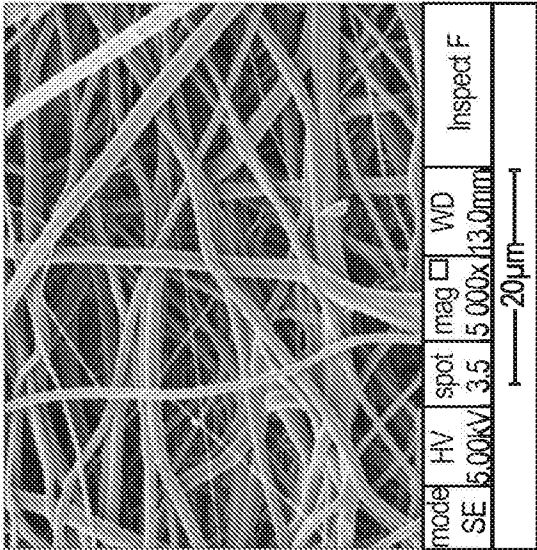
Fig. 8A



A



D



B

Fig. 9

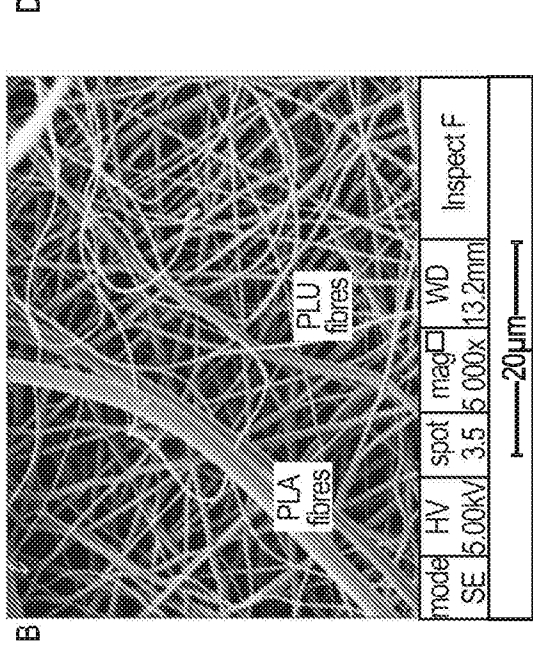
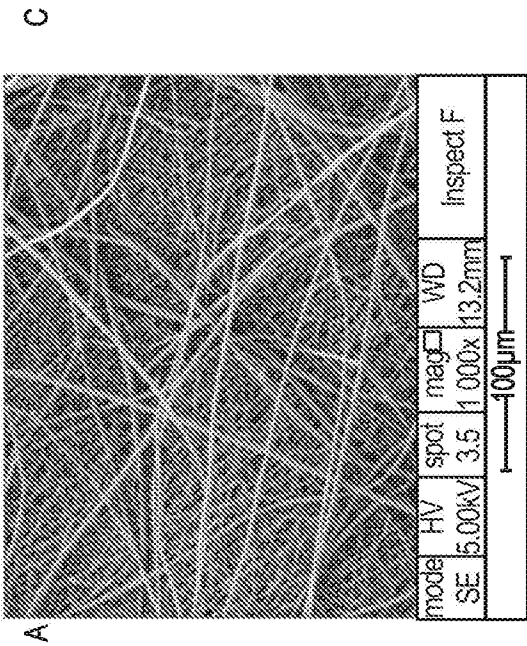
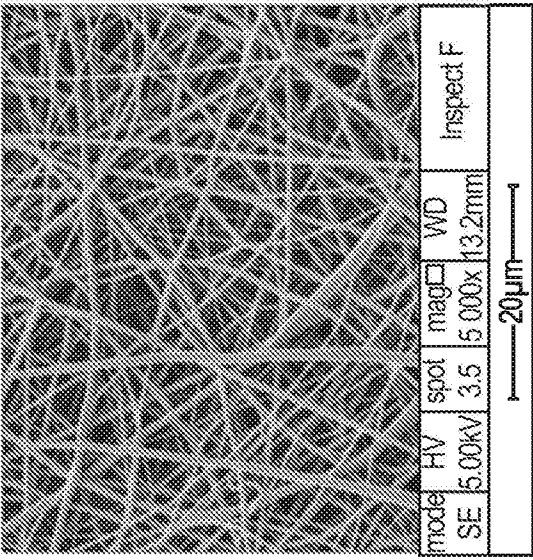
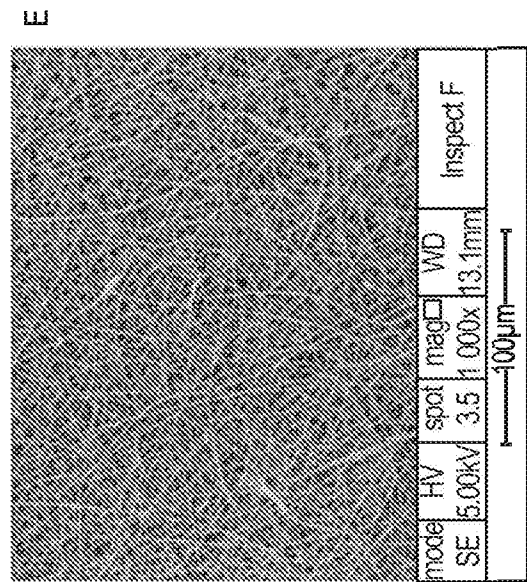
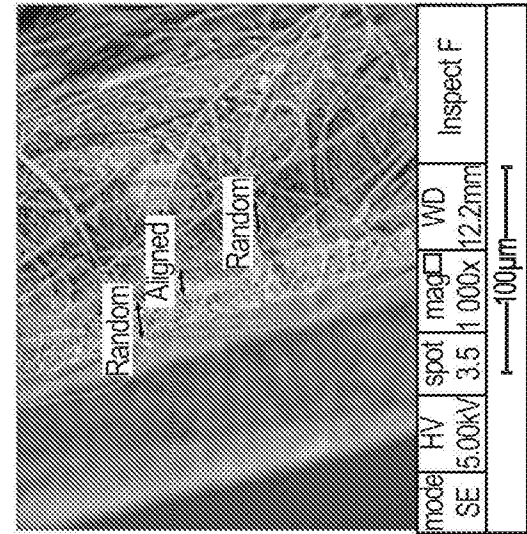


Fig. 10

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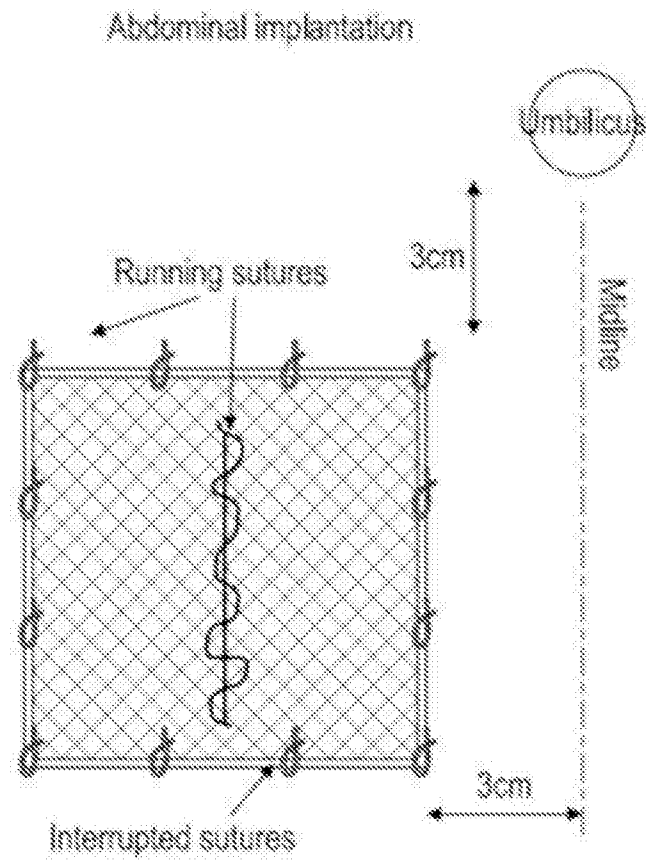


Fig. 11

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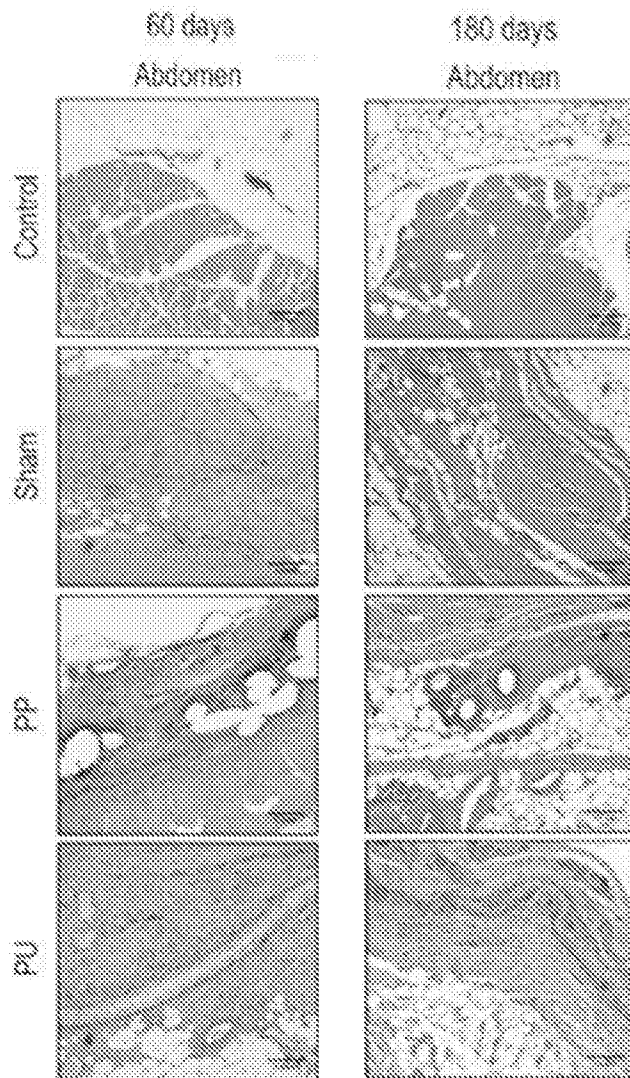


Fig. 12

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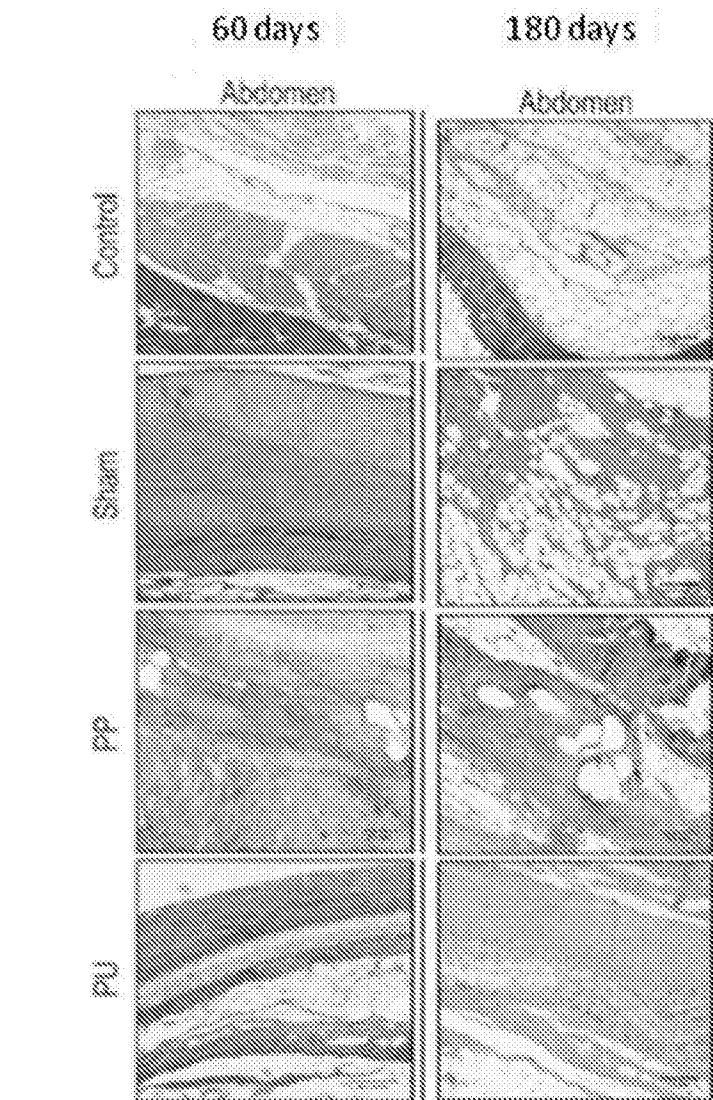


Fig. 13

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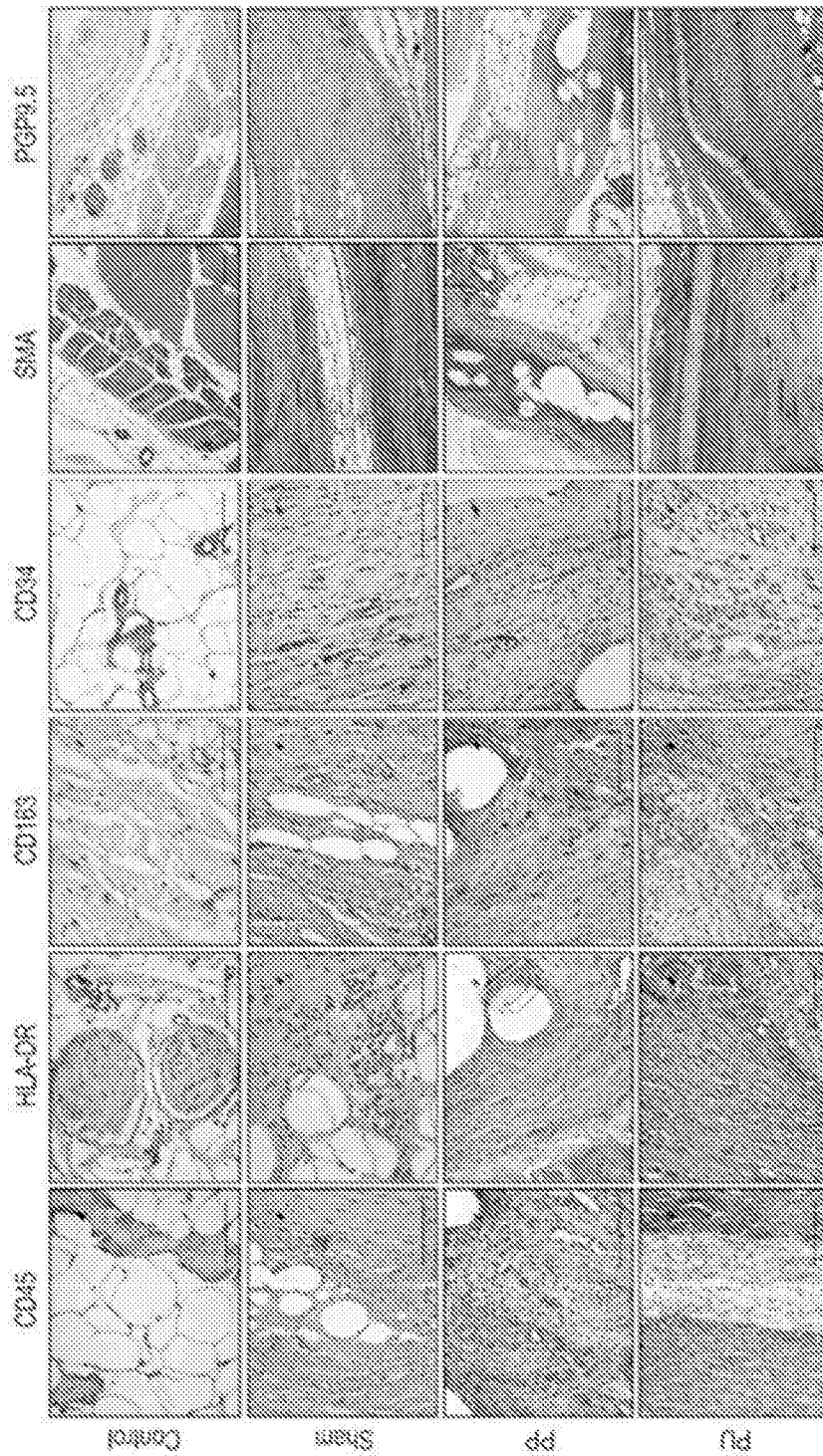


Fig. 14

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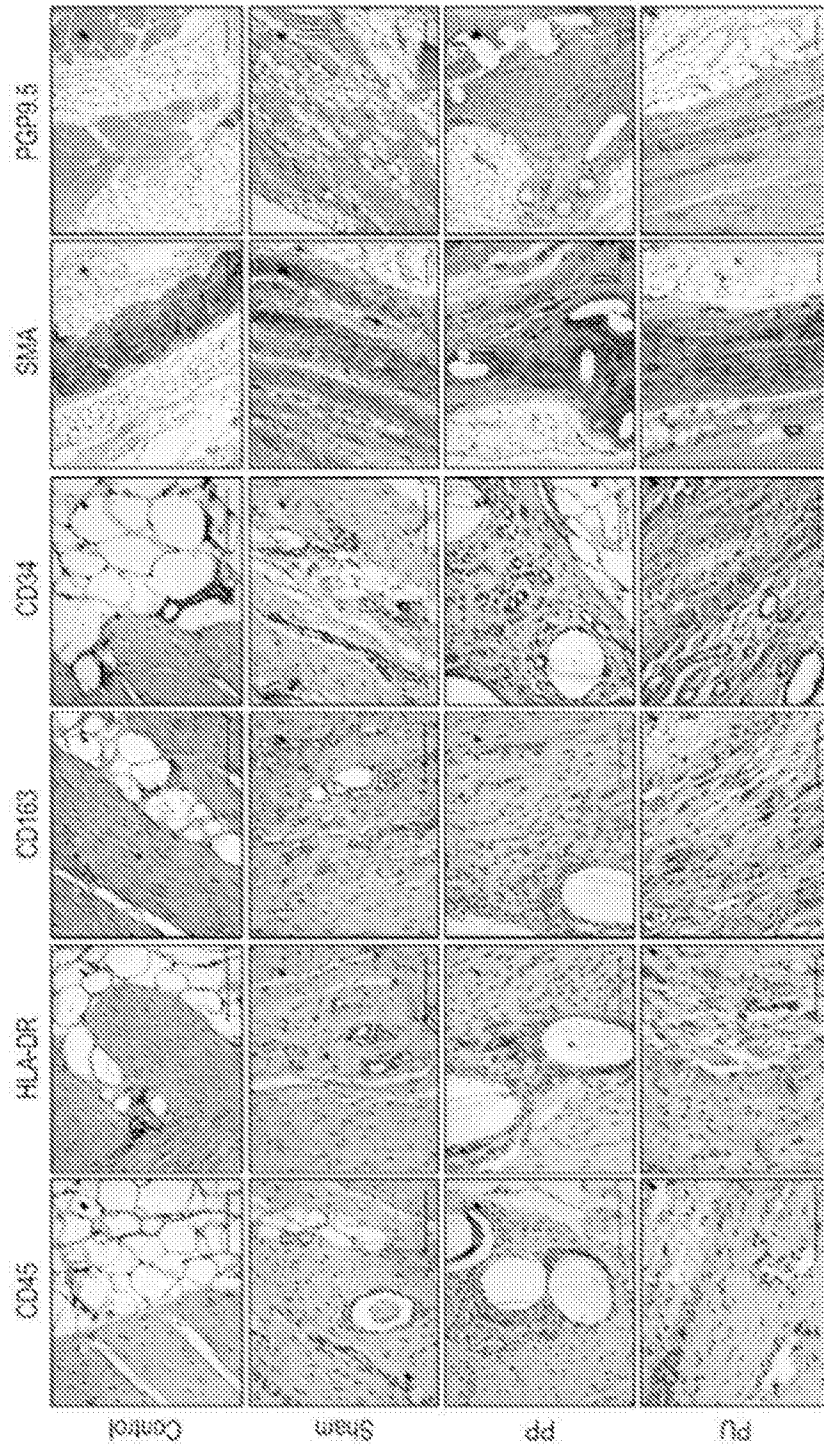


Fig. 15

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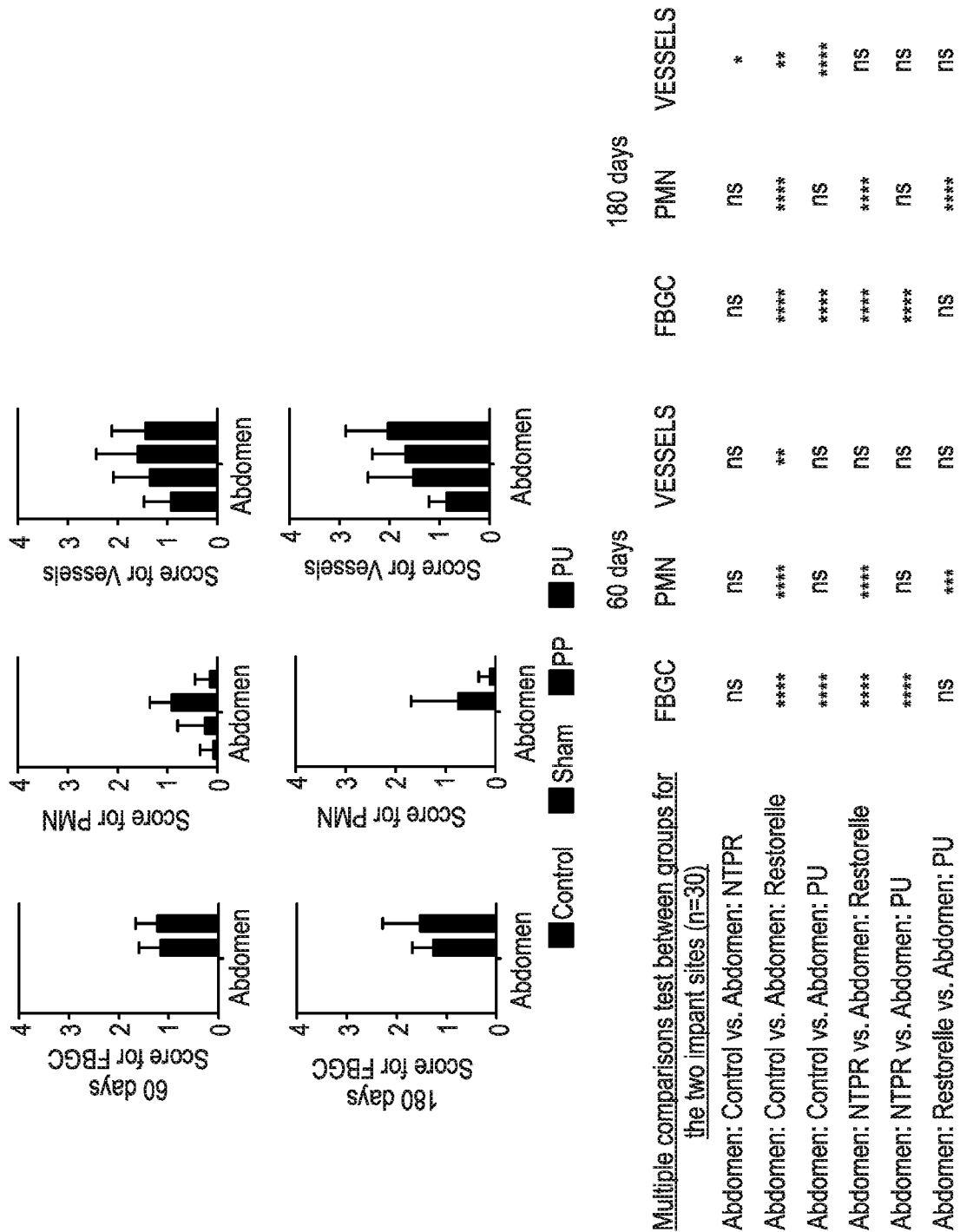
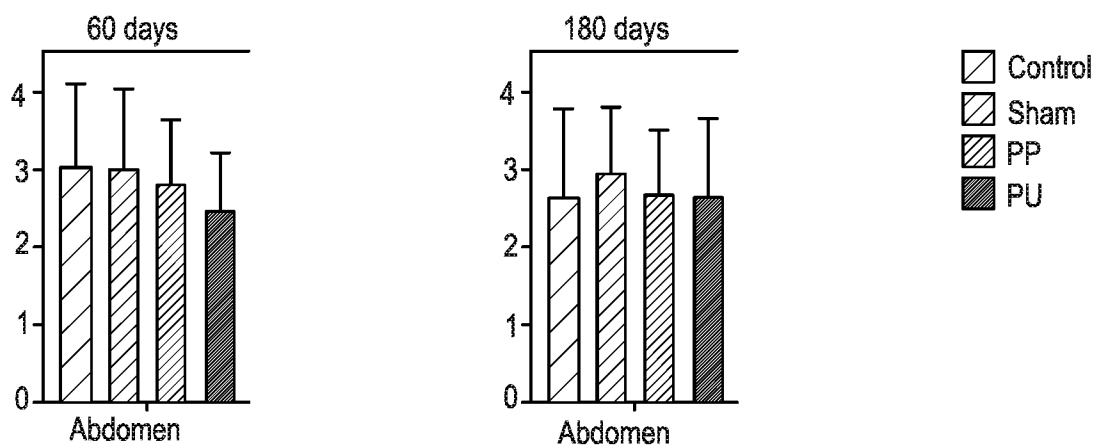


Fig. 16

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Multiple comparisons test between groups for the two impant sites (n=30)	60 days	180 days
Abdomen:Control vs. Abdomen: Sham	ns	ns
Abdomen:Control vs. Abdomen: PP	ns	ns
Abdomen:Control vs. Abdomen: PU	ns	ns
Abdomen:Sham vs. Abdomen: PP	ns	ns
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Abdomen:PP vs. Abdomen: PU	ns	ns

Fig. 17

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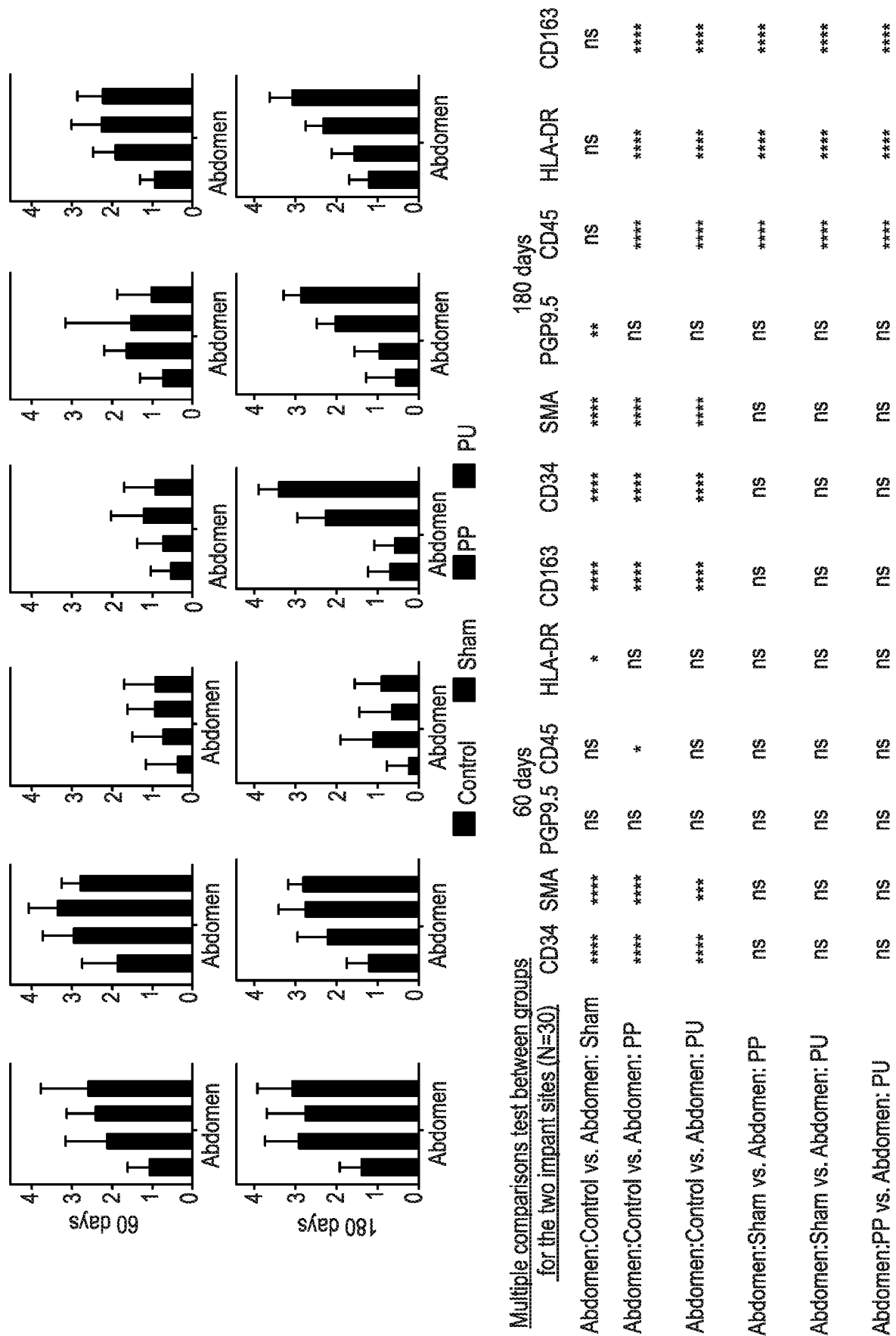


Fig. 18

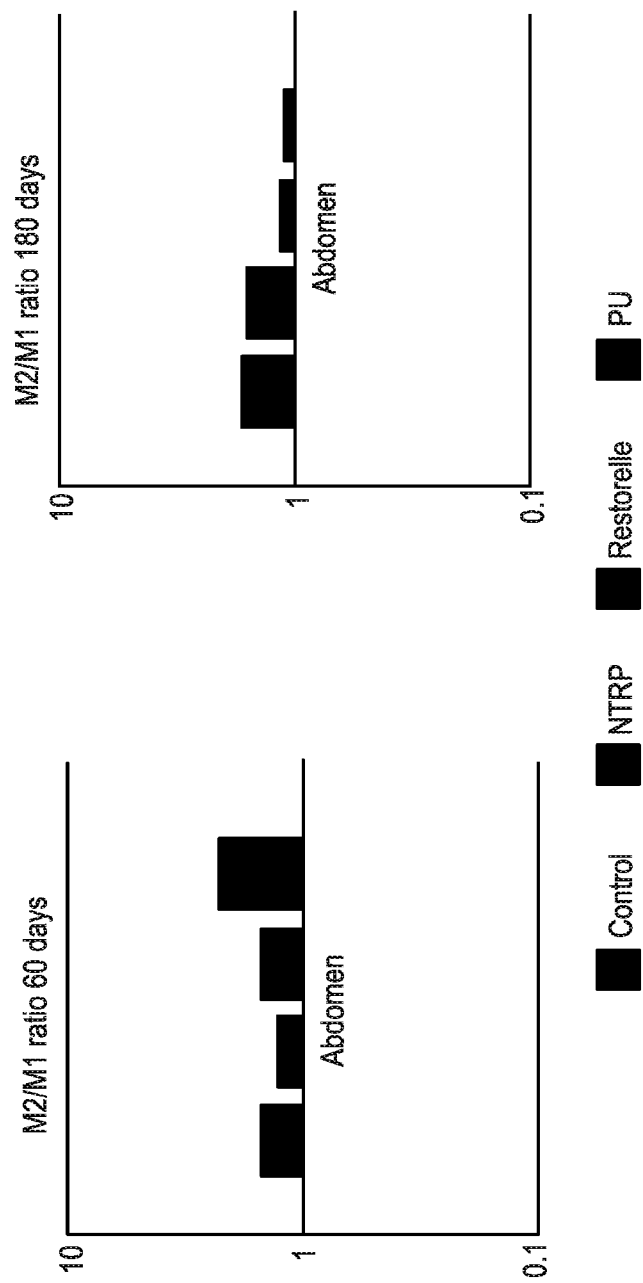


Fig. 19

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2019/053351

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61L27/18 A61L27/56
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2016/045296 A1 (GUO ZEYUE [CN]) 18 February 2016 (2016-02-18) page 1, paragraphs 0010, 0012 page 2, paragraphs 0052, 0054 page 3, paragraphs 0060, 0064-0067 page 4, paragraph 0094 page 5, paragraphs 0099, 0102-0104, 0108, 0110, 0113 examples 1, 8 ----- -/--	1-29



Further documents are listed in the continuation of Box C.



See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search

14 February 2020

Date of mailing of the international search report

21/02/2020

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Dudás, Eszter

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2019/053351

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHRISTOPHER J. HILLARY ET AL: "Developing Repair Materials for Stress Urinary Incontinence to Withstand Dynamic Distension", PLOS ONE, vol. 11, no. 3, 16 March 2016 (2016-03-16) , page e0149971, XP55502323, DOI: 10.1371/journal.pone.0149971 Methods; page 1 Introduction; page 2, paragraph 1 Polymers; Electrospinning; page 3 Electrospinning of Scaffolds; page 5 -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2019/053351

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 2921136 A1	23-09-2015
		JP 6140295 B2	31-05-2017
		JP 2016502426 A	28-01-2016
		KR 20150086227 A	27-07-2015
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		WO 2014075388 A1	22-05-2014
