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***In vivo* performance of a bilayer wrap to prevent abdominal adhesions**

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Abstract

There is a high prevalence of intra-abdominal adhesions following bowel resection which can result in chronic pain, bowel obstruction, and morbidity. Although commercial adhesion barriers have been widely utilized for colonic resections, these barriers do not prevent anastomotic leakage resulting from reduced healing of the anastomosis which can result in long-term health problems. To address this limitation, we have developed an adhesive bilayer wrap with selective bioactivity to simultaneously prevent intra-abdominal adhesion formation and promote anastomotic healing. Reactive electrospinning was used to generate a crosslinked gelatin mesh to serve as a cell-instructive substrate to improve anastomotic healing. A coating of poly(ethylene glycol) (PEG) foam was applied to the bioactive mesh to generate an antifouling layer and prevent abdominal adhesions. After *in vitro* confirmation of selective bioactivity, the composite wrap was compared after 2 weeks to a commercial product (Interceed®) in an *in vivo* rat colonic abrasion model for prevention of intra-abdominal adhesions. The composite bilayer wrap was able to prevent intra-abdominal adhesions when clinical placement was maintained. The composite bilayer wrap was further modified to include tissue adhesive properties for improved efficacy. Preliminary studies indicated that the adhesive composite bilayer wrap maintained a maximum shear strength comparable to Interceed® and greater than fibrin glue. Overall, this work resulted in an initial proof-of-concept device that was shown to effectively prevent adhesion formation *in vivo*. The

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Declaration of interests

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composite bilayer wrap studied here could lead to an improved technology for improved healing of intestinal anastomoses.

1. INTRODUCTION

Intestinal anastomoses following bowel resection are among the most frequently performed procedures in general surgery [1]. Despite advancements in suturing and stapling techniques, there is a high incidence of post-operative complications with up to 30% of patients presenting with anastomotic leakage and up to 90% of patients developing intra-abdominal adhesions [2–5]. Anastomotic leakage is of particular concern due to its associated high morbidity and mortality rates as a result of infection, peritonitis, and sepsis [4, 6, 7]. Treatment of anastomotic leakage often requires prolonged hospitalization and a secondary operation to minimize infection. Intra-abdominal adhesions form vascularized fibrotic bridges between adjacent tissues that can restrict normal organ movement and blood supply leading to chronic pain and bowel obstruction [8, 9]. The severity and widespread occurrence of these two complications underscore the need for a method to improve patient outcomes after these surgical procedures.

The two most prevalent approaches to reduce anastomotic leakage are reinforcement of the anastomotic closure with surgical sealants (*i.e.*, fibrin and cyanoacrylate glues) and an adjunct wrap placed over the anastomosis. Despite *in vivo* experimental efforts to develop a method using surgical sealants to strengthen the suture line, results have displayed high variability of outcomes that were attributed to dosage variations and inconsistencies in application [10–12]. As an alternative, several groups have evaluated tissue-derived wraps to externally reinforce the anastomosis and enhance anastomotic healing including patient-specific omentum, bovine pericardium, human amniotic membrane, and porcine collagen foams [13–18]. Bovine pericardium, in particular, has been shown to improve wound healing with significant increases in burst pressures of the treated anastomoses [16]. Although these materials effectively enhance healing and reduce anastomotic leakage, none have gained commercial success possibly due to the increased incidence and severity of intra-abdominal adhesions associated with their use [19, 20].

To address the complications of intra-abdominal adhesions after intestinal surgeries, several adhesion barriers have been developed and commercialized. Adhesion barriers act to physically separate the injured tissue from the adjacent viscera during the critical period of postoperative healing (5–7 days), thereby reducing the formation of fibrotic adhesions [21]. Seprafilm®, composed of sodium hyaluronate and carboxymethylcellulose, and Interceed®, composed of an oxidized regenerated cellulose, are adhesion barriers that effectively reduce the incidence and severity of adhesions both experimentally and clinically [8, 22, 23]. Despite established efficacy, these barriers have limitations. The efficacy of Interceed® is significantly reduced in the presence of blood due to fibrin deposition and fibroblast penetration which reduces the capacity to prevent adhesions [24–26]. Application of Seprafilm® immediately after anastomoses delays inflammatory-mediated processes that promote healing of the suture line which leads to an increased rate of anastomotic leakage

[25–27]. As a result, several clinical studies have recommended that surgeons avoid applying Seprafilm® directly onto fresh suture lines [22, 27, 28].

To date, current treatments to enhance anastomotic healing to prevent leakage often result in increased adhesions; whereas, barrier materials to reduce adhesions can delay anastomotic healing and increase anastomotic leakage. These findings suggest that therapeutic strategies that only address one of these complications independent of the other will ultimately fail. Our laboratory developed a new approach to concurrently address these complications using a composite bilayer wrap with selective bioactivity to both reduce intra-abdominal adhesions and enhance anastomotic healing. The inner layer consists of an electrospun gelatin mesh to promote cell adhesion and remodeling [29]. Reactive electrospinning developed in our laboratory was utilized to modulate the degree of gelatin crosslinking and the corollary resorption rate. In order to resist adhesion formation, a resorbable poly(ethylene glycol) (PEG) hydrogel was selected as an outer layer due to its inherent non-fouling properties [30]. Iterative design of hydrogel and electrospun mesh formulations was utilized to identify a bilayer structure with requisite surgical handling characteristics and retention of wrap integrity after swelling. Candidate bilayer wraps were then evaluated to confirm selective bioactivity over two weeks. As an *in vivo* proof-of-concept study, adhesion prevention was assessed using a rat colonic abrasion model in comparison to a clinical control using Interceed®. Based on initial *in vivo* results, the bilayer wrap was further modified to include an additional tissue adhesive component to prevent displacement after implantation. Collectively, these studies provide an initial evaluation of a new bilayer wrap design to address the multimodal complications associated with intestinal anastomoses.

2. MATERIALS & METHODS

2.1 Materials

All chemicals were purchased from Sigma Aldrich (Milwaukee, WI) and used as received unless otherwise noted.

2.2 Polymer Synthesis

Poly(ethylene glycol) diacrylate (PEGDA) was synthesized according to a method adapted from Hahn, *et al.* [31]. Briefly, acryloyl chloride was added dropwise to a solution of PEG 2 kDa or 6 kDa diol and triethylamine in dichloromethane (DCM) under nitrogen. The molar ratio of PEG, acryloyl chloride, and triethylamine was 1:2:4, respectively. After the addition of acryloyl chloride, the reaction was stirred for an additional 24 hours at room temperature. The resulting solution was then washed with 8 molar equivalents of 2 M potassium bicarbonate to remove acidic byproducts. The product was then precipitated in cold diethyl ether, filtered, and dried under vacuum.

PEGDA with thio-β esters (PEGDTT) was synthesized as previously described [30]. d,l-dithiolthreitol (DTT) and triethylamine (TEA) were added dropwise to a solution of PEGDA (2 kDa) in DCM. The molar ratio of DTT, PEG and triethylamine was 3:2:0.9, respectively. After the addition of the DTT and triethylamine, the reaction was stirred for 24 hours at room temperature. The resulting solution was then precipitated in cold diethylether,

washed, filtered, dried under ambient conditions for 24 hours then placed under vacuum to remove any excess solvent.

Characterization of PEGDA and PEGDTT was confirmed using proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy. Proton NMR spectra of control and functionalized polymers were recorded on a Mercury 300 MHz spectrometer using a TMS/solvent signal as an internal reference. Percent conversions of PEG diol to acrylate endgroups was greater than 85%. $^1\text{H-NMR}$ (CDCl_3): δ 3.6 ppm (m, $-\text{OCH}_2\text{CH}_2$), 5.8 ppm (dd, $-\text{CH}=\text{CH}_2$), 6.1 (dd, $-\text{CH}=\text{CH}_2$) and 6.4 ppm (dd, $-\text{CH}=\text{CH}_2$).

2.3 Reactive electrospinning of gelatin

In situ crosslinking of electrospun gelatin was performed as previously reported [29]. Briefly, a double-barrel syringe with an attached mixing head and a diisocyanate crosslinker were utilized to generate electrospun scaffolds that crosslink during the electrospinning process via reaction of the isocyanate with the pendant amines of lysine residues in gelatin. Bovine-derived gelatin, 1,4-diazabicyclo[2,2,2]octane (DABCO) and hexamethylene diisocyanate (HDI) were each dissolved in 2,2-trifluoroethanol (TFE). The concentration of HDI was determined such that the crosslinker density would equal a 5:1 ratio of isocyanate:amine. Double-barrel syringes were loaded with 12 wt% gelatin in TFE and 5 wt % (of solids) DABCO solution in one barrel and HDI in TFE solution in the other. The double barrel syringe solutions were pumped through a mixing head into an 18-gauge blunted needle at a rate of 3.0 mL/hr. The needles were placed 12 cm away from the collector to allow for adequate solvent evaporation and fiber drying. A high voltage of 10 kV was then applied to the needle. Meshes were electrospun for 3 hours and stored under vacuum for at least 24 hours prior to further use.

2.4 Bilayer wrap fabrication

A PEG-based hydrogel coating method was selected through iterative design using 3 distinct methods. Namely, a bulk hydrogel coating, an electrosprayed hydrogel coating, and a hydrogel foam coating were tested. Bulk hydrogel coatings were fabricated from a 10 wt% PEGDTT:PEGDA 75:25 solution in distilled water. A photo-initiator solution (1 mg Irgacure 2959 per 10 μl 70% ethanol) was added at 1 vol% of precursor solution. The PEG hydrogel precursor solution was pipetted between 1-mm spacer plates and exposed to UV light to initiate crosslinking. After 45 seconds of UV illumination, a 0.2-mm thick gelatin mesh was placed on top of the surface of the hydrogel. Samples were then exposed to UV light for 5 additional minutes to complete hydrogel formation. Bilayer wraps were swollen in reverse osmosis water overnight followed by lyophilization before further use.

Electrospraying precursor solutions were prepared from 30 wt% 75:25 PEGDTT:PEGDA in 70:30 DCM:EtOH solvent. A photo-initiator solution (1 mg Irgacure 2959 per 0.01 ml 70% ethanol) was added at 3 vol% of precursor solution. The hydrogel solution was then added to a syringe and electrospun gelatin meshes were attached to a copper plate as the collector. A voltage of 7.5 kV was applied to the needle tip and -2 kV was applied to the collector, which was set 13 cm away from the needle tip to allow for solvent evaporation and particle drying. The polymer solution was dispensed at a rate of 1.0 mL/hr using a syringe pump. A

UV lamp was placed above the electrospraying set up to enable *in situ* curing. The hydrogel solution was electrosprayed for 3 hours, after which the resulting bilayer wrap was vacuumed for at least 24 hours prior to characterization.

Finally, PEG-based hydrogel foam coatings were prepared using an emulsion templating method developed previously in our laboratory. Hydrogel precursor solutions were prepared by mixing 20 wt% PEGDTT:PEGDA 75:25 in a 10 wt% solution of Pluronic F-68 in water, and a photo-initiator solution added at 1% of the total polymer of lithium phenyl-2,4,6-trimethylbenzoylphosphinates (LAP). Precursor solutions were made with and without 10 wt% trimethylolpropane ethyodolate triacrylate (TMPE-TA) as an additional crosslinker. Once thoroughly mixed, light mineral oil was added at volume fractions of 50% of the continuous polymer precursor phase. The water and oil composition was mixed at 2500 rpm for 5 minutes using a FlackTek Speedmixer DAC 150 FVZ-K. Electrospun gelatin meshes were affixed to 1.0-mm spacer plates and hydrogel emulsions were then transferred into the mold. Samples were exposed to UV light for 6 minutes to complete foam formation. Wraps were then subjected to a series of 3 1-hour soaks to remove the oil content: 1) 100% DCM; 2) 50/50 DCM/ethanol; 3) 100% ethanol. After the last ethanol soak, wraps were soaked in distilled water overnight prior to lyophilization before further use.

2.5 Bilayer wrap characterization

Scanning electron microscopy (SEM) (JOEL 6500) was utilized to image specimens. Lyophilized samples were stored under vacuum prior to imaging. A total of 4 composite bilayer wraps were fabricated for each composite formulation. One specimen was taken from each wrap to yield 4 specimens/group. Specimens were coated with 4 nm of gold using a sputter coater (Sputter Coater 108, Cressington Scientific Instruments). Confirmation of two distinct layers was performed by evaluating the chemical structure of each using attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy (Nicolet iS10 (Thermo Scientific) at a resolution of 2 cm^{-1} for 32 scans.

A previously described, 4 composite bilayer wraps were fabricated for each composite formulation. One dog-bone specimen was cut from each wrap to yield 4 specimens/group in accordance with ASTM D1708. Specimens were strained to failure at a rate of 100 %/min based on the initial gauge length using an Instron 3345 uniaxial tensile tester equipped with a 100-N load cell and pneumatic side action grips (Instron 2712-019). The elastic modulus, tensile strength, and ultimate elongation were calculated from the resultant engineering stress-strain curves. A secant modulus at 2% strain was calculated for the elastic modulus and subsequently referred to as “modulus”. Toughness was calculated as the area under the stress-strain curve.

Selective bioactivity over time was assessed via cell adhesion. Adult human dermal fibroblasts (hDFs, isolated from adult skin, Invitrogen) were cultured in growth media containing 16.5% fetal bovine serum (FBS, Atlanta Biologicals), 1% L-glutamine (Life Technologies), and Minimum Essential Media α (MEM α , Life Technologies) to 80% confluence and utilized at Passage 5. A total of 4 hydrogel foam fiber composite bilayer wraps were fabricated and 8 mm-disks were removed from each wrap using a biopsy punch (Integra Miltex). Specimens were placed in a tissue cultured 48-well plate (Corning) and

were sterilized for 30 minutes under UV. They were then immersed in PBS and incubated at 37 °C for 2 weeks. PBS was selected as the hydrogel component is hydrolytically degradable and incubation in PBS is a common analogue for real-time hydrolytic degradation under physiological conditions (e.g. pH and ion concentration) [32–34]. Specimens (n=4/group) were then taken at 1, 7, and 14 days to test selective bioactivity. Human dermal fibroblasts were seeded upon either the hydrogel foam layer or the gelatin layer at 10,000 cells/cm² in growth media supplemented with 1 vol% penicillin-streptomycin. Following a 3-hour incubation, cells were fixed and stained with rhodamine phalloidin (F-actin/cytoplasm, Biotium) and SYBR green (DNA/nucleus, Thermo Fisher Scientific). A 3-hour culture period was selected as it is considered a typical length of time to allow cell attachment [35–37]. Cell adhesion was calculated from manual cell counts of images obtained through raster patterning of 4 specimens/group/end point using a fluorescence microscope (Nikon Eclipse TE2000-S).

2.6 Animal Care

All procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC, Animal Use Protocol# 2017-0316).

2.7 In vivo assessment

First, *in vivo* resorption rates for each component of the bilayer mesh was assessed. Sprague-Dawley rats (n = 3/group) were randomly assigned to 1 of 2 groups: 1) hydrogel foam or 2) electrospun gelatin mesh to assess degradation. Animals were anesthetized with isoflurane in oxygen and maintained on isoflurane in oxygen via a nose cone. The ventral abdomen was clipped and prepped for surgery with chlorhexidine and 70% ethanol. The abdomen was draped with a sterile paper drape and a 2-cm ventral mid-line incision made in the central third of the abdomen. The appropriate dried mesh or gel was paced in the abdomen in a sterile manner. The body wall was closed with 5-0 PDS in an intradermal pattern and the skin was closed with 5-0 PDS in a simple continuous pattern. A circumferential abdominal bandage was placed and all animals were administered buprenorphine (0.01 mg/kg, subcutaneously) immediately post-operatively. At each time point, the rats were euthanized via carbon dioxide asphyxiation. The abdomen was opened and entire abdomen thoroughly explored macroscopically to identify remaining specimens or confirm full resorption.

The effect of treatment on the formation of intra-abdominal adhesions was assessed using a rat cecum abrasion model as previously described [38]. Sprague-Dawley rats (n = 5/group) were randomly assigned to 1 of 6 groups: 1) cecum abrasion with no treatment; 2) cecum abrasion with fibrin glue applied; 3) cecum abrasion with Interceed ® applied; 4) cecum abrasion with gelatin mesh only applied; 5) cecum abrasion with hydrogel foam only applied; 6) cecum abrasion with bilayer wrap applied. Specimens were sterilized using ethylene oxide and allowed to vent for 3 days prior to use. Twelve-week-old rat were anesthetized and prepared for surgery as described above. A 2-cm ventral mid-line incision made in the central third of the abdomen. The cecum was exteriorized and a template used to abrade the cecal serosa in a standard location, size (1 x 2 cm²), and severity of abrasion (40 strokes of a dry gauze with mild pressure). Similarly, a 1 x 2 cm² area of body wall was

resected from the right side of the abdomen centered 1 cm dorsal to the incision. A single simple interrupted suture (5-0 monocryl) was placed between the cecum and the body wall at a site approximately 1 cm distant (cranial) to the abraded area of both the cecum and body wall. The treatments were then applied. Briefly, Tisseel® tissue sealant was used as fibrin glue (Baxter healthcare) and was thawed in a water bath 1 hour prior to use and used within 4 hours of thawing. Approximately 200 µL of fibrin glue was applied to the abraded surface of the cecum for every group except PBS and Interceed®. The appropriate mesh was then immediately applied to the area making every effort to cover the abraded area with the mesh. For Interceed®-treated rats, fibrin glue was not applied and instead Interceed® was applied according to manufacturer's directions (applied dry to a non-bleeding surface). Similarly, approximately 500 µL PBS was pipetted onto the damaged portion of the cecum 3 times for the PBS group. The cecum was then returned to the abdomen. The body wall was closed with 5-0 PDS in a simple continuous pattern and the skin was closed with 5-0 PDS in a simple continuous pattern. A circumferential abdominal bandage was placed and all animals were administered buprenorphine (0.01 mg/kg subcutaneously) immediately post-operatively.

At each time point, animals were euthanized via carbon dioxide asphyxiation. The abdominal wall was opened at the left flank to avoid disturbing site of adhesion and retracted to expose the right body wall. Adhesions were photographed and scored discretely for size, strength, and maturity as described in Supplemental Table 1. One observer blinded to treatment group scored all of the animals. As the small scale used to determine adhesion scores (0-3, 0-5) does not capture the differences between large, mature adhesions, and smaller, less cohesive adhesions, a composite score was assigned to each rat based on the following calculation:

$$\text{Composite score} = \text{size} \times (\text{strength} + \text{maturity})$$

2.8 Dopamine tissue adhesive coating

Dopamine hydrochloride was dissolved at 50 mg/mL in 10 mM tris. 1 mL of solution was then carefully pipetted onto the gelatin layer of a swollen bilayer wrap. After 24 hours, the solution was aspirated and 1 mL of 3 wt% sodium periodate in distilled water was applied to the bilayer wrap. After 1 minute, the sodium periodate solution was aspirated and wraps were washed in 50 mL of distilled water for 5 hours with water changes every hour. Samples were subsequently frozen and lyophilized prior to characterization. The dopamine-modified gelatin layer was affirmed using ATR-FTIR spectroscopy at a resolution of 2 cm⁻¹ for 32 scans.

2.9 Adhesion strength measurement

Adhesive properties of the dopamine-coated gelatin layer were determined using lap-shear tensile stress measurements in accordance with the ASTM standard F2255-05. Fresh split thickness porcine skin was cut into 2.5 x 5 cm² segments with uniform thickness and hydrated in PBS for immediate use. A total of 4 un-modified and adhesive gelatin meshes were fabricated and stored in a dry location at room temperature. A 2.5 x 1 cm² specimen was removed from each mesh to account for batch variability. Interceed® (Ethicon) sheets

were cut into comparable dimensions for comparison to the un-modified and adhesive gelatin meshes. Interceed® was selected as a clinical control due to its ability to securely adhere to the anastomosis without sutures when meticulous hemostasis has been achieved [24]. The porcine test substrates were removed from PBS and blotted with sterile gauze to remove excess PBS prior to coming into contact with the un-modified gelatin specimens, adhesive gelatin specimens, or Interceed®. The specimens were positioned between two tissue substrates with a 1 cm overlap. They were then compressed with a force of approximately 1 N to allow for the bond to set. Specimens were further conditioned in a humidity chamber at 37°C for 30 minutes prior to being strained to failure at a rate of 5 mm/min using an Instron 3345 uniaxial tensile tester equipped with a 100-N load cell and pneumatic side action grips (Instron 2712-019). The maximum strength and failure strain were recorded. The adhesive strength was calculated by maximum strength divided by the initial bond area.

2.9 Statistical Analysis

Data are displayed as mean $\pm$ standard deviation for each composition. Statistical analysis was performed utilizing a standard one-way ANOVA unless otherwise noted in the figure captions. Statistical significance was accepted at $P < 0.05$.

3. RESULTS

3.1 Bilayer wrap optimization

Multiple bilayer formulations were investigated for their suitability to produce an optimized adhesion barrier with enhanced handleability and stability *in vitro*. The proposed PEG-based hydrogel layer for adhesion prevention was applied to the inner electrospun gelatin layer in 3 forms: a hydrogel layer, an electrosprayed layer, and a foam layer (Figure 1A–C). Each of the 3 bilayer wrap fabrication methods were assessed for handling in terms of tensile properties and stability via delamination assessment upon swelling. The hydrogel bilayer wrap displayed the most brittle behavior, as evidenced by the stress-strain curves in Figure 1C. The electrosprayed bilayer wrap had the lowest toughness ($0.4 \pm 0.2 \text{ MJ/m}^3$) and reached tensile failure at $3.5 \pm 1.2\%$ elongation (Table 1). The hydrogel foam composite bilayer wrap displayed the highest toughness of $23.6 \pm 1.7 \text{ MJ/m}^3$ and reached an elongation of $41.0 \pm 4.0\%$. As such, this composite formulation was selected for future testing. However, swelling analysis indicated delamination upon immersion in water. In order to decrease the degree of swelling and thereby resist delamination, the crosslinker, trimethylolpropane ethoxylate triacrylate (TMPE-TA), was added to the foam precursor solution. The TMPE-TA containing bilayer was again evaluated to ensure maintenance of handleability and stability *in vitro*. The formulation displayed a further significant increase in toughness, from 23.6 ± 1.7 to $28.8 \pm 1.5 \text{ MJ/m}^3$ (Table 1). Furthermore, the formulation displayed no evidence of delamination upon swelling. This optimized bilayer wrap, containing an electrospun gelatin layer and PEG-TMPETA foam was subjected to further characterization prior to assessment *in vivo*.

3.2 Bilayer wrap characterization

ATR-FTIR spectroscopy was utilized to evaluate the chemical composition of each layer in the selected bilayer wrap. Spectral analysis indicated that although the hydrogel was visible on both layers, as demonstrated by the ether peak at 1110 cm^{-1} , the amine stretch at 3350 cm^{-1} of gelatin was only present on the inner layer (Figure 2A). Scanning electron microscopy of the selected bilayer wrap, displayed in Figure 2B, demonstrated that the inner gelatin layer consisted of homogenous, smooth fibers while the outer hydrogel foam layer displayed a porous intraarchitecture encased between the fibrous layer and a smooth, outer film.

Next, the potential to maintain selective bioactivity over the desired application period (target 2 weeks) was evaluated by assessing hDF adhesion on each layer at selected time intervals. Bilayer wrap samples were incubated in PBS for 2 weeks and removed at 1, 7, and 14 days to prior to culturing with cells to determine the effect of degradation on bioactivity. After each time point, hDFs were seeded onto each side of the bilayer wrap for 3 hours and cell adhesion was evaluated. There was significant cell adhesion on the electrospun gelatin layer throughout the 14-day period, (Figure 2C). In contrast, the anti-fouling properties of the hydrogel foam layer prevented cellular adhesion resulting in cells being washed away prior to analysis. Overall, these results suggested that selective bioactivity was maintained.

3.3 Assessment of in vivo degradation

Prior to evaluating the efficacy of the bilayer wrap, an *in vivo* degradation scouting study was conducted. Samples of each composition were implanted within the abdominal cavity of rats and degradation was assessed visually after 7, 14, and 21 days. Initial studies indicated that gelatin meshes were present at 7 days and had lost mechanical integrity by 14 days. Hydrogel foam specimens remained at 21 days, suggesting that a longer study must be conducted to fully evaluate the *in vivo* degradation rate. These initial results indicated that the degradation profiles of each component were within range of the targeted rate, permitting initial evaluation for adhesion prevention.

3.4 In vivo evaluation of intra-abdominal adhesion formation

Intestinal adhesion formation between the cecum and peritoneum of rats was evaluated by observation of abdominal wound sites within a rat abrasion model after 14 days (Figure 3). Initial scouting studies indicated that applying the bilayer wrap to the wound site with sutures resulted in strong adhesion formation initiated by leakage, and ultimately infection. As a result, fibrin glue was used to affix the bilayer wrap to the abraded area. The adhesions were evaluated macroscopically to discretely evaluate their area, strength, and maturity (data not shown). A summary of the adhesion scoring method is described in Supplemental Table 1. In addition to the adhesion scoring system, a composite score was also calculated in an attempt to better capture the differences in adhesions by considering the correlation between size, strength, and maturity. Figure 3B displays a summary of the composite scores as well as representative images from each group (Figure 3C–H). Control rats, and rats with only fibrin glue application, showed the development of strong, thick adhesions between the cecum and peritoneum in all instances, with composite scores of 18 ± 9 and 20 ± 4 , respectively. Interceed® was utilized as a clinical control and showed complete prevention

of adhesions in all specimens (composite score = 0 ± 0). Gelatin meshes applied alone saw a range of adhesion formation in the rats, with a composite score of 12 ± 10 that was statistically greater than Interceed®. This was expected as gelatin is known to promote cell adhesion, which the bilayer aims to utilize to promote healing. Hydrogel foam control and bilayer wrap treated rats had composite scores of 8 ± 7 and 8 ± 5 each. Though these scores were higher than that for Interceed® treated rats, they were not statistically different. The higher scores were attributed to a subset of rats in both groups presenting with immature adhesions at the wound site. It was noted that the foam and bilayer wraps had been displaced from the wound site in each of the rats where adhesions were reported. Few adhesions were found in rats where the foams or wraps remained in place. As such, it was concluded that the fibrin application was insufficient to maintain placement and that wraps needed to be formulated such that they adhered to the wound bed for further studies.

3.5 Development of a tissue-adhesive bilayer wrap

Dopamine modification of the electrospun gelatin was investigated as a method to confer adhesive properties to the bilayer wrap to prevent displacement. Electrospun gelatin meshes were coated with dopamine through the reported dip-coating method (Figure 4A). Dopamine-modified gelatin meshes were then oxidized and lyophilized to preserve their oxidized state. Infrared spectral analysis indicated the presence of the dopamine-specific peaks in the modified gelatin meshes (Supplemental Figure 1). Lap shear testing was utilized to evaluate the adhesive properties of the dopamine-modified gelatin mesh as compared to un-modified gelatin, Interceed®, and fibrin glue. Specimens were applied to split-thickness porcine skin and subjected to tensile testing as depicted in Figure 4B. The mean shear strength of the dopamine-modified electrospun gelatin layer was 11.6 ± 2.9 kPa. This value is significantly higher than values reported for fibrin glue (4.6-6.9 kPa) and comparable to that obtained from commercial Interceed® (Figure 4C) indicating that dopamine can potentially prevent displacement [39, 40]. The shear strength of the un-modified gelatin mesh was not included as, compared to other groups, the hydrated gelatin wrap had shear strength associated with forces that were below the detection limit of the load cell.

4. DISCUSSION

It remains a challenge to improve healing of intestinal anastomoses complicated by anastomotic leakage. Current treatments to improve anastomotic healing often result in increased adhesions [19, 26]. Adhesion development typically occurs within the first 5 days after surgery and is driven by the natural healing cascade. After damage at the peritoneum, macrophages and mesothelial cells secrete cytokines and other inflammatory mediators to initiate re-epithelialization [41]. During this process, fibrin is deposited at the injured site, forming a matrix to facilitate repair. At this time, fibrin bridges may form between two adjacent damaged surfaces. Typically, fibrin is then broken down by fibrinolytic enzymes leaving behind a healthy mesothelial layer. However, the activity of these enzymes can become compromised due to surgical damage limiting fibrin breakdown. This results in an influx of fibroblasts, capillaries, and nerves that lead to the development of permanent fibrous connective tissue, also known as adhesions [42]. Adhesion barriers such as Seprafilm® and Interceed® typically prevent adhesion formation by physical separation

between tissues during the first 5 days. Contrarily, there is concern with the use of adhesion barriers as adhesion formation has an integral role in the 1 to 2 week critical re-epithelialization phase of the anastomotic healing [26, 43]. Prevention of adhesion formation has been shown to delay anastomotic healing leading to increased incidences of leakage [19, 26, 43]. Thus, barrier films that solely address adhesion prevention fail to holistically address the clinical needs and can lead to more severe complications associated with anastomotic leakage. Key requirements for an improved adhesion barrier include that it promotes anastomotic healing, is simple to apply, is stable in aqueous environments, degrades within the targeted time-frame, and maintains its position during treatment [44].

To this end, we have developed a resorbable bilayer wrap that resists adhesion formation while maintaining the potential to promote healing of the anastomosis site. The inner layer consists of a crosslinked gelatin electrospun mesh. Gelatin, a natural polymer, inherently promotes cellular adhesion through its RGD (arginine-glycine-aspartate) ligand [45]. The fibrous structure of the electrospun mesh also promotes cellular integration by mimicking the native structure of the extracellular matrix [46]. As the gelatin layer of the bilayer wrap is present to promote this re-epithelialization, the targeted degradation time for the wrap is 2 weeks to provide a buffer for delayed healing. Previous work demonstrates a methodology to crosslink gelatin during the electrospinning process with a diisocyanate for improved fiber morphology retention and controlled enzymatic degradation. Enzymatic degradation facilitates cleavage of peptide bonds within the gelatin structures resulting in resorption byproducts that are cytocompatible and cleared from the body [29, 47]. *In vitro* studies demonstrated that the gelatin layer reached full degradation in enzymatic solution by 14 days [29]. Furthermore, the outer layer of the wrap is a degradable PEG-based hydrogel foam. PEG-based hydrogels have an intrinsic resistance to cell adhesion and thereby have the potential to prevent adhesion to surrounding tissue. This was demonstrated by West *et al.* who utilized an *in situ* crosslinked polyethylene glycol-co-lactic acid diacrylate hydrogel to effectively reduce the adhesion formation by 60% in a similar rat model [48]. Although adhesion formation can be prevented during the first 5 days by the PEG-based foam, it was hypothesized that the PEG layer degradation rate should be slower than that of the gelatin layer (targeted 3 weeks). PEGDA degradation is primarily mediated by hydrolysis of the esters within the acrylate endgroups. As there are low numbers of esters present in PEGDA, the *in vivo* degradation rate is slower than desired [32, 49]. A modified PEGDA-based foam with thio- β ester linkages was utilized to control hydrolytic degradation to achieve a targeted degradation rate. The incorporation of thio- β ester linkages increases the positive atomic charge on the carbonyl carbon of proximal acrylate ester bonds which heightens ester hydrolysis via nucleophilic reactivity. Higher stoichiometric monomer ratios of thio- β ester linkages to PEGDA results in more hydrolytically labile sites leading to faster degradation [50]. Previous *in vitro* work demonstrated that the modified PEG hydrogel degraded via hydrolysis within 3 weeks and that degradation byproducts were cytocompatible [30, 50].

In contrast to other PEG-based formulations, we hypothesized that the PEG foam would provide the requisite toughness and elongation for improved handleability and ease of application of the bilayer wrap. The favorable mechanical properties of the foam-based bilayer wrap were attributed to the foam's microarchitecture (*i.e.*, porosity and pore size) which were consistent with mechanical properties of hydrogel foams possessing the similar

microarchitecture [51, 52]. It has been noted that hydrogel-based foams with similar porosity and pore sizes have relatively lower tensile strength and modulus with corollary enhanced elongation [52]. Equally important to the ease of application is the stability of the bilayer wrap in terms of delamination. The bilayer wrap displayed delamination which was attributed to appreciable hydrogel layer swelling. TMPE-TA was added to the hydrogel foam precursor solution as it is well established in literature that the addition of a crosslinker reduces scaffold swelling [53]. The addition of TMPE-TA further increased the strength of the bilayer wrap improving handleability and making it more conducive for clinical application.

A common drawback of commercial adhesion barriers is that they compromise anastomotic healing. An advantage of the bilayer wrap is that it has distinct layers to impart selective bioactivity. Although ATR-FTIR analysis indicated that hydrogel was present on both layers, the presence of hydrogel on the inner layer does not preclude the cell adhesive characteristics of gelatin [54]; therefore, these results were considered to be acceptable. Fibroblasts were selected for evaluation of selective bioactivity as they are the most prevalent cell type during the healing process and are commonly utilized as a screening method for adhesion evaluation [55]. Cell adhesion primarily on the gelatin layer indicated that cell adhesion was unaffected by the presence of a minuscule amount of PEG foam. This was expected as gelatin is widely incorporated into PEG-based scaffolds to enhance cellular adhesion [56, 57]. The presence of hydrogel on the gelatin layer is preferred over the presence of gelatin on the hydrogel layer as the gelatin would reduce the adhesion resistant properties of the hydrogel layer [54].

In vivo evaluation of the bilayer wrap in a rat abrasion model demonstrated that the selected compositions of gelatin and PEG-based hydrogel foam were within the targeted degradation time-frame. The bilayer wrap resulted in reduced adhesion formation. Further reduction, closer to that of Interceed®, is expected provided that there is maintenance of the position of the bilayer wrap relative to the wound site. Wraps noted as being displaced resulted in minor adhesions and indicated a need to more securely affix the wraps to the wound. Recent studies have reported that dopamine layers can be added to substrates in order to impart tissue adhesive properties through simple dip-coating [40]. This technology is derived from mussels, which are known to adhere to all types of organic and inorganic surfaces under wet conditions. Dopamine hydrochloride (dopamine) has been identified as the compound which facilitates mussel adhesion to substrates through both covalent and noncovalent interactions [58]. As a result, dopamine has become a popular candidate to promote tissue adhesion in devices [59]. We hypothesized that a dopamine coating could facilitate crosslinking with the wound surface as the catechol transitions into a quinone upon oxidation, which can then form a covalent bond with primary amines through nucleophilic attack [39, 60]. This reaction would permit adhesion between the dopamine-coated gelatin layer and the wound surface. *In vitro* evaluation of the adhesive gelatin layer demonstrated marked tissue adhesion as compared to that of un-modified gelatin, comparable to that of Interceed®, and higher than adhesion strengths reported for fibrin glue [25–27]. These findings indicate that the adhesive dopamine coating can be implemented to effectively prevent displacement of the bilayer wrap during healing to prevent adhesion formation.

Overall, these unique wraps utilize material properties to maintain selective bioactivity throughout degradation in a controlled manner. By combining the native properties of PEG hydrogels and gelatin, this bilayer approach could be used to simultaneously enhance anastomotic healing while preventing abdominal adhesions, unlike any current clinical standard. As a first step towards this goal, the presented studies provide an initial evaluation of the bilayer wrap with optimized handling, controlled degradation, and tissue adhesiveness. Despite promising initial results, there were limitations in the current study that necessitate future research. Regarding the *in vitro* characterization, only hydrolytic degradation was considered during evaluation of the selective bioactivity despite enzymatic degradation being the primary mechanism of gelatin degradation. *In vivo* evaluation of gelatin control meshes did confirm that enzymatic degradation would not impede the cell instructive behavior of gelatin as adhesions readily formed over 2 weeks. In addition, future studies are needed that provide *in vitro* characterization of the dopamine-modified bilayer wrap to ensure that the mechanical properties and selective bioactivity is maintained. The adhesive strength of dopamine-modified gelatin meshes was also not experimentally evaluated against fibrin glue but the shear strength of fibrin glue is well documented in the literature using the ASTM F2255 – 05 standard. Following validation of the dopamine-modified bilayer wrap, prevention of intra-abdominal adhesion with the dopamine adhesive-modified bilayer wrap will need to be evaluated *in vivo*. Most notably, the current studies did not evaluate the efficacy of the bilayer wrap to promote anastomotic healing. The demonstration of reduced adhesions from this study using an abrasion model provides the requisite proof of principle for future study in the more rigorous anastomoses animal model. These future animal studies will need to assess anastomotic healing by evaluating the tensile strength and burst strength of the treated anastomoses as well as collagen deposition and organization at the site. Moreover, results will need to be compared to Interceed® in order to assess improvement upon clinical standards. The full *in vivo* degradation rate and biocompatibility assessment of each layer of the bilayer wrap will also need to be evaluated. If the degradation profile significantly differs from our initial scouting, the rates may be adjusted according to crosslink density (gelatin layer) and DTT content (hydrogel layer). Collectively, this tunable system has the potential to have a direct impact upon patients through rational design of a more effective adhesion barrier with the capacity to enhance anastomotic healing.

5. CONCLUSIONS

In this study, we successfully fabricated a bilayer wrap that retains the advantages of each respective material to form a composite device for the simultaneous treatment of adhesion formation and anastomotic healing. Specifically, the completed studies establish initial efficacy to prevent surgical adhesions with the potential to enhance anastomotic healing. This wrap has been optimized for easy surgical handling, tailored degradation to match the physiological cascade, selective bioactivity to control cellular behavior, and tissue adhesiveness for facile application. This work resulted in an initial proof-of-concept device that was shown to effectively prevent adhesion formation *in vivo*. Future studies will evaluate the effect of improved retention at the injury site on reducing adhesions and the benefit of the gelatin matrix in improving anastomotic healing. Given the prevalence of these

procedures and the high morbidity and healthcare costs associated with complications, the proposed bilayer mesh could have a strong clinical impact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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7. REFERENCES

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A) Hydrogel-fiber composite formulations

B) Tensile testing setup

C) Stress-strain curve

Figure 1.

Iterative design of a composite bilayer wrap with requisite mechanical properties. A) Schematic of composite bilayer wrap fabrication for bulk hydrogel-fiber composite, electrospayed hydrogel-fiber composite, and hydrogel foam-fiber composite. B) Set up for mechanical testing of the three composite formulations and C) resulting stress-strain response for each composite bilayer wrap (arrows indicate tensile failure at the associated strain). *Figure created with [BioRender.com](https://www.biorender.com/).

Figure 2.

Characterization of the hydrogel foam + TMPETA-fiber composite. A) ATR-FTIR of the gelatin layer (gray line) and the hydrogel layer (black line). B) Cross-sectional SEM depicting the intra-microarchitecture of each layer (scale bar = 30 μm). SEM blowouts display the plan view of each layer (scale bar = 100 μm). C) Representative images of hDF attachment over 14 days (scale bar = 200 μm) and quantified cell adhesion on each layer. Cells stained with rhodamine phalloidin (F-actin/cytoplasm) and SYBR green (DNA/nucleus). * indicates statistical differences with respect to the hydrogel foam at each time point, (two-way ANOVA with Sidak's analysis, $P < 0.05$).

A) Rat abrasion model

B) Composite adhesion score

Figure 3.

In vivo intra-abdominal adhesion study results. A) Schematic of rat *in vivo* abrasion model utilized to assess adhesion formation. B) Composite adhesion scoring for all treatments, red data points indicate observed displacement of the treatment specimen. * indicates statistical differences as compared to Interceed®, ($P < 0.05$). Representative images taken from site where treatments were applied: C) PBS treated, D) Tisseel® treated, E) gelatin mesh treated, F) hydrogel foam treated, G) composite bilayer treated, H) Interceed® treated. *Figure created with [BioRender.com](https://www.biorender.com)

A) Dopamine coating process

B) Lap shear set-up

C) Tissue adhesion strength

Figure 4.

Effect of dopamine coating on tissue adhesion to the bilayer wrap. A) Schematic of dopamine coating process and hypothesized reaction of dopamine coating with the wound site. B) Schematic depicting the lap shear test set-up and C) average maximum shear strength of the dopamine coated bilayer wrap on porcine substrates as compared to Interceed®. Un-modified gelatin control was not included in graph due to shear forces below

the detection limit of the instrument. * indicates statistical differences as compared to Interceed®, (student's t-test, $P<0.05$). *Figure created with [BioRender.com](https://www.biorender.com).

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Table 1.

Tensile properties of composite bilayer wrap formulations.

Composite formulations	2% Secant modulus (MPa)	Ultimate tensile strength (MPa)	Elongation (%)	Toughness (MJ/m ³)
Bulk hydrogel - fiber composite	22.8 ± 4.8 *	0.6 ± 0.2	2.5 ± 0.5 *	1.0 ± 0.4 *
Electrosprayed hydrogel - fiber composite	4.8 ± 0.7	0.1 ± 0.1 *	3.5 ± 1.2 *	0.4 ± 0.2 *
Hydrogel foam - fiber composite	4.7 ± 0.7	0.7 ± 0.1	41.0 ± 4.0	23.6 ± 1.7
Hydrogel foam + TMPETA - fiber composite	4.1 ± 0.2	0.9 ± 0.1	48.3 ± 3.4 *	28.8 ± 1.5 *

* indicates statistical differences as compared to the hydrogel foam-fiber composite, ($P < 0.05$).