Biocompatible surgical meshes based on decellularized human amniotic membrane

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ABSTRACT

Meshes play important roles to repair human tissue defect. In this work, human amniotic membrane (HAM) was decellularized and explored the efficacy as an implantable biological mesh. Surfactant, hypertonic saline, lipase and DNAase were used individually or collectively to remove all cell components and remain the extracellular matrix. Results of H&E and DAPI staining demonstrated that the method of surfactant and lipase combining with DNAase is the most effective treatment for HAM decellularization. Primary smooth muscle cells were seeded to evaluate the decellularized HAM's (dHAM) *in vitro* cytocompatibility. The *in vivo* test was performed *via* implantation at rabbits' uterus with clinic polypropylene mesh (PP) as the control. The results indicated that dHAM possessed good biocompatibility and will be a potential candidate for biological mesh.

Keywords: Biological mesh; Human amniotic membrane; Decellularization, Biocompatibility.

1. Introduction

With the development of technology in tissue engineering, biological meshes play more and more crucial roles in repairing of abdominal wall hernia, vaginal, uterus and bladder prolapse, pelvic floor defects, and interventional devices like vascular/endovascular grafts, biological substitutions for resected tissues [1-5]. Most traditional surgical meshes were made from artificial materials or animal tissues to provide permanent reinforcement or temporary substitutes. For

example, polypropylene mesh (PP) is used for urological and urogynecological surgery and polyester mesh (PE) used for hernia repair [6,7]. However, these implants have been associated with inflammation, infection, tumor or even carcinogenicity due to the material's hydrophobicity and consequently poor tissue integration [8-10]. Researchers start to study on improving meshes' chemistry via using plant-/animal-derived materials or surface crosslinking with hydrophilic components to enhance the meshes' wettability [11,12].

Human amniotic membrane (HAM) is a translucent thin membrane derived from the most inner layer of placenta, with no blood vessels, nerves and lymph vessels. It possesses properties like low-inflammation, low-immunogenicity and inhibition of leukocyte infiltration, and thus has been explored for a variety of clinical applications like grafts for eye injuries and dressing for integumentary wounds [13-15]. The basic structure of HAM consists of three layers: epithelial, basement layer and avascular stromal layer. In order to diminish the immunogenicity originated from cells and improve the *in vivo* biocompatibility, people always separated its basement layer (containing epithelial cells) and conduct decellularization performance to obtain a denuded human amniotic scaffold with ~50 μm thickness [16]. Some attempts have been made to decellularize HAM [17]. For example, surfactant like sodium dodecyl sulfate (SDS), ethylenediaminetetraacetate dehydrate (EDTA), sodium hydrochloride or mechanical scraping were used [18,19]. However, these treatments cannot remove amniotic epithelial cells completely and shall be optimized the detail procedures.

In this work, we focused on decellularization of HAM and *in vitro/in vivo* testing, aiming at producing a biocompatible mesh. In order to settle the HAM's problems like over-softness and weak mechanical properties, Poly (ester urethane) (PU) was selected as physical support because of PU's non-toxicity and biodegradability with good mechanical properties like strength, elasticity and stability, which was much proved in our previous studies or other's work [20-24]. Decellularized HAM (dHAM) was expected to provide biocompatibility while PU provided physical strength. The dHAM/PU/dHAM hamburger composite (abbreviated as dHAM/PU) was therefore fabricated. It was supposed to possess both anti-inflammation and good mechanical strength. This kind of HAM-based scaffold was not reported in literatures. *In vitro* and *in vivo*

evaluations were performed on dHAM/PU membrane. The results demonstrated the enhanced interaction with smooth muscle cell and high integration with rabbit abdominal tissue. This dHAM/PU was anticipated to serve as an implantable biological mesh in future.

2. Experimental section

2.1 Materials

Poly(ester urethane) (PU, 58213 NAT 022) was purchased from Estane Co., China. Triton X-100 (Solarbio, China), Antibiotics Solution (Penicillin and streptomycin, Beyotime Institute of Biotechnology, China), DNAase (Sinopharm Chemical Reagent Co., China), lipase (Solarbio, China), Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA), fetal bovine serum (FBS, TransGen Biotech, China), Methylthiazolyldiphenyl-tetrazolium bromide (MTT, BioDee, China) were used as obtained.

Fresh HAM was collected immediately after delivery from the Affiliated Hospital of Ningbo University, with prior consent of the pregnant women. All HAMs used in experiments were obtained from healthy pregnant.

2.2 Decellularization of HAM

The fresh HAM was separated from placenta, rinsed away all blood and other dirties in phosphate buffered saline(PBS), rapidly sterilized in 75% alcohol/water for 10 s, and washed away the alcohol with PBS. It was then put in PBS containing antibiotics (200 U/mL) under shaking for 1 d with changing the PBS for each 12 h.

These pretreated HAMs were divided into 4 groups for decellularization performance; (1) Surfactant + DNAase (SD): HAM was immersed in 1% Triton X-100 for 1, 2 or 3d, respectively, followed by treatment with DNAase/PBS solution(2000U/L) for 3h at 37°C; (2) Hypertonic saline + DNAase (HD): HAM was incubated in 1M, 2M or 3M NaCl/PBS solution separately for 1, 2 or 3d, followed by DNAase treatment as above; (3) Lipase + DNAase (LD): HAM was firstly incubated in lipase/PBS solution (concentration of 100, 200, 500, 800, 1000,1200 1500, 1800, 2000 and 2500 U/L was explored individually to select a favorable solution) for 10 h at 37 °C. It was then treated in DNAase solution as above. (4) Surfactant + Lipase + DNAase (SLD): HAM

was firstly treated in 1% Triton X-100 for 14 h (changed the solution at 7h), followed by incubation in 2000 U/L lipase solution for 10 h at 37 °C. Finally, it was incubated in 2000 U/L DNAase/PBS for 3 h at 37 °C. PBS rinsing (for 24 h with changing PBS at 12 h) was performed in each procedure. All PBS was supplemented with 200 U/mL antibiotics.

All samples after decellularization were fixed in 4% paraformaldehyde/PBS for the following characterizations.

2.3 HAM characterizations

These fixed samples were rinsed by water completely, dehydrated with a graded series of ethanol, embedded in paraffin wax on Paraffin Embedding machine (EC350, Thermo Scientific, USA), and sectioned at 5.0 µm on Microtome (HM325, Thermo Scientific, USA). Then the slices were mounted on glass, dewaxed by dimethylbenzene, rehydrated, and stained with hematoxylin for nucleic acid and eosin for cytoplasm. They were observed on Inverted Phase-contrast Microscope (FV1000, Olympus, Japan) to monitor the decellularizing effectiveness. The clean HAM was examined as the control reference.

These decellularized HAM (dHAM) was stained by 4',6-diamidino-2-phenylindole (DAPI, Sigma) to expose the existed nuclear matters. dHAM was ruptured in 0.5% Triton X-100 for 20 min., and then dipped in DAPI/PBS (5µg/mL) for 10min. The samples were examined under Fluorescence Inverse Microscope (FV2000, Olympus, Japan). The DNA content in cell nucleus will display as blue fluorescence.

The HAM morphology was examined on Scanning Electron Microscope (SEM, Hitachi S3400, Japan) after samples were surface metalized by gold on Ion Sputter (E-1010, Hitachi, Japan).

2.4 Fabrication of dHAM/PU composite mesh

PU was dissolved in 1,4-dioxane to get the concentration of 0.125 g/mL. This PU solution was cast on smooth glass to obtain a membrane with ~0.3 mm thickness. The wet dHAM (treated with SLD method, i.e. 1% Triton X-100 for 1 d, 2000 U/L lipase for 10 h and 2000U/L DNAase for 3 h) was coated evenly on PU membrane and dried in oven for 10 h at 37 °C. The coating was performed repeatedly on the other side to get the dHAM/PU/dHAM hamburger composite

(abbreviated as dHAM/PU).

2.5 *In vitro* cytocompatibility evaluation

Primary smooth muscle cells (SMCs) were digested and proliferated from rabbit esophagus [20]. Membranes of dHAM/PU and PU were punched into a disc with the diameter of 6.4mm. These round membrane were sterilized in 75% ethanol for 4 h and then rinsed ethanol away with sterile PBS. SMC ($3 \sim 5^{\text{th}}$ passages) were seeded on membranes at the density of 4×10^4 /mL, 200 μ L per well in 96-well plate (Corning Co., USA). SMCs were cultured in DMEM containing 10% FBS and 100 U/mL antibiotics in a CO₂ incubator (HF90, Heal Force, China) with 5% humidity at 37 °C. The culture medium was changed every 2 d.

To observe the cell morphology under optical microscopy, the cultures were stained with hematoxylin and eosin dye because of the poor transparency of the composite membrane. Cells were rinsed with PBS and fixed in 4% paraformaldehyde for 4 h, rinsed by PBS to completely remove the residual paraformaldehyde, and immersed in 0.5% triton-X100/PBS for 15 min and rinsed with water. Cells were stained with hematoxylin for 30 min, rinsed in 1% hydrochloric acid/alcohol for 10 s to remove the excess dye. Subsequently, cells were stained in eosin for 1 min.

For mitochondrial activity assay, cells were seeded on dHAM/PU and PU membrane (used as control), and kept in 96-well culture plate with tissue culture polystyrene plate (TCPS) as the positive reference. After 1, 3, 7 or 10d, respectively, the cell-seeded membranes were transferred into a new plate. MTT/PBS solution (20 μ L, 5 mg/mL) was added to each sample well (in 96-well plate) and incubated at 37 °C for 4 h, followed by addition of 150 μ L dimethyl sulfoxide (DMSO) to dissolve the purple formazan crystal. The absorbance at the wavelength of 490 nm (OD) was determined on an ELISA colorimeter (MaxM5, Labsystems Spectra, USA). The absorbance of the same solvent in an unseeded well (without formazan crystal) was used as blank reference. OD values were averaged from 5 repeats for each sample type.

2.6 *Immunohistochemistry staining*

Immunohistochemistry staining was performed on the cultures using anti- α -Smooth Muscle Actin (SMA, Boster, China) as the primary antibody. A standard immunostaining protocol as manual introduced was used. Samples were washed with PBS and fixed in 4% paraformaldehyde

for 30 min. They were first incubated in 0.1% Triton X-100 for 10 min, rinsed triply with PBS for 10 min each time, and blocked in 4% goat serum for 1h to minimize non-specific binding. Cells were then incubated in mouse anti- α -SMA (dilution 1:200 in PBS) for 1h at 37 °C. After rinsing with PBS triply, the samples were incubated in goat anti-mouse IgG (H+L) secondary antibody conjugated with FITC (1:50 dilution in PBS, Boster) for 1 h. Rinsing with PBS triply, samples were dipped in DAPI/PBS (3 μ g/mL) for 5 min to reveal the nuclei. In the stained image, SMC's actin displayed green fluorescence and nucleus displayed blue.

2.7 In vivo testing

In order to enhance the tissue integration, dHAM/PU membrane was manually punched multi-holes with stainless steel needle (14G, inner/external diameter, 1.45/1.8 mm, ratio of pore/membrane, 1:1, Figure 9 a0, inserted). 2 adult rabbits (1 year-old, 4-6 kg) were used in this experiment. The rabbits were anesthetized with 3% pentobarbital sodium (intravenous injection, 12 mg/kg). dHAM/PU composite mesh (1 $\times$ 1 cm) was implanted at uterus through rabbit's abdomen (Figure 9 a0, black arrow). Polypropylene mesh (PP, Marlex) supplied by Affiliated Hospital was used as the control (Figure 9 a0, red arrow). After 2 or 10 months, dHAM/PU and PP mesh was explanted with small amount of tissue, fixed by 4% paraformaldehyde, sectioned at 6 μ m, and performed H&E staining.

The animals used in this study were treated as required by the Ethical Committee of Ningbo University and in accordance with NIH's Principles of Laboratory Animal Care.

2.8 Statistical Analysis

Statistical analyses were performed with ANOVA software to evaluate the differences between groups. p values less than 0.05 were considered to be significant. Data were presented as mean $\pm$ standard deviation (SD).

3. Results and discussions

3.1 Decellularization and the topography of HAM

Methods like SD, HSD, LD and SLD with variety of solution concentrations, processing intervals and temperatures were explored to establish an effective approach to peel off all

epithelial cells but maintain the ECM integrity. Results of HE and DAPI staining for cell cytoplasm and nucleus show that HAM consists of epithelium and mesenchymal connective tissue (Figure 1 a0, arrow and triangle), which is the amnion histology as reported [25]. Cuboidal epithelial cells live compactly on basement membrane to line the HAM topology (Figure 1 a0, inserted). Blue fluorescence in Figure 2 a0 displayed the orderly-arranged epithelial nuclei. These epithelial cells were decellularized using 4 methods separately, i.e. SD, HD, LD and SLD (the detailed procedures were described in “Experimental Section”). The processing parameters were compared among reagent selection, concentration of solutions, treatment time and temperatures. For the method of SD, HD or LD, the results of both HE and DAPI staining showed cells were not entirely removed; some nuclei or debris exposed (Figure 1, a1-a3 and Figure 2, a1-a3). Comparatively, the fewest nuclei or fragments left after HAM was treated by LD method, i.e. lipase/PBS (2000u/L) for 10h and DNAase (2000U/L) for 3h at 37°C (Figure 1 a3 and Figure 2 a3). As literature [26] reported, Triton X-100 is a kind of non-ionic surfactant and able to denaturalize protein gently. It was found that Triton X-100 destroyed the normal structure of the amniotic epithelial cell membrane to let DNAase infuse in and enzymolyzed the nuclear apparatus. Lipase, an enzyme able to catalyze the hydrolysis of lipids, was used to digest the lipids of epithelial cells. But it is not sufficient enough to enzyme all lipids by itself [27]. Thus, some nuclei or DNA debris were still existed in the treated HAM. Moreover, the extracellular matrix (ECM) was over-loosened, particularly under the rigorous treatment parameters like longer treatment time (2 or 3 d for Triton X-100) and greater concentration of solution (2500 U/L for lipase). Excessive enzyme reaction in method SD, HD or LD must have occurred not only on cell components but also on the ECM, resulting in the destructive mechanics of HAM.

Hopefully, we modified the methods, combined Triton X-100 and lipase to denature the cell membrane, and then used DNAase to digest all nuclear matters. Expectedly, epithelial cells were removed effectively and completely after treatment with method SLD, i.e. 1% Triton X-100 for 1 d, 2000 U/L lipase for 10 h and 2000U/L DNAase for 3 h, as shown in Figure 3; seldom nucleus exhibited and ECM appears integration from HE and DAPI staining. Here, Triton X-100 combined with lipase ruined the epithelial cell's membrane. Thus, DNAase can permeate into

nuclei membrane to enzymolyze the DNA content.

SEM examination revealed the topographic alteration (Figure 4). The apparent ECM fibers exhibited on dHAM (Figure 4b) but plumpy and cobblestoned topology on the original HAM (Figure 4a). Therefore, we treated HAM with method SLD (1% Triton X-100 for 1 d, 2000 U/L lipase for 10 h and 2000U/L DNAase for 3 h) to obtain the decellularized HAM (dHAM). It was then combined with PU membrane to prepare dHAM/PU/dHAM hamburger composite mesh. After H&E staining (Figure 5 a), the pink dHAM was covered tightly at both sides of PU membrane. It seems there was no interspace between PU and dHAM (Figure 5 a, arrow in the inserted picture). However, under higher resolution (SEM observation), there was still a small gap in the interface (Figure 5 b, arrow in the inserted picture). Moreover, PU membrane surface was not very smooth. There existed some micro-pores originated from solvent evaporation during PU membrane fabrication using solution casting method. It had been observed in our previous experiment [28]. We thus inferred that dHAM and PU was connected via some physical interaction due to the different surface energy of these two materials. This kind of interaction was considered to be fragile. We are now digging for more effective method to connect HAM and PU.

3.2 Evaluation of in vitro cytocompatibility

The cytocompatibility of dHAM/PU composite was evaluated via primary smooth muscle cell culture. Figure 6 shows the proliferation and mitochondrial activity of SMC on dHAM/PU composite membrane, with PU as the negative control and TCPS as the positive reference. During the 10-day culture interval, cells proliferated on all matrices, particularly rapidly increased at the first 7 d (OD values increased). There was insignificantly difference between OD values of dHAM/PU and of PU during the 10-day culture interval ($p>0.05$). However, OD value was higher significantly at day 3 than that at day 1 on both materials ($*p<0.05$ in Figure 6). After then, the values on these two materials still increased with the time past. The general activities on both dHAM/PU and PU were lower than those of cells on TCPS, particularly after 7 or 10 d ($^{\&}p<0.05$, Figure 6). That is, cells proliferated on PU or dHAM/PU as the time went on, but they did not do as fast as those on TCPS because the cell adhesion and proliferation properties of dHAM or PU surface are poorer than those of TCPS. It is reasonable since both dHAM's oversoftness and PU's

hydrophobicity are disadvantageous for cells' attachment.

From H&E staining, cells exhibited the spindle or triangular smooth muscle morphology (Figure 7).. The appearance or general density of cells on dHAM/PU increased during the culture time (Figure 7 a1-a4), just similar to the OD value tendency. At day 7, cells spread well and reached nearly confluence (Figure 7 a3). That's why the cell density (or OD value) increased slowly from day 7 to day 10 due to the cells' contact control. It is worth noting that cells on PU membrane displayed high growth and activities, which were lower insignificantly than those of cells on dHAM/PU. This is the motivation for us to select PU as the physical support to offer the mechanical strength while the biocompatibility from dHAM was maintained. The not bad cytocompatibility of PU material has repeatedly proved in our previous work [20-22].

The expression of smooth muscle α -actin (α -SMA), a major constituent of the contractile apparatus of SMCs, was examined too. SMCs cultured on dHAM/PU had the higher α -SMA fluorescence intensity with the longer culture time, suggesting the SMC phenotype and function were well maintained (Figure 8 a1-a4). Cells were able to reach confluence at day 10 (Figure 8 a4), which is of great importance once they are implanted *in vivo*. This tendency is coincident with the result of mitochondrial activity assay and morphology observation. Altogether, the combination of dHAM and PU was demonstrated to be a stable and straightforward treatment for implantable and cytocompatible mesh for clinic surgery purpose.

3.3 *In vivo* testing

On the basis of those *in vitro* cytocompatibility evaluations, dHAM/PU composite was implanted into uterine site through rabbits' abdomen with clinic PP mesh as the control, which would supply sufficient information for dHAM to be used as surgery mesh in future, The composite mesh was manually punched multi-poles in order to promote tissue infiltration and get the close appearance as PP mesh (Figure 9 a0). The implanted rabbits were raised normally after the operation. The wound on the skin cured and the fur regenerated completely after one month post operation. Rabbits were sacrificed and examined at the 2nd month. Viewing from eyesight, the implanted site was clear and no inflammation was obvious for both meshes, except for the adhesion of PP to the periphery viscera. H&E staining of where mesh was originally implanted

displayed that tissues were already infiltrated into the mesh pores (Figure 9 a and b). Tissues around dHAM/PU mesh indicated clear configuration (Figure 9 b1); the regenerated tissue integrated with the material while PU started to degrade due to fresh tissue invasion (Figure 9 b2, arrow), which is apparent in comparison of the appearance of PU before implantation (Figure 5a, inserted). The tissue enclosing PU was supposed to have regenerated freshly after implantation because there existed cell nuclei (b2, blue purple). The dHAM (without cell nuclei) must have been degraded and disappeared.

Whilst, tissues around PP fibers exhibited a disordered arrangement; excessive nuclei in cells suggested inflammation still existed around the fiber circumference after implantation for 2 months (Figure 9 a, inserted), which was caused by hydrophobicity and bio-inertia of PP material. After 10 months, the regenerated tissue around PP fibers showed the similar tissue hierarchy as that at 2nd month (Figure 10 a). Interestingly, at site of dHAM/PU implantation, tissues remodeled orderly and normally. Some pores generated from microtome procedure during HE staining suggested dHAM/PU has been degraded into fragments (Figure 10 b1, arrow). The tissue around the fragments (Figure 10 b2, inserted) arranged orderly and almost similar to the neighbouring tissue (Figure 10 b2). The degradation of PU had once been investigated *in vitro* and *in vivo*. Generally, PU degraded more quickly *in vivo* than *in vitro*. It degraded into peeling off at day 100 when implanted subcutaneously in rat, but degraded into fragments at day 80 when implanted as muscular scaffold at porcine esophagus [21,22]. Mechanical stress during food ingestion and digestive enzymes brought by food bolus from mouth or the occasional backflow of gastric fluids from stomach were the reasons to have accelerated PU degradation. We thus considered that it was reasonable for PU to have degraded at 10 month in rabbit abdomen.

4. Conclusions

In summary, we established a technique to decellularize HAM with surfactant combining with lipase and DNAase, where surfactant and lipase were used to denature cell membrane and DNAase was used to enzymolyze DNA. Biodegradable poly(ester urethane) (PU) was used as a physical support for soft HAM because of its good biocompatibility. Results of *in vitro*

cytocompatibility tests demonstrated that dHAM/PU composite can support primary SMC to grow and differentiate, with high proliferation, mitochondrial activity and special protein expression (α -SMA) after SMCs were cultured on dHAM/PU for 10 d. The results of 10-month implantation revealed that dHAM/PU possessed much better biocompatibility than PP did. All these results convinced us that dHAM/PU was promising to be used as biological mesh in clinic surgery. A more comprehensive study with different animals (even human beings) and variety of implantation sites will be processed in near future.

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Captions:

Figure 1 H&E staining of HAM after decellularization with methods of SD, HD, and LD. (a0) the original HAM (cross section), arrow shows the amniotic epithelium and triangle indicates connective tissue. The inserted is the topology of amniotic membrane stained by H&E with nucleus displayed as purple particles. (a1) Treated with SD method: 1% Triton X-100 for 1d and DNAase (2000 U/L) for 3h at 37°C; (a2) Treated with HD method: NaCl solution (3M) for 1d and DNAase (2000 U/L) for 3h at 37°C. (a3) Treated with LD method: lipase/PBS (2000 U/L) for 10h and DNAase (2000 U/L) for 3h at 37°C. The inserted is the HAM treated by lipase/PBS (1000 U/L) and DNAase. Scale bar, 20µm.

Figure 2 Nuclei exhibitions after DAPI staining (membrane topography) of HAM decellularized with methods of SD, HD, and LD. (a0) Original HAM. (a1)~ (a3) the same treatment as Figure 1 description. Scale bar, 100µm.

Figure 3 H&E (a1, cross-section) and DAPI staining (a2) of HAM treated with SLD method: 1% Triton X-100 for 14 h + 2000 U/L lipase solution for 10 h + 2000 U/L DNAase/PBS for 3 h at 37°C.

Figure 4 Topography of HAM (a) and dHAM (b) treated with SLD method, observed under SEM.

Figure 5 H&E staining of dHAM/PU (a, the inserted is magnified and arrow pointed the interface) and SEM observation (b) of dHAM/PU composite. The upper layer is dHAM and the underline is PU in (b). The inserted is a magnified picture.

Figure 6 Mitochondrial activity assay. Cells were seeded at the density of 4.0×10^4 /mL and cultured for 10d. $p > 0.05$ for OD value difference between PU and dHAM/PU, but $p < 0.05$ between day 1 and day 3 on both materials (*), and between materials and TCPS after culture for 7 or 10 d (&).

Figure 7 Cell morphology after cells were stained with H&E dye. SMCs were seeded on dHAM/PU at the density of 4.0×10^4 /mL and cultured for 1d (a1), 3 d (a2), 7d (a3) and 10d (a4). Scale bar, 100µm.

Figure 8 Immunofluorescent staining of SMCs. Cells were cultured on dHAM/PU for 1d (a1), 3 d (a2), 7d (a3) and 10d (a4), respectively. Scale bar, 50µm.

Figure 9 H&E staining of PP (a) and dHAM/PU mesh (b) implanted at uterine site for 2 months. b2 is magnified from b1. (a0) Overview of implanting operation, red arrow shows PP (upper inserted) and black indicates dHAM/PU mesh (lower inserted). The inserted displayed the appearance of clinic PP and dHAM/PU mesh. Arrow in (a) pointed PP fiber, and the inserted is a magnified picture showing the tissue microscopy around PP fiber. Scale bar in (a) and (b1) is 200 μ m and in (b2) is 20 μ m.

Figure 10 H&E staining of PP (a) and dHAM/PU mesh (b) after implanted for 10 months. b2 (scale bar, 100 μ m) is magnified from b1(scale bar, 200 μ m), square site. Arrow indicates the pore originated from PU fragment. The inserted in b2 showed the tissue around PU fragment with higher magnification.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Graphical Abstract: