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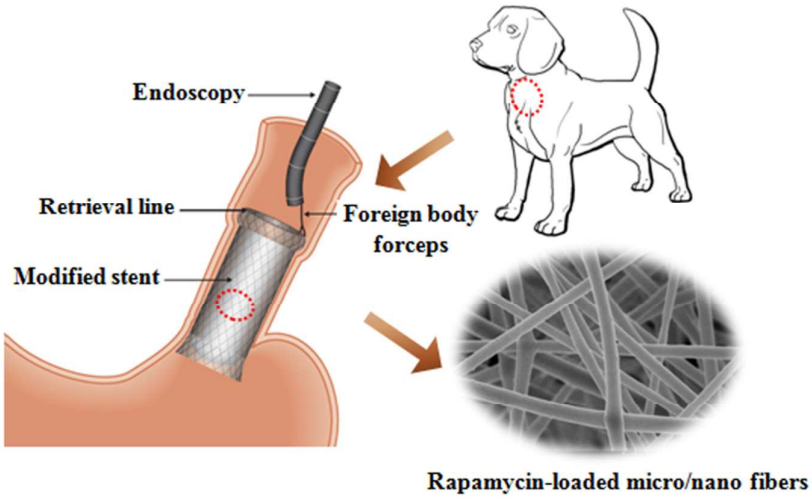

Diagram of the process used to fabricate non-biodegradable metal stents with an outer layer of Rapa-loaded fibrous membrane by using the electrospinning process. With the release of Rapa, the stents are expected to inhibit fibroblast proliferation and tissue hyperplasia, therefore treating a benign cardia stricture.

In vitro and *in vivo* evaluation of Rapamycin-eluting nanofibers coating on cardia stents

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ABSTRACT:

It is known that a Rapamycin (Rapa)-coating on stents can be potentially applied to treat benign cardia stricture, it is, however, difficult to be used as a delivery system for controlled and sustained release particularly in acidic environment. The aim of this study was therefore to explore the potential of self-expandable metal stents covered with Rapa-loaded electrospun poly (ε-caprolactone) (PCL) fiber membranes for controlled Rapa delivery to inhibit inflammation and tissue hyperplasia. It was found that stents coated with the membranes had comparable mechanical properties including expansion properties, mechanical strength and rigidity with bare cardia stents. In addition, Rapa were found to be released from the membrane coated stents in a controlled and sustained manner, and via varying the concentration of Rapa incorporated in the PCL membranes, the drug release rates could be controlled. The released Rapa was also found to inhibit the proliferation of smooth muscle cells. Moreover, the site implanted with the stents coated with the Rapa loaded PLC membranes showed reduced tissue inflammation and collagen fiber deposition compared to those with no coating. The above results have therefore suggested that the new Rapa-coated stents developed in this study have great potential to treat benign cardia strictures.

KEYWORDS: Rapamycin; drug-coating; stent; cardia stricture; fiber.

1. Introduction

Benign cardia stricture, an uncommon disorder, is defined as the inability of the lower esophageal sphincter (LES) to relax resulting in physical obstruction or the absence of esophageal pertalisis. The disorder can be initiated by idiopathic achalasia and pseudoachlasia diseases. In 2003, we wrote a paper describing retrievable stents that could be used for curing achalasia patients.[1] In 2010, we further developed the retrievable stents to include superior caliber and structure, an antireflux valve, and an acid-resistant coating.[2] These improvements have made the retrievable stents particularly suitable to combat benign cardia strictures. However, our previous study indicated that after retrievable stent placement, there was a tendency for fibroblast proliferation and tissue hyperplasia, greatly limiting the stent retention time. Scaring was also observed following the extraction of the stents, which was found to facilitate an increased recurrence of benign cardia strictures.[3, 4]

Rapamycin (Rapa) has proven to prevent the recurrence of arterial stenosis by inhibiting the inflammation and growth of smooth muscles cells (SMCs).[5, 6] Rapa is commonly administered orally or via injection.[7, 8] For example, Rapa was administered orally to treat in-stent re-stenosis for patients with coronary heart disease.[9] Also, intravenous injection of Rapa bonded to a perfluorobutane gas microbubble carrier was used for the treatment of neointimal formation in a porcine coronary re-stenosis model.[10] The efficacy of using these common routes of administering Rapa has been studied in detail; however, they are subject to several problems as follow: (1) unexpected medication of surrounding healthy tissue; (2) difficulty of guaranteeing sustained and controllable medication reaching the target site; (3) necessity of frequent doses; (4) repeated physical suffering of patients. To address these problems, we expect that an advanced topical

administration route can be used.

Currently stents have been developed with coatings to facilitate the delivery of biologically active agents and drugs to the target site to prevent the negative effects of the stent placement including increased fibroblast growth which may cause inflammation and re-stenosis.[11] Jeon *et al.* added a paclitaxel-silicon coating to an esophageal stent which showed in an animal study to significantly decrease abnormal multiplication of cells and other tissue disturbances.[12] Guo *et al.* demonstrated that a coating of ethylene-vinyl acetate (EVA) and 5-fluorouracil (5-FU) applied to an esophageal stent by hot-pressing.[13, 14] was able to alleviate re-stenosis as it allowed a high concentration of 5-FU close to the target site. However, our group has found that when using a drug-coated stent, it is difficult to avoid burst release of the drug when the stent is applied *in vitro* or *in vivo*.[2,3] Therefore, we focus our attention on looking for a way to reduce this phenomenon in order to avoid short retention time on stent and reduce the recurrence rates for more effective treatment of benign cardia strictures.

Electrospun poly (ε-caprolactone) (PCL) nanofibers have valuable and unique properties including high surface area to volume ratio, large length/diameter ratio, and superior tensile strength and flexibility.[15] Due to their unique properties, the use of electrospun fibers has become increasingly popular in tissue engineering and drug delivery.[16] Therefore, we expect that the stents coated with electrospun fibers would be a good alternative to the currently available stents that are coated with or with no drugs. Here we present *in vitro* and *in vivo* test results which prove the superior qualities of a stent, designed by the current authors, coated using electrospun fibers loading Rapa. The study was done by treating benign cardia strictures using a dog model (Figure 1). The following properties of the new coated stents were evaluated to demonstrate its clinical

potential: mechanical properties, release of Rapa (*in vitro*), inhibition of SMC proliferation (*in vitro*), and success of implantation (*in vivo*).

Materials and methods

Materials.

Poly(ε-caprolactone) (PCL, Mw=50 kDa, Mw/Mn=1.76) was purchased from Daigang Co., Jinan, China. Rapamycin (Rapa) was purchased from Fusheng Pharmaceuticals Inc., Dalian, China. The solvents, all of reagent grade, were obtained from Guoyao Regents Company, Shanghai, China. Beagle dogs of both sexes were used to establish the benign cardia stricture model. National Tissue Engineering Center of China (Shanghai, China) kindly provided SMCs from porcine femoral veins.

Electrospinning.

The detailed electrospinning procedure has been described in previous literature.[17] Briefly, 0 (control), 5, 10 and 20 mg of Rapa were each dissolved in 1.0 g methanol, and then added to a PCL solution (1.0 g PCL dissolved in a mixed solution of 2.0 g dichloromethane and 1.0 g ethanol) to prepare the electrospinning solution. Electrospinning parameters were: 16 kV voltages, 0.6 ml/min solution feed rate, 15 cm collect distance and 0.8 mm syringe needle diameter. The resultant solution was then electrospun and layered onto a stent by rotating it as a collection plate to produce Rapa-loaded PCL electrospun fibrous membrane coating. Coated stent samples were titled PFM00, RPFM05, RPFM10 and RPFM20 for incorporating 0, 0.5, 1, and 2% Rapa electrospun membranes respectively. All samples were dried in a vacuum for one day around

22°C.

Characterization of fibrous membranes.

The width and length of the membranes were measured using a micrometer. The surface morphology of the membranes was examined using a scanning electron microscope (SEM, Quanta 200, FEI, Netherlands). Crystalline phase of the Rapa-loaded membranes (20×20 mm^2) was studied using X-ray diffraction (XRD, Philips X'Pert PRO, the Netherlands) over the 2 theta range from 5° to 70° with a scanning speed of 0.35°/min (λ=1.54060 Å). To examine stent resilience and usability, all samples were tested on bending, extrusion, and bundling for 100 times. The surface wettability of the electrospun PCL membranes was examined at room temperature using a water contact angle (WCA) method using a Kruss GmbH DSA 100 Mk 2 goniometer (Hamburg, Germany).[18] The compression-recovery characteristic of the membranes was investigated using a purpose mechanical testing machine (Instron 5567, Norwood, MA).

***In vitro* release and degradation.**

Small square samples (2×2 cm^2) of Rapa-loaded electrospun fibrous membrane, each with a total mass of ca. 50 mg, were immersed in 20 ml pH 4.0 and 7.4 phosphate buffered saline solution (PBS) containing 0.02% sodium azide. This pH was chosen to mimic the *in vivo* environment as the site of implantation of the stents was very close to the stomach whose pH ranges between 3 and 5. The suspension was maintained at 37 °C in a shaking water bath at 100 cycles/min. At predetermined time intervals, 2.0 ml of the buffer was removed for biological analysis and replaced with 2.0 ml fresh PBS for further incubation.[19]

High-performance liquid chromatography (HPLC) was used to examine the structural integrity of the Rapa released from the electrospun fibers. Drug peak time appeared at 15.1 min when using a C18 stationary phase with a 5μm particle size and column dimensions of 4.6×150 mm i.d. at 25 °C for sample and 40 °C for column temperature. The mobile phase was acetonitrile and distilled water (75/25 v/v) at a flow rate of 1.0 ml/min. The analysis time used was 23.0 min. The concentration of released Rapa was determined using a UV spectrometer at 278 nm absorbance (Waters 2695 and 2487, Milford, MA) via comparison with standard samples with a concentration from 0, 0.75, 1.5, 3, 6, 12 to 24 μg/ml.

Cell culture.

SMCs, isolated from a porcine femoral vein, were grown in the culture medium in a humidified 5% CO_2 incubator at 37°C. SMCs were identified with the expression of anti-smooth muscle actin and their typical elongated swirling and overlapping morphology. The cells were seeded at a density of 2.5×10^4 cells/well in a 12-well plate and incubated for 24 hrs. The culture medium was then replaced with a fresh medium containing Rapa released from PCL fibers as described above (1.5 mg fibers/150 μl cell culture medium). These cells were then cultured for another 9 days. Proliferation of the cells was observed using a CCK-8 assay for cell counting (Cell counting kit-8, Dojindo, Kumamoto, Japan). 100 μl of the incubated medium was pipetted into a 96-well plate and the absorbance at 450 nm was measured using a microplate reader (Thermo Lab Systems, Helsinki, Finland).[20]

Histological examination in dog models.

This experiment was carried out based on standards from the International Council for Animal Care. It was approved by our institution's animal research committee. 30 beagle dogs (male and female) had benzyl-dimethyl tetradeacyl ammonium chloride (BAC, 4 mmol/l, 12ml) injected circumferentially into their LES to induce benign cardia strictures (Agronomic School of Shanghai Jiao Tong University, Shanghai, China; license SCXK 2007-0004). The 30 dogs were subsequently randomly split into three groups: control (no stent insertion, n=10), bare stent (BS, bare stent insertion with 1-week retention, n=10), and coated stent (PFM20, coated stent insertion with 1-week retention, n=10).

At each follow-up analysis, five dogs from each group were sacrificed. Their cardias were resected and prepared for histological examination to compare the effects of the different treatments on tissue reaction. First, the samples were submerged for 48 hrs in 10% neutral-buffered formalin, they were then passed through a series of increasingly graded ethanol solutions (70% to 100%), and finally embedded in paraffin. The thickness of the paraffin-embedded dog cardia cross-sections ranged from 30 to 50 μm. The samples were stained with Masson's trichrome in order to evaluate the amount of collagen proliferation in the submucosa layers (blue, magnification ×400). The samples were also incubated with mouse anti-proliferating cell nuclear antigen (PCNA) antibody (at 1:100 dilution; Cell Signaling Technology, Inc. Boston, Massachusetts, USA) and examined using the Elivision immunohistochemical technique. Proliferation was calculated as the percentage of the PCNA-positive cells over the total number of cells. The percentage of PCNA-positive cells and the area of collagen expression were calculated using Image-Pro Plus Version 6.0 software (Media Cybernetics, Inc., Bethesda, MD) using at least 20 randomly selected high-power (×400) tubulointerstitial fields in each section.

Statistical analysis.

The data analyzed in this experiment was obtained in triplicate. The data is expressed as mean value with standard deviations (SD). Two-tailed Student's t-test was used to see the statistical differences between groups. One-way ANOVA was used to compare the PCNA proliferation index, smooth muscle α-actin positive area and collagen area at each follow-up.

Results

Morphology and usability properties.

As shown in Figure 2a, the Rapa-loaded electrospun fibrous membranes were tightly and completely wrapped around the bare metal stents. The fibrous membrane has a uniform thickness of ~ 100 μm. All electrospun fibers exhibited smooth morphology and uniform size with randomly interconnected structures (see example in Figure 2 b). The measured diameters of the fibers of all formulations were comparable, for PFM00, RPFM05, RPFM10 and RPFM20 with 1.5 ± 0.7, 1.4 ± 0.5, 1.3 ± 0.4 and 1.4± 0.5 μm, respectively.

To confirm the function and stability of the developed stents, these samples were subjected to bending (Figure 2c), extrusion (Figure 2d and e), and bundling (Figure 2f), each repeated 100 times. It was found that the electrospun fibrous membrane coated stents displayed good elastic deformation properties with their ability to spring back after compression with no membrane tearing or ablations after 100 repeated compressions. These data suggested that the electrospun fibrous membrane coated stents possessed good flexibility and elasticity and should be stable against these forces when used in the body.

Characterization of fibrous membranes.

To examine the effects of the drug incorporation on the surface properties of the electrospun fibers, WCAs of the surface of the electrospun fibrous scaffolds were measured. It was found that the WCAs of these scaffolds were 130.1±4.1, 133.5±3.2, 135.1±3.6 and 135.9±3.3° for PFM00, RPFM05, RPFM10 and RPFM20, respectively. The WCA of the scaffolds increased with higher amounts of the drug incorporated, possibly due to the hydrophobicity of the drug although statistical difference was not found between these samples.

The crystalline phase of the drug incorporation into the electrospun fibers was examined using XRD (Fig. 3A). The crystalline phase of the electrospun PCL fibrous membranes showed characteristic peaks at 21.4° and 23.8° (see Figure 3A. a-d). During the crystallization of pure Rapa, many major peaks were present between 0 to 20° (Figure 3Ae), whereas no Rapa peaks could be found in the drug loaded fibrous membrane samples (Figure 3Ab, c and d).

Characterization of the compression-recovery properties of the stents revealed that both the bare stents and PCL coated stents possessed excellent recovery properties (see example in Fig. 3B). It was found that the recovery curves of all samples had inflexion point both in compression and recovery process, and the inflexion point was the same for 50 times press.

***In vitro* release and degradation behavior.**

As stents are mostly displaced in the distal esophagus and in which the pH values mostly ranged from 5.0-6.8, pH value less than 4.0 is often considered as presence of food reflux [21]. Therefore, the pH 4.0 PBS was chosen to study the *in vitro* Rapa release from the electrospun

fibrous membranes. It was found that pure Rapa, Rapa in electrospun fibers and Rapa released into the release medium all exhibited the maximum absorbance peak at 278 nm, and the elution time of 15.1 min (data not show), suggesting that the Rapa in the electrospun fibers after electrospinning and incubation in the release medium was not chemically modified.

Figure 4a showed the profiles of Rapa release from the electrospun fibers in PBS of pH 7.4. Similar release behaviors were observed between different formulations. The percentage of total drug released plateaued around 33, 37 and 38% for RPFM05, RPFM10 and RPFM20, respectively after 30 days of incubation. When the pH of the immersion medium was reduced to 4.0, initial burst and higher drug release rates could be clearly noticed (compare Figure 4 a and b). The percentage of total amount of drug released was 45, 52, and 53% for RPFM05, RPFM10 and RPFM20, respectively after 30 days of incubation, approximately 1.4 times higher than that of drug release in the pH 7.4 buffer solutions. The drug entrapped in the fibers after 30 days of incubation might not diffuse out until significant degradation of the polymer matrix.[19]

The drug release rate could be adjusted via using different types of polymers. [19,22,23] Figure 4c showed the morphology of medicated fibers after 32 days of incubation in PBS. We found that the fibers has maintained their structures after 32 days of drug release, and the fiber diameter increased from 1.46 ± 0.52 μm to 2.58 ± 0.37 μm before and after incubation. This result indicated that the drug could be released from the puffing bigger fibrous matrix because of water penetration. Therefore, the Rapa has the higher release rate than the PCL degradation because of slow degradation rate of PCL, and most drugs could be released from fibrous matrix with water penetration.

***In vitro* assay of SMCs culture.**

PFM00 and Rapa/PCL fibrous membranes presented an obvious reduced SMC proliferation on the 6th and 9th day of culture, indicating that Rapa was effective to inhibit SMC growth (Figure 5). There was a significant difference ($p<0.05$) in cell proliferation levels between PFM00 and the control (tissue culture plastic), and between Rapa/PCL and PFM00 on the 6th and 9th day of culture.

Histological examination.

Figure 6 showed PCNA-positive cells were present in the epithelial lamina and submucosa of the site treated using control (no stent), bare stents and stents with Rapa coating after 1 month. The percentages of PCNA-positive cells were highest after 1 month treatment in the BS and PFM20 group. Quantification of the PCNA-positive cell proliferation index at 1 month revealed markedly lower cell proliferation in the PFM20 group than that in the BS group ($p<0.05$) .

Masson's trichrome staining demonstrated the collagen fiber proliferation gradually increased during the follow-up period and became stable ~3 months after treatment (Figure 7). At 3 and 6 months follow-up, the area of deposited collagen in the BS group was significantly higher than that in the PFM20 group ($p<0.05$), suggesting that the PFM20 may be able to treat benign cardia stricture via inhibiting inflammation and reducing subsequent collagen deposition (scar formation).

Discussion

Currently, self-expandable/metallic stents have been used in practices to treat benign cardia

strictures but with inflammation and scar formation. [3, 24-26] As a result, various drugs such as paclitaxel mototic inhibitor, various protein-pump inhibitors (PPIs) and H_2 antagonists have been employed to reduce fibroblast proliferation and tissue hyperplasia. [27, 28] Among these drugs, Rapa was found of particular usefulness in prevention of re-stenosis following stenting in arteries via inhibiting inflammation and proliferation of SMCs.[5, 6] Therefore, in this study, Rapa was incorporated in a electrospun PCL fibrous membrane coating on self-expandable stents to treat benign cardia strictures.

Rapa-loaded electrospun fibrous membrane can be tightly wrapped on the entire surface of a self-expandable metallic stent. This stent demonstrates the stability which would be required against potential radial forces from the outside by successfully conducting 100 repetitions of bending, extrusion and bundling, each with no alteration to the stent. Electrospun fibrous membranes displayed good elastic deformation properties with the stent compression and release, and no membrane tearing or ablations were observed after hundreds of times repeated compressions. It can therefore be concluded that the Rapa/PCL micro/nano-fibrous membrane-covered self-expandable metallic stents maintained the original mechanical strength and rigidity of the bare stent. In addition, the PCL electrospun fibrous membrane surface properties, crystalline state and fiber diameters were not modified with the incubation of Rapa.

The electrospun fibers can provide added functionality to control the release of a combination of drugs.[29] The porous structure of the microporous fibrous membranes allow for two different steps of drug release. First, drug molecules are able to diffuse from the outer layer, then making it possible for a constant release from the inner layer. Rapa-loaded fibrous membranes showed burst release in pH 4.0 as well as sustained release in pH 7.4 solutions. With

decrease in the pH of the buffer solution, the drug release rate was accelerated. Therefore, depending on the pathological conditions, the following may be achieved in a lower pH environment: larger initial burst release, faster release and higher sustained release rate. The *in vitro* and *in vivo* results also confirmed the advantages of using Rapa for prohibiting scar development. After about 6 months, the stent still maintained its original position, which suggested that it was still preventing the esophageal restenosis.

Therefore, Rapa-loaded fiber covered stents have shown potential as a device for esophageal stenosis therapy. These stents would allow for a convenient operation helping patients overcome discomfort and pain. The operation was safe and effective for the treatment of a dog model with benign cardia stricture, and was successful in avoiding inflammation and scar formation.

Conclusion

In this study, we developed controlled Rapa-releasing electrospun fibers built to form a layer on the self-expandable/metallic stent surface with the use of a rotor electrospinning technology. Wrapping the stents using the Rapa releasing electrospun fibrous membranes had no influence on the mechanical properties of the original bare stent. The release of Rapa from the fibrous membranes could be readily controlled via varying the concentration of the incorporated. The released Rapa could significantly inhibit the proliferation of SMCs. The implanted Rapa-loaded PCL micro/nano fibers layered stents could successfully inhibit inflammation and scar formation. It can therefore be concluded that the drug-loaded electrospun fibers have great potential to treat benign cardia stricture and this drug releasing membrane can be used as a model for treatment of other diseases.

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Figure legends

Figure 1. Diagram of the process used to fabricate non-biodegradable metal stents with an outer layer of Rapa-loaded fibrous membrane by using the electrospinning process. With the release of Rapa, the stents are expected to inhibit fibroblast proliferation and tissue hyperplasia, therefore treating a benign cardia stricture.

Figure 2. Images showing the macro/micro morphologies and mechanical properties related to the usability of the Rapa-loaded PCL electrospun fiber coated metal cardia stents. (a) Macro morphology of the modified stents; (b) Representative SEM image of Rapa-PCL fibers; (c) bending; (d) compression; (e) rebound; (f) fasten.

Figure 3. (A) X-ray diffraction profiles of PFM00 (a), RPFM05 (b), RPFM10 (c), RPFM20 (d) and Rapa (e). (B) Compression-recovery curve in repeated compression test for RPFM20 (n=50, Constant pressure=10N).

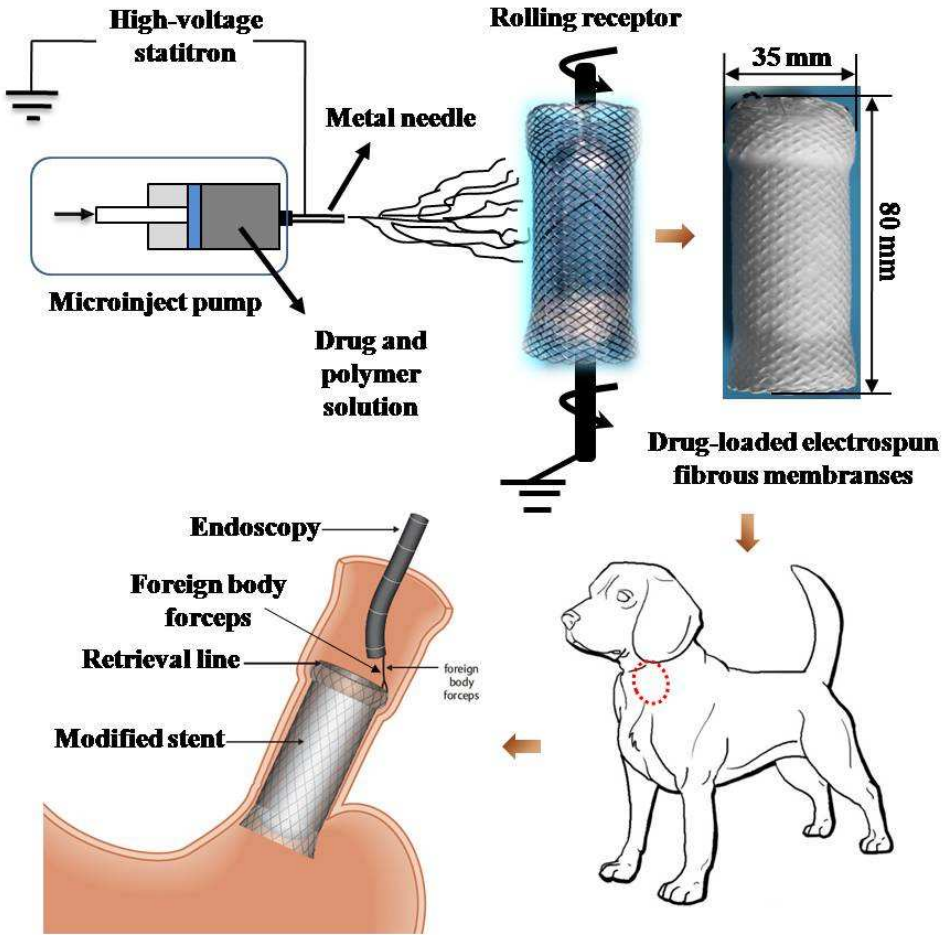
Figure 4. *In vitro* Rapa release curves of cumulative percentage of Rapa release from RPFM05, RPFM10 and RPFM20 after immersion in PBS of pH=7.4 (a) and pH=4.0 (b) at 37 ℃, SEM image (c) of RPFM20 after 32 days immersion in pH=4.0 PBS.

Figure 5. SMC proliferation on PFM00, RPFM05, RPFM10 and RPFM20 fibrous membranes after 1, 3, 6, and 9 days of culture. The control was tissue culture plastic. * represents $p<0.05$ with respect to the PFM00 fibrous membrane.

Figure 6. Inflammation after treatment using control (no stent), BS and PFM20 at different follow-up intervals. PCNA staining (a, b and c) was observed in the cubicula layer after 1 month (brown, magnification ×40; red arrows). (d) Percentage of PCNA positive cells after treatment

using different stents. *p < 0.05 for comparison with the BS group, §p < 0.05 for comparison between groups.

Figure 7. Scar formation after treatment of control, BS or PFM20 at different follow-up intervals. Masson's trichrome staining indicated that collagen fiber deposition in the submucosa (blue, magnification ×400; red arrows) stabilized ~3 months after treatment. (d) Collagen fiber proliferation. *p < 0.05 for comparison between the BS and PFM20 group, §p < 0.05 for comparison between groups.



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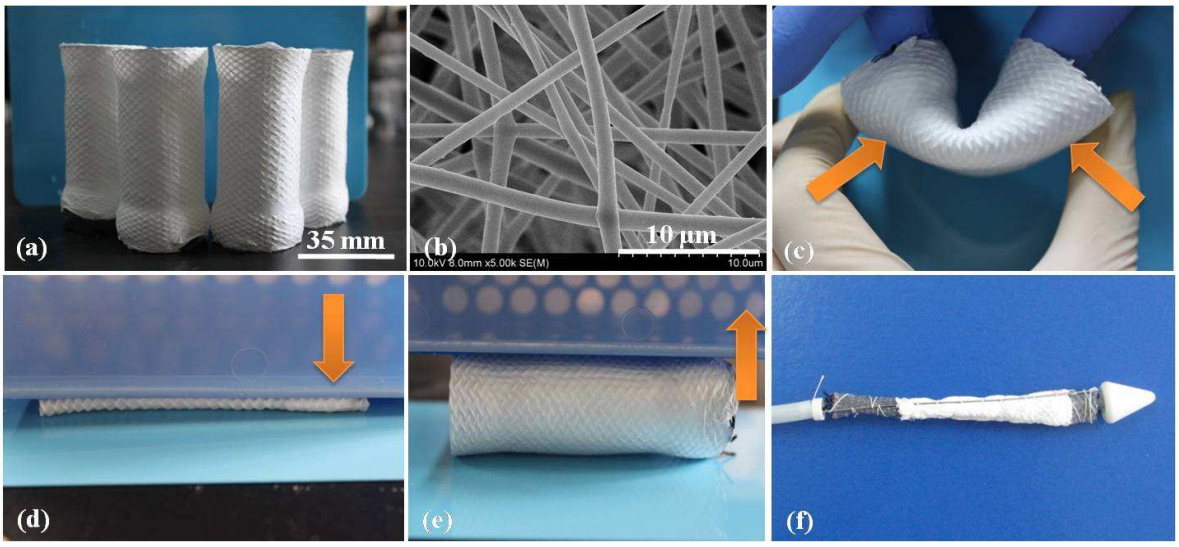


Figure 2. Images showing the macro/micro morphologies and mechanical properties related to the usability of the Rapa-loaded PCL electrospun fiber coated metal cardia stents. (a) Macro morphology of the modified stents; (b) Representative SEM image of Rapa-PCL fibers; (c) bending; (d) compression; (e) rebound; (f) fasten.

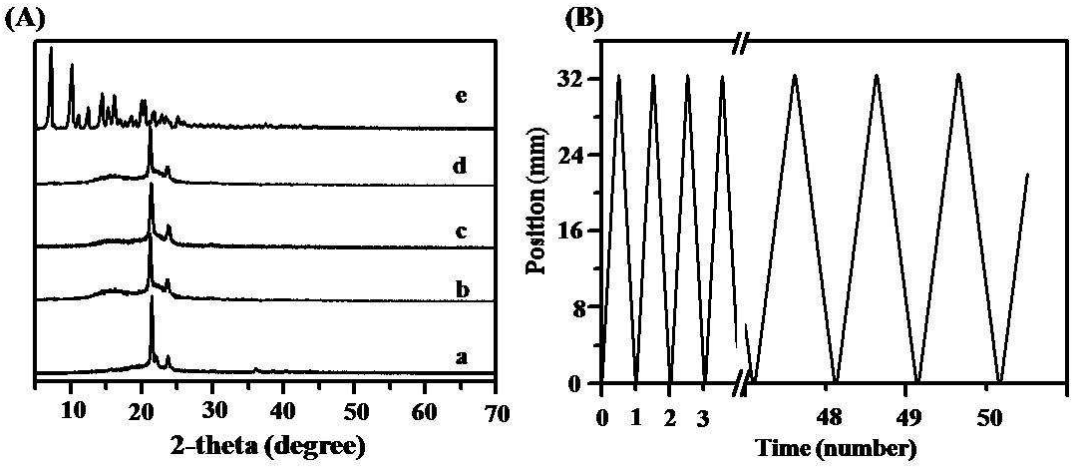


Figure 3. (A) X-ray diffraction profiles of PFM00 (a), RPFM05 (b), RPFM10 (c), RPFM20 (d) and Rapa (e). (B) Compression-recovery curve in repeated compression test for RPFM20 (n=50, Constant pressure=10N).

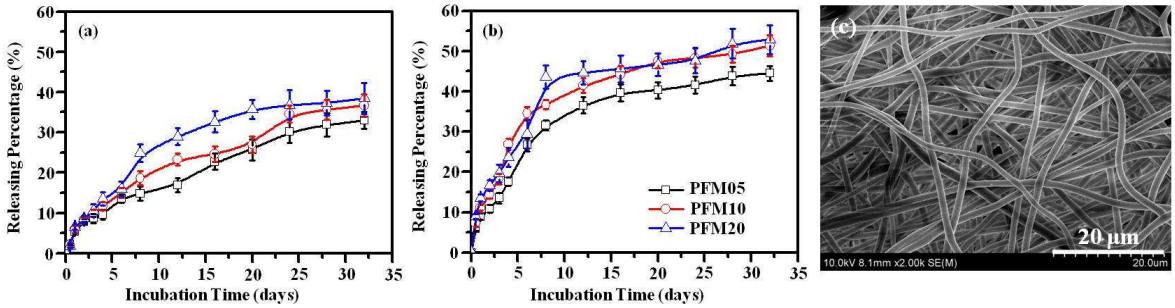


Figure 4. *In vitro* Rapa release curves of cumulative percentage of Rapa release from RPFM05, RPFM10 and RPFM20 after immersion in PBS of pH=7.4 (a) and pH=4.0 (b) at 37 °C, SEM image (c) of RPFM20 after 32 days immersion in pH=4.0 PBS.

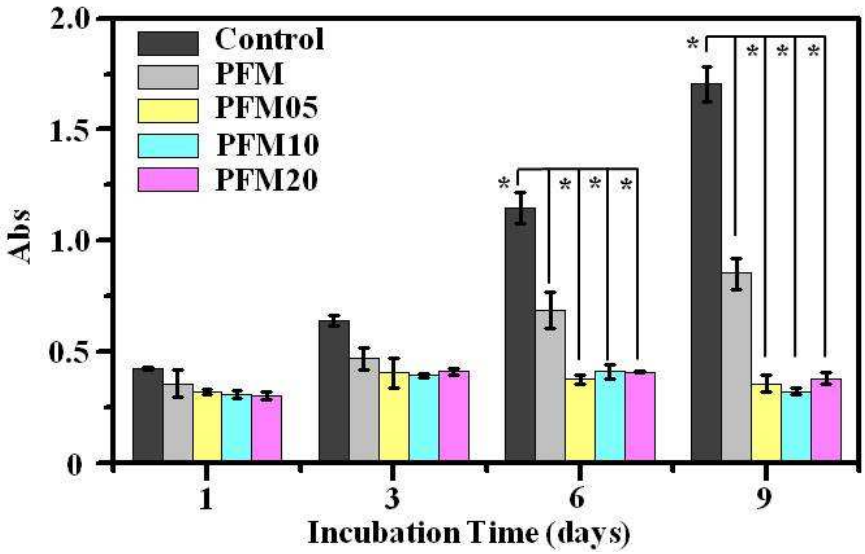


Figure 5. SMC proliferation on PFM00, RPFM05, RPFM10 and RPFM20 fibrous membranes after 1, 3, 6, and 9 days of culture. The control was tissue culture plastic. * represents $p<0.05$ with respect to the PFM00 fibrous membrane.

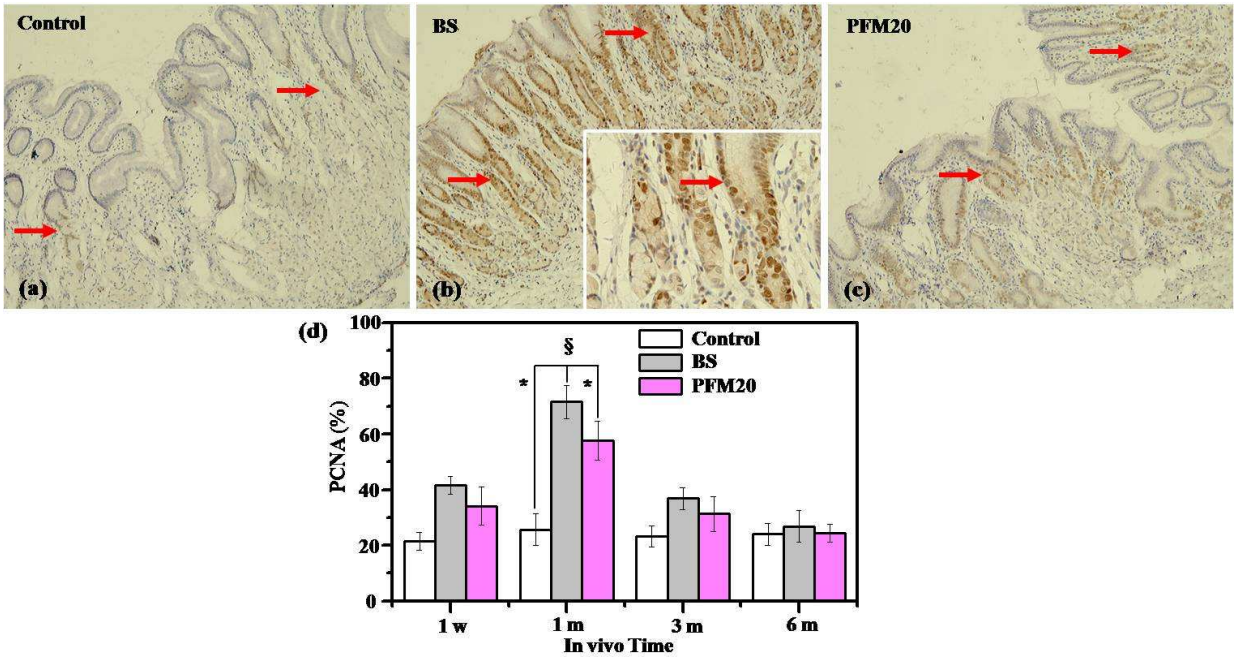


Figure 6. Inflammation after treatment using control (no stent), BS and PFM20 at different follow-up intervals. PCNA staining (a, b and c) was observed in the cubicula layer after 1 month (brown, magnification ×40; red arrows). (d) Percentage of PCNA positive cells after treatment using different stents. *$p < 0.05$ for comparison with the BS group, §$p < 0.05$ for comparison between groups.

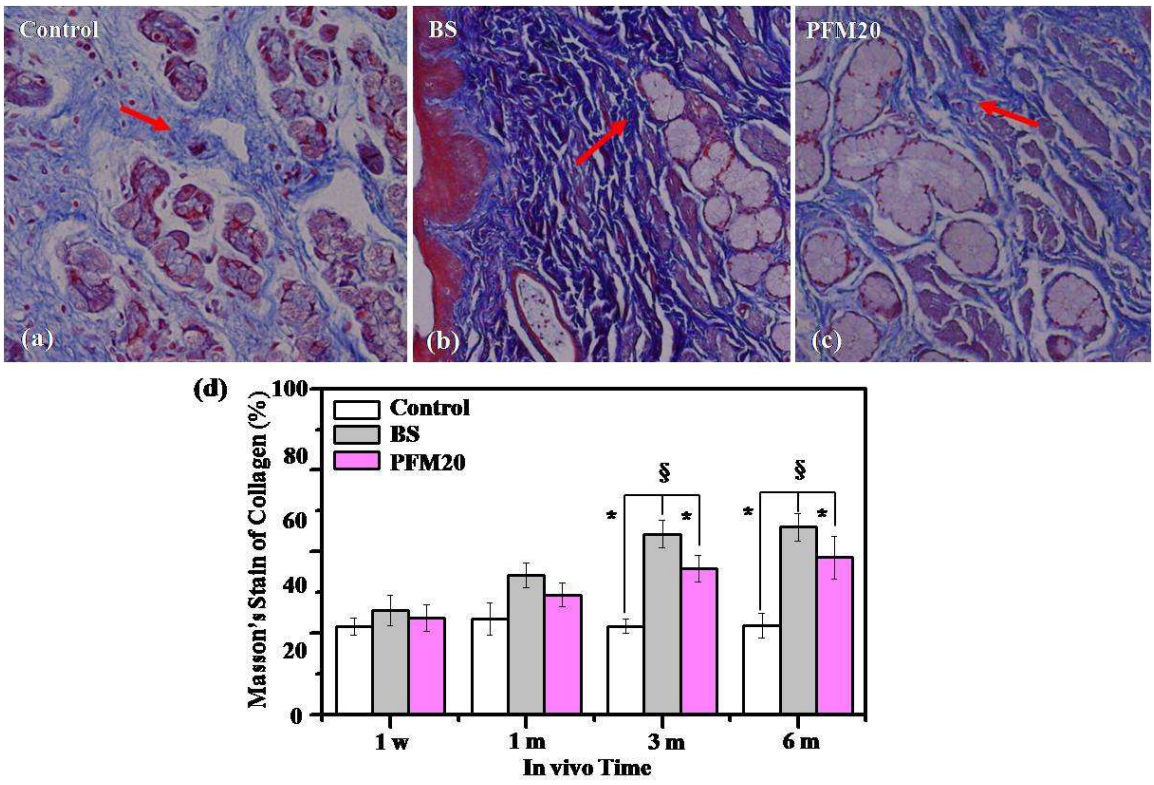


Figure 7. Scar formation after treatment of control, BS or PFM20 at different follow-up intervals. Masson's trichrome staining indicated that collagen fiber deposition in the submucosa (blue, magnification ×400; red arrows) stabilized ~3 months after treatment. (d) Collagen fiber proliferation. *p < 0.05 for comparison between the BS and PFM20 group, §p < 0.05 for comparison between groups.

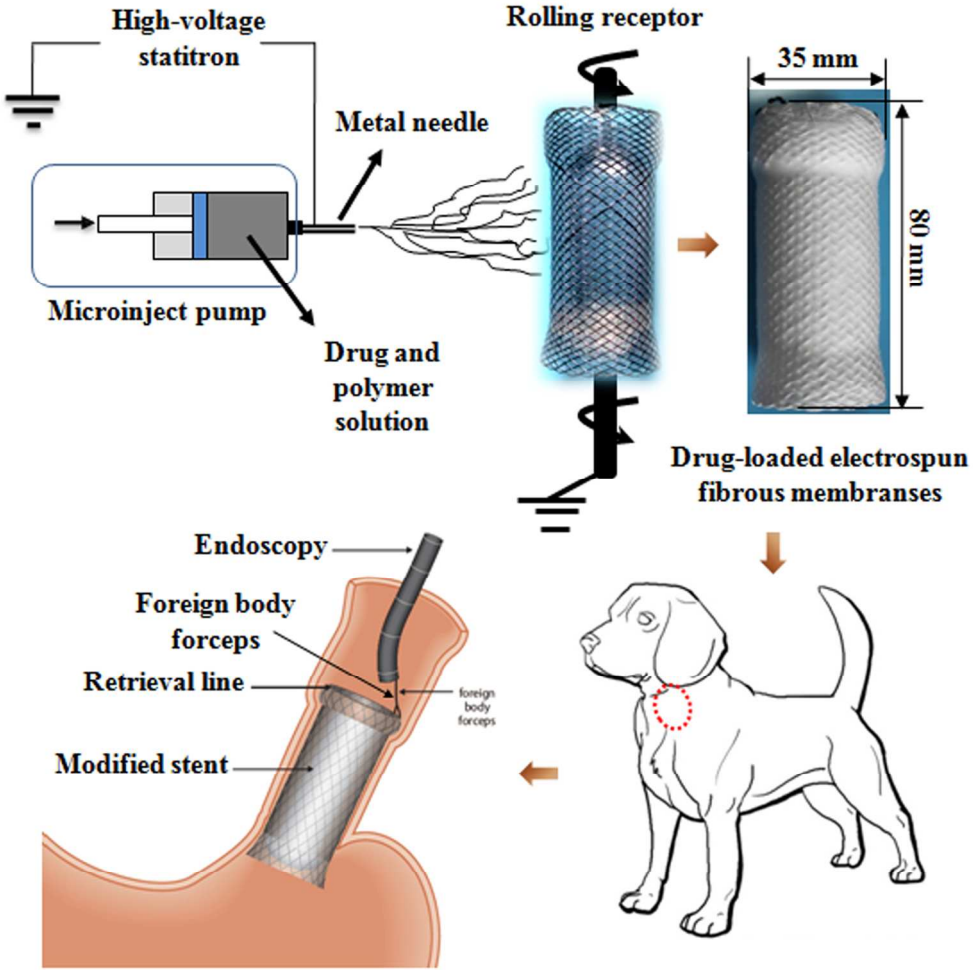
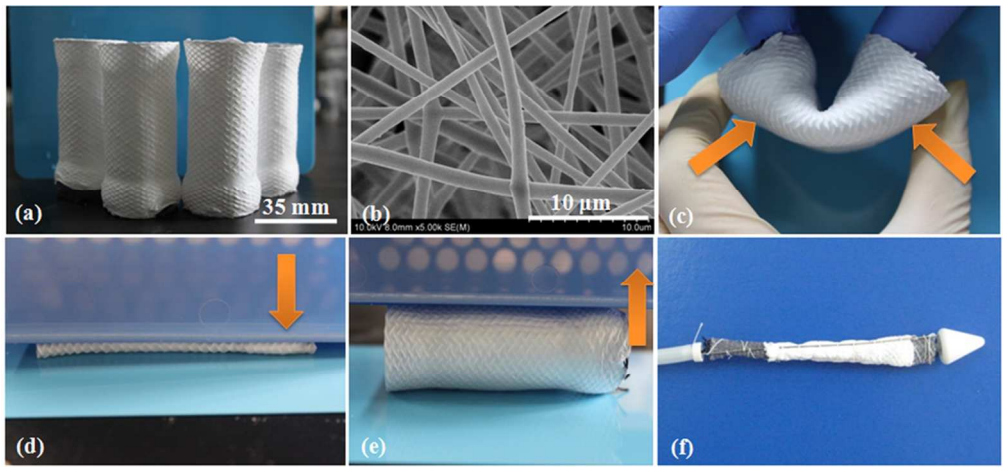


Figure 1. Diagram of the process used to fabricate non-biodegradable metal stents with an outer layer of Rapa-loaded fibrous membrane by using the electrospinning process. With the release of Rapa, the stents are expected to inhibit fibroblast proliferation and tissue hyperplasia, therefore treating a benign cardia stricture.
68x67mm (600 x 600 DPI)



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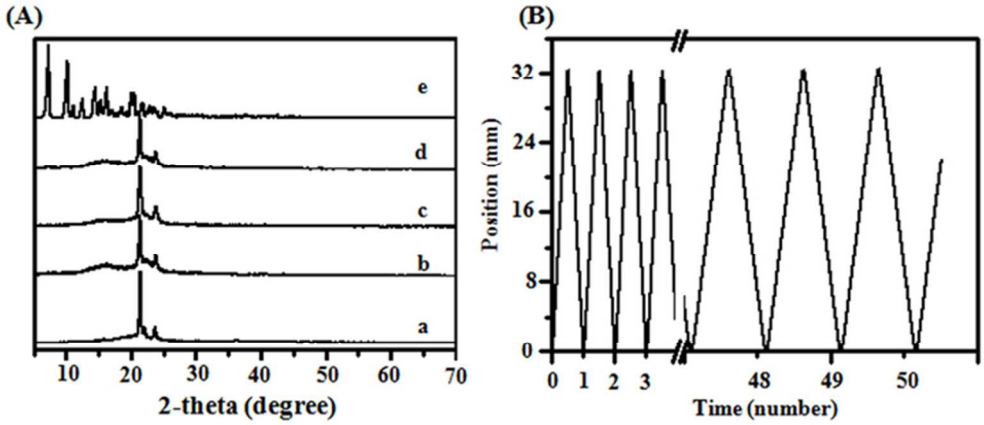
45x21mm (600 x 600 DPI)



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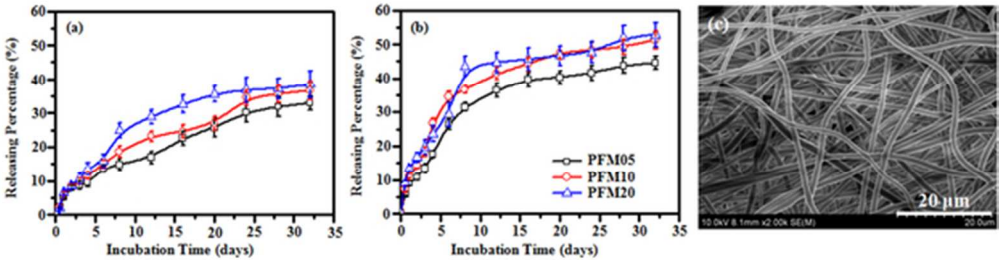
30x13mm (600 x 600 DPI)



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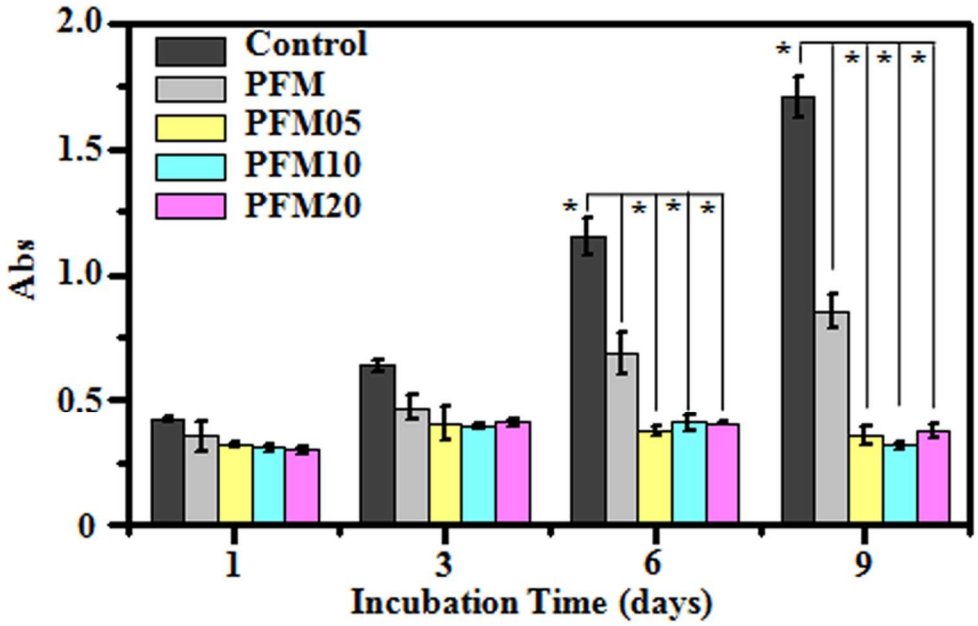
26x6mm (600 x 600 DPI)



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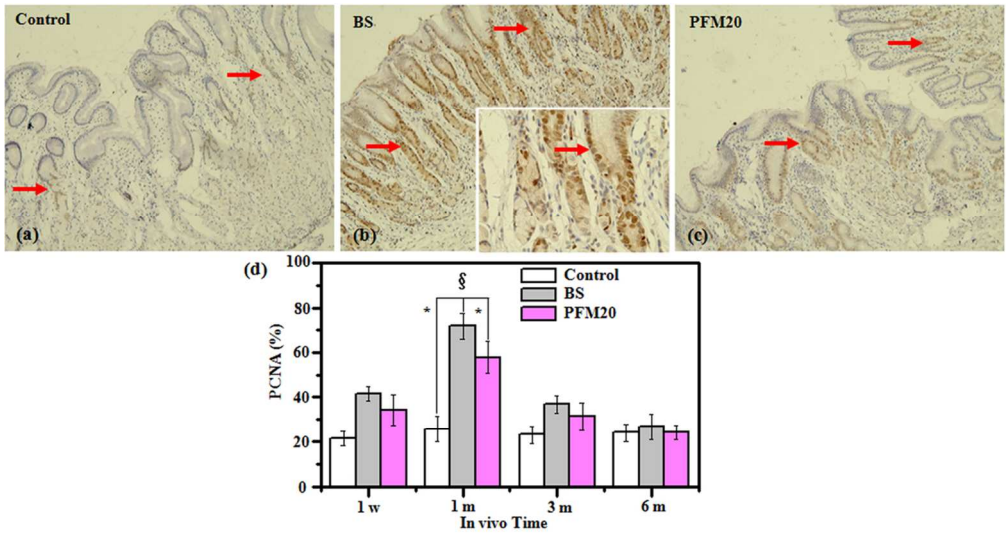
44x28mm (600 x 600 DPI)



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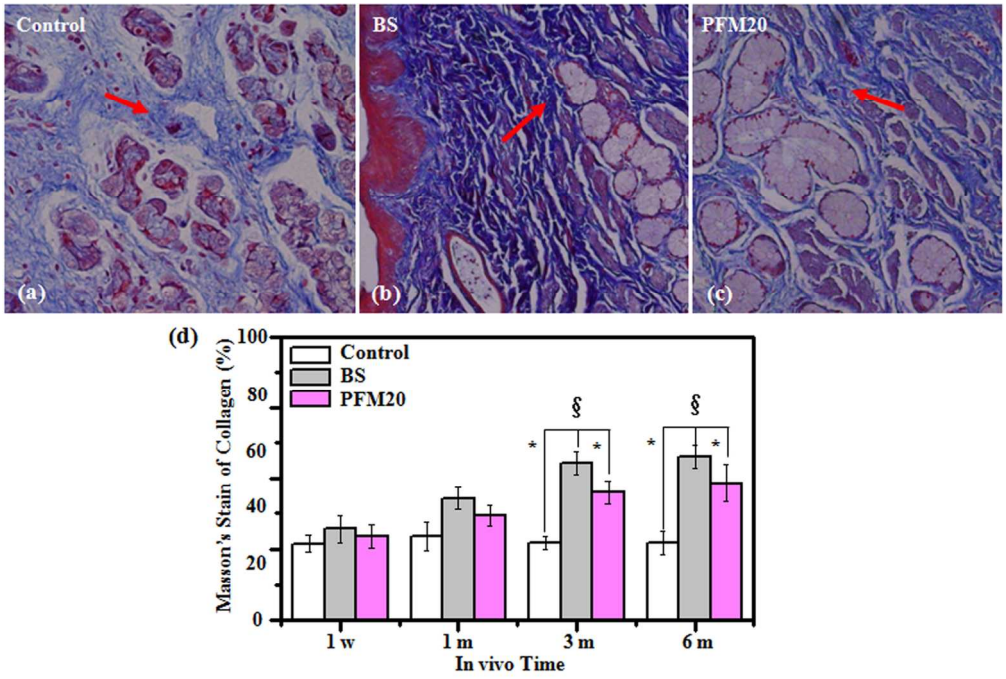
53x28mm (600 x 600 DPI)



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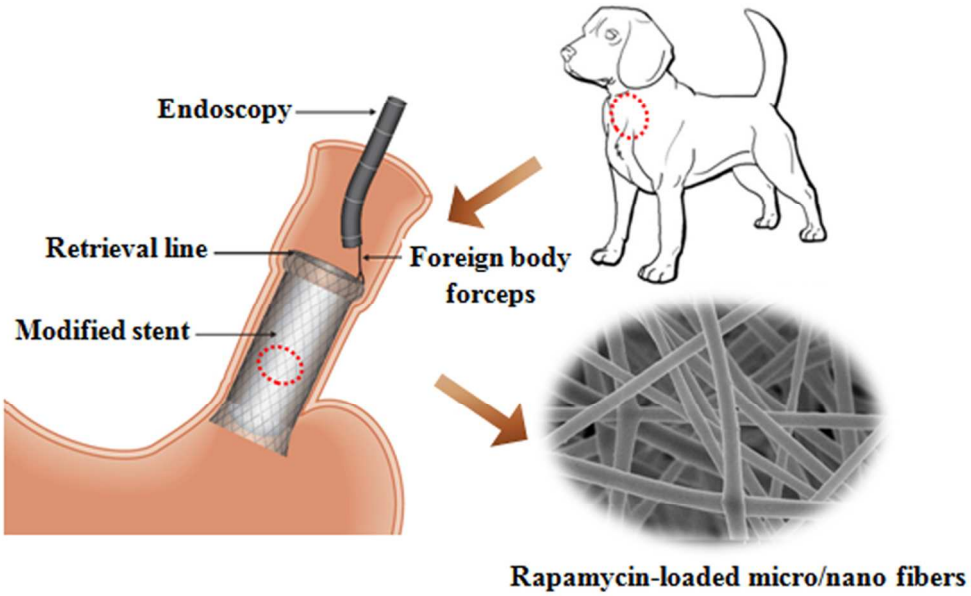
67x45mm (600 x 600 DPI)



42x25mm (600 x 600 DPI)