
Article:
Pensabene, V orcid.org/0000-0002-3352-8202, Patel, PP, Williams, P et al. (4 more authors) (2015) Repairing Fetal Membranes with a Self-adhesive Ultrathin Polymeric Film: Evaluation in Mid-gestational Rabbit Model. Annals of Biomedical Engineering, 43 (8). pp. 1978-1988. ISSN 0090-6964

https://doi.org/10.1007/s10439-014-1228-9

TITLE PAGE

Title: Repairing fetal membranes with a self-adhesive ultrathin polymeric film: evaluation in mid-gestational rabbit model

Authors: Virginia Pensabene[1], PhD, Premal P Patel [1], Phillip Williams[2], Assoc. Prof., Trisha L. Cooper[3], MSM PA-C, Kellye C. Kirkbride[1], PhD, Todd D. Giorgio[1], Prof., Noel Tulipan[3], Prof.

[1]Department of Biomedical Engineering, Vanderbilt University, Nashville, TN, USA

[2]Department of Surgery, Vanderbilt University Medical Center, Nashville, TN, USA

[3]Department of Pediatric Neurosurgery, Monroe Carrell Jr Children's Hospital at Vanderbilt, Nashville, TN, USA

Abbreviated title: Self-adhesive ultrathin film to repair fetal membranes

Corresponding author:

Virginia Pensabene,

Department of Biomedical Engineering

Vanderbilt University

5824 Stevenson Center, Nashville, TN (USA) 37235-1592

e-mail: virginia.pensabene@vanderbilt.edu

Phone: +1 615 525 7253, Fax: +1 615 343 7919

Abstract

Preterm premature rupture of membranes (PPROM) causes 40% of all preterm births, affecting 150,000 women each year in the United States. Prenatal diagnostic procedures and surgical interventions increase incidence of adverse events, leading to iatrogenic membrane rupture after a fetoscopic procedure in 45% of cases. We propose an ultrathin, self-adherent, poly-L-lactic acid patch ("nanofilm") as a reparative wound closure after endoscopic/fetoscopic procedures. These nanofilms are compatible with application in wet conditions and with minimally invasive instrumentation.

Ex vivo studies to evaluate the nanofilm were conducted using human chorion-amnion (CA) membranes. A custom-built inflation device was used for mechanical characterization of CA membranes and for assessment of nanofilm adhesion and sealing of membrane defects up to 3 mm in size. These ex vivo tests demonstrated the ability of the nanofilm to seal human CA defects ranging in size from 1 to 3 mm in diameter. In vivo survival studies were conducted in 25 mid-gestational rabbits, defects were created by perforating the uterus and the CA membranes and subsequently using the nanofilm to seal these wounds. These in vivo studies confirmed the successful sealing of defects smaller than 3 mm observed ex vivo. Histological analysis of whole harvested uteri 7 days after surgery showed intact uterine walls in 59% of the nanofilm repaired fetuses, along with increased uterine size and intrauterine development in 63% of the cases. In summary, we have developed an ultrathin, self-adhesive nanofilm for repair of uterine membrane defects.

Keywords

Fetal surgery, IPPROM, amnion, chorion, ultrathin polymeric film, poly L-lactic acid

Introduction

Fetal membranes (the chorion and the amnion) develop immediately after embryo implantation at six days of gestation. The fetal membrane continues to grow and change during the entire pregnancy protecting the fetus during its development inside the uterus. After 12 weeks of gestation, the amnion is clearly distinguishable from the chorion as a thin layer of epithelial cells in direct contact with the amniotic fluid. The chorion is a thicker membrane that is in close contact with the decidual layers and the endometrial tissue and at this stage is considered part of the placenta. The human fetal membranes are not innervated and the chorion is poorly vascularized. These membranes remain intact until the end of the pregnancy when estradiol secretion from the placenta stimulates the uterus to produce more oxytocin receptors and to release prostaglandins, which initiates uterine contractions and triggers mechanical fetal membrane rupture. The natural rupture of these membranes is part of the sequence of term delivery.

Serious complications occur when fetal membrane rupture occurs prior to term. In 3% of all pregnancies, the membranes rupture prior to term, causing as many as 40% of all preterm births (1). Intrauterine rupture of the chorion-amnion (CA) membrane in the first three months of pregnancy (generally resulting in a miscarriage) can be distinguished from preterm premature rupture of the membrane (PPROM) which occurs when the CA membrane mechanically fails prior to the completion of 37 weeks of gestation. PPROM affects 150,000 women per year in the United States (2). Prenatal diagnostic procedures, such as chorion villi sampling and amniocentesis, and prenatal surgical interventions are currently possible. These procedures, however, significantly increase the incidence of adverse events and can often lead to iatrogenic membrane rupture (IPPROM). Prenatal rupture of the fetal membranes was found to occur after a fetoscopic procedure in 45% of cases (3, 4).

The natural mechanism of repairing and sealing the fetal membranes is described as retraction, sliding, contraction and/or scarring in the myometrial and decidual layers of the uterus without involving inflammation, scar formation, and tissue regeneration (5). A fundamental reason for poor outcomes of existing techniques for repairing fetal membrane ruptures is based on the requirement for an active response by the tissue. Attempts to suture these unique membranes using bioactive sealants (such as injectible fibrin or collagen-based sealants) have been studied, but remain to be confirmed using consistent animal models (3). In fact, in most cases the mechanical properties of current materials do not match the mechanical characteristics of the fetal membranes due to the fact that they change continuously during gestation. For example, the application of sealant preparations containing blood components (such as fibrin, thrombin, fibrinogen, or platelets) was shown in human trials to prolong pregnancy, but a significant perinatal mortality rate (70%) remained. Extracellular matrix, maternal blood or collagen plugs have been compared in rabbit models in vivo and do not show significant improvements in the closure of fetoscopy access sites. Laser welding has been limited by heterogeneous thermal properties of the tissues as well as by the risk of thermal injury to the membranes. Finally, single synthetic components that self-polymerize to form a biocompatible hydrogel have been shown to restore the amniotic epithelial continuity, allowing compression and contraction of the tissue in the chorion layer.

A recent review reported the diameter of the surgical instruments and the number of entries into the uterine cavity as the two main factors leading to IPPROM (6). Physical access and time constraints are critical to successful suturing of fetal tissues. Therefore, a primary goal of repair mechanisms is to reduce the duration and invasiveness of the surgical intervention as much as possible. In addition, the method used to suture the wound must not hinder the development of the fetus; therefore, the repair mechanism must be non-toxic, rapidly bioabsorbed, of minimal size, easily applied and adherent to the uterus.

To solve these current problems, we have developed an innovative ultrathin polymeric self-adherent patch as a repair method to use during fetal surgeries and invasive endoscopic/fetoscopic procedures. The methodology was developed to provide efficient, non-disruptive, non-toxic bonding to fetal membranes and to be compatible with deployment in wet conditions and with minimally invasive tools.

The principal innovative aspect of these ultrathin films (also called "nanofilms") compared to existing materials and techniques is their self-adhesive properties. The patch does not require glue or clips to adhere to wet surfaces since adhesion is primarily due to the nanometric thickness of the patch and secondarily to the polymeric composition of the film. Previous studies from the laboratory of Shinji Takeoka demonstrate excellent sealing efficacy of a poly-L-lactic acid (PLLA) nanofilm for gastric incisions as a novel wound dressing material that did not require adhesive agents, induce scars or adhere to surrounding tissues (7). This same group also demonstrated the efficacy of PLLA sheets as a method for fixing a polypropylene mesh in the peritoneal cavity without inducing visceral adhesions during open surgery (8).

To determine if PLLA nanofilm technology could be applied to the repair of uterine and fetal tissues and to determine the performance characteristics, we performed ex vivo studies using human fetal membranes and in vivo studies in mid-gestational rabbits. This project initially proposes to verify adhesion of the nanofilm patch on the target tissues ex vivo and then advances to a set of in vivo trials and survival tests to validate the performance of the patch and the proposed approach.

Materials and Methods

Nanofilm preparation

Poly-L-lactic acid (PLLA) (molecular weight ~60 kDa; 2 wt% solution in dichloromethane; Sigma-Aldrich) and poly (vinyl alcohol) (PVA; molecular weight ~13-23 kDa, 98% hydrolyzed; 1 wt% in deionized water; Sigma-Aldrich,) were used for nanofilm fabrication. Preparation procedures are described in (9). Briefly, 3" silicon wafers (N/Ph <100> 5-10 ohm-cm; 381±25 µm Thick SSP Prime grade Si wafers w/ Primary Flat only; NOVA Electronic Materials, TX, USA) were cut into 2x2 cm pieces using a diamond cutter. PLLA was spin-coated onto the first layer at 3000 rpm for 30 sec. After drying the sample at 80°C for 1 min the PVA layer was dropped onto the PLLA covered silicon wafer and allowed to dry for 24 hours. Sterilization of the nanofilm, prepared in a Class 100 clean room and thus closed in polystyrene Petri dishes was performed either by ethylene oxide sterilization or by UV exposure for 30 minutes. Characterization of the nanofilm thickness and roughness by atomic force microscopy was performed by a Digital Instruments Nanoscan III AFM operating in tapping mode in order to avoid artifacts and adhesion between the nanofilm and

the tip (tip with elastic modulus of 20–80 N/m, resonance frequency in the range of 235–317 kHz and average tip radius of 8 nm).

Human fetal membranes mechanical design

A custom built device was modified from that described by Perrini and colleagues (12) to test biaxial inflation/bursting of the tissue. The device is shown and described in Figure2a. Briefly, the device contains three distinct layers with a circular 14 mm-diameter hole. Tissue can be sandwiched between the pieces creating two 1 mL separated chambers. Two rubber O-rings provide an airtight sealing for the membrane. The bottom chamber has two ports allowing for water to fill one side by using Master Tygon tubing (6411-13, 4 mm outer diameter) connected to a peristaltic pump (Rabbit Plus, max flow rate of 20 mL min-1) while the liquid pressure is measured on the other end with hydrostatic pressure sensor (digital

manometer, LEX 2, -1 to 3 bar, accuracy within 1%). The tissue is mechanically stretched under liquid pressure consistently higher than the ~30 mbar measurement of the amniotic fluid pressure at term (15). The flow rate was kept constant during the inflation at 2.5 mL min-1. For calibration purpose, an elastomeric tape (3M™ VHB™ Tape 4910, clear, general purpose acrylic adhesive, thickness 1 mm) was used, as previously described (12).

Applying a hemispherical bubble assumption, the Laplace's law for failure stress can be simplified as:

$$\sigma_f = \frac{P_f R}{2t_0} \quad (1)$$

where σ_f is the failure stress, P_f is the pressure for rupture, R is the radius of the "bubble" prior to bursting and t_0 is the initial thickness of the membrane (with t_0<R). Assuming R is equal to the radius of the exposed membrane, eq. 1 can be used to evaluate the bursting tension as:

$$T = \frac{P_{max} R}{2} \quad (2)$$

Ex vivo tests

All procedures related to the consent and collection of tissues were approved by the Vanderbilt University Institutional Review Board. Ten de-identified placentas were collected from patients who underwent elective cesarean sections between 37 and 39 weeks of gestation. Preterm contractions, PROM, diabetes, and evidence of infection were considered as exclusion criteria. Tissues were kept at 4 °C and tested within 12 hours of the surgery. For experiments, 4x4 cm specimens were obtained from each placenta. In order to standardize the preparation protocol, samples were cut at least 2 cm from the placenta border (12). For these experiments, fetal membranes were kept as intact chorion-amnion (CA) or separated in

amnion (A) and chorion (C). All tissues were kept hydrated by spraying with fresh physiological saline solution during the experimental session.

In vivo studies using a mid-gestational rabbit model

Adapting a model already proposed in the literature (17), all procedures followed a specific protocol approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC) and were carried out in compliance with institutional guidelines. Female New Zealand, time-date pregnant rabbits were purchased in five separate groups for a total of 25 from Charles Rivers Laboratories (USA). They were received on day 16-20 of gestation and housed for seven days allowing for acclimation in a quiet room with normal diet. Preemptive analgesia, buprenorphine (0.02 mg kg-1 IM) and prophylactic antibiotics (cefazolin, 10 mg kg-1 IM) were administered once before the surgery. General anesthesia was induced with ketamine and acepromazine (35 and 1 mg kg-1 IM, respectively) and supplemented with inhaled anesthetics isoflurane and 0_2 by mask. The animals were intubated and placed on a ventilator and general anesthesia was maintained with isoflurane and O_2. The abdominal area was shaved, washed and prepared as a sterile surgical site. Pulse oximetry was utilized to assess heart and respiratory rate, O_2 saturation and $ETCO_2$. Body temperature was assessed with a rectal probe. Following preparation and stabilization of a surgical plane of anesthesia an 8 cm lower midline laparotomy was performed and the left or right uterine horn was exposed through the incision. After exposure of a segment of the uterus corresponding to a single fetus and visualization of movement of the fetus in the sac, a defect was induced in the myometrial tissues with an electric pencil. In order to confirm perforation of all the membranes from the uterus to the amnion, the sac was gently pressed until drops of amniotic liquid were observed from the defect.

Uterine wall repair was performed with both a polymeric patch and standard suturing techniques with polypropylene suture. An unrepaired defect was used as a positive control and non−manipulated fetuses served as negative controls. The nanofilm, 1.5 x 1.5 cm, was immediately placed in contact with the tissue to cover the defect. Once the uterus was exposed and the myometrial defect and membrane perforations were performed, a nanofilm was peeled off of the silicon wafer with sterile tweezers and placed directly over the defect. Sterile warm saline solution was dropped on the film with a 10 mL disposable syringe to dissolve the supporting layer of PVA. With this step the remaining nanofilm can conform to the tissue surface, thus covering and adhering completely to it.

As discussed in the Results, several factors were considered during the surgery:

- Adhesion to the fetal membrane, i.e. adhesion to the exposed chorion, adhesion to the uterus;
- Instantaneous sealing of the defect, by monitoring amniotic liquid escaping or bleeding at the site of the defect after repair;
- Therapeutic potential, i.e. handle feasibility using standard tools such as syringes and tweezers to deploy the nanofilm;
- Timing, i.e. time required for the film to adhere to the tissues, time for the supporting PVA layer to dissolve;
- Compatibility of open vs minimal invasive surgery i.e. size of the nanofilm vs size of the defect.

Upon completion of the procedure, the uterus was placed back into the abdomen and the abdominal incision was closed. Medroxyprogesterone was administered intramuscularly for uterine relaxation, at the time of closure of the abdominal incision. The animals were anesthetically recovered and upon sternal recumbency were transferred to housing with post-operative administration of analgesics.

On GA 30−33 (7 days post−surgery) the animals were euthanized and post-surgical observations were performed as well as collection of tissues for histological analysis. The whole uteri were harvested and evaluated for increased size of the fetuses, movement of the fetuses inside the sacs and integrity of the uterine wall at the rupture site. Tissues were then fixed with 10% formalin. Histological analysis was performed by hematoxylin and eosin staining.

Results

Adhesive and sealing performance on human chorion-amnion

Polymeric ultrathin films were made using a previously established method that was optimized for deployment on wet tissues. Figure 1 represents the film ready to be applied. As shown, the thick PVA layer works as a water soluble supporting structure, which peels off of the silicon with the nanometric PLLA film, thus allowing easy handling and deployment. Characterization of the nanofilm by atomic force microscopy confirmed previously published data for similar films (9, 10, 11), showing thickness of the PLLA film of 120±25 nm and average roughness R_A below 10 nm. Next, to test the ability of the nanofilm to repair uterine defects, we developed a modified version of the device described by Perrini and colleagues (12). As shown in Fig. 2.a, the aluminum device consists of three distinct layers containing a 14 mm circular hole in which a 2x2 cm tissue sample can be sandwiched between the pieces to create two 1 mL separated chambers. The bottom chamber has two ports allowing for water to fill one side and the liquid pressure is measured on the other end. The fetal membranes are mechanically stretched under liquid pressure levels that are consistently higher than the ~30 mbar measurement of amniotic fluid pressure at term (15). This device allowed for the biaxial inflation/bursting tests of the fetal membrane. As shown in Fig. 2.c,

there is a linear and clear relationship between membrane (elastomeric tape as a control) and critical pressure to rupture.

Human fetal membrane samples were used in initial experiments to collect data on their basic tensile properties. Membrane thickness was approximated using both measured values ("measured" in Fig. 3.a) and values commonly used in the literature ("predicted" in Fig. 3.a). Structure and thickness of the membranes generally remain constant in the last five months of human gestation (18, 19), but there are substantial differences within patients and discrepancies that depend on the measuring technique used (imaging analysis of fixed tissues versus digital caliper). Values of thickness in the literature range from about 250 µm (measured after separation from the uterine wall during labor) to 560 µm (18). Moreover, thickness and structure of the chorion is substantially different in close proximity to the placenta.

Biaxial inflation involves the inflation of a membrane until failure. The membrane is fixed and a circular region is exposed. The membrane is then inflated from one side using fluid pressure until it bursts. The pressure required to rupture the membranes during controlled inflation were collected. Using measured pressures and equation 2 (refer to Materials & Methods), values of strength were calculated. As summarized in Fig. 3.a, the strength of the amnion (~4.6 MPa) is significantly higher than that of the chorion (~0.8 MPa) and CA membranes (~1.4 MPa), confirming existing data in the literature (20-22). Since the early 1960s, mechanical tests on amnion and chorion revealed that the amnion, although it is thinner, can withstand higher pressures than the chorion (23-27).

Different theories are used to explain the mechanism of fetal membrane rupture due to the fact that testing intact healthy membranes at different stages of gestation is difficult to do. Given the fact that fetal membranes are soft, hydrated biological materials, we observed time-dependent changes in the measurements, confirming the viscoelastic properties of these

membranes. As a result, a second set of measurements were obtained to observe and evaluate the adhesive strength of the film on both intact tissue and on perforated fetal membranes. The film was initially applied to the fetal membrane and the tissue was then inflated until rupture. The data demonstrate that the film adhesion was stable until bursting and did not alter the fetal membrane inflation (Fig. 3.b). This was similar to other observations in the literature (14, 21). In general, the mechanical properties of failed membranes repaired with the nanofilm do not differ from intact membranes.

Next, we wanted to assess the ability of the nanofilm to repair fetal membrane damage. To do this, fetal membranes were wounded with biopsy punches ranging in size from 1 to 3 mm, repaired with a PLLA film (either 10x10 mm or 20x20 mm in size) and inflated until bursting. The PLLA nanofilms are able to efficiently seal defects of 1 and 2 mm in diameter (Fig. 3.c). The values of critical pressure to rupture are lower than those for intact membranes, but still within the range of chorion properties. Interestingly, no leakage was observed during liquid inflation of the membranes at the site of the defect or at the margins of the film, regardless of the size of the defect. When comparing the two different sizes of the film, no significant differences were observed when larger films are used to seal the defects (9 to 15% higher pressure to rupture was registered with the 10x10 mm film). This trend is also preserved when the defect size was varied. These measurements suggest that the contact area is not a determinant parameter for adhesion.

In vivo study observations and histology

Pregnant rabbits at mid-gestation are considered reasonable models for studying fetal membrane defects and for testing closure techniques after fetal surgical procedures (15, 16). The duration of a rabbit's pregnancy is short, 32 days, and the mean litter size is eight. Starting from gestational age (GA) of 19 days, organogenesis is achieved and most of the

fetuses show a completed fetal appearance. A single amniotic sac reaches a size of 4-5 cm at a GA of 23-26 days, which is compatible with tests of fetal membrane repair. Following existing published protocols (15), multiple fetuses in each animal were treated with the nanofilm patch in the first 3 groups (Table I). This treatment approach appeared to be too stressful for both the animal and the untreated fetuses- after the surgery more than one fetus was found reabsorbed or dead. These outcomes made it difficult to understand whether this was due to the failure of the suture technique or if unsuccessful repair of one of the fetuses led to failure for all the other sacs.

To compare the use of the nanofilm with traditional wire suturing, a second group of animals had one fetus treated with the nanofilm and one with polypropylene wire. The use of wire is currently not a very successful technique for suturing the amnion and the chorion, due to the extremely thin and fragile nature of these membranes making them difficult to handle and their tendency to tear if perforated. After experiencing these difficulties, the last 3 groups were treated only with the patch, thus limiting the pain and stress on the animal.

Upon exposure of a segment of the uterus corresponding to a single fetus, a defect was formed in the myometrial tissues with a fine-tipped electrocautery pencil. The tissue was gently pressed in order to ensure that the perforation went through all of the membranes, including the amnion. A 1.5 x 1.5 cm film was used for the surgery, in accordance with the ex vivo results (Fig. 4.a). The PLLA film was immediately placed in contact with the tissue covering the defect. As suggested by previous studies (9), the adhesion of the nanofilm is mainly ascribed to the Van der Walls forces between the film surface and the tissue. The film adhered to the uterine wall as soon as the thick supporting layer of poly vinyl alcohol (PVA) was completely dissolved using saline solution, leaving the ultrathin film free to bend, cover and easily form to the tissue contours (Fig. 4.b). Visual observation indicated that the PVA was completely removed after two minutes; however, five minutes was considered a reliable

duration for a complete adhesion of the film. Shorter times and manipulation of the uterus and the fetus indicated that blood or amniotic fluid would leak from the edge of the nanofilm or the nanofilm would become displaced, thus suggesting that the adhesion was not firm. Consistent with previous reports (8), when the repaired tissue was placed back inside the abdominal cavity the film did not stick to any opposing tissues and remained adherent strictly to the treatment site.

Due to concerns for the mother's health, five animals had to be euthanized prior to postoperative day 7 because of decreased dietary intake, lethargy, self-mutilation of digits, or presence of blood in the urine. In addition to pathological analysis of these lost animals, abdominal ultrasound analysis was performed on one group of rabbits prior to surgery and just prior to euthanasia in order to verify fetal activity and fetal heart rate before harvesting the tissues. Unexpectedly, some of the fetuses in a single mother were no longer alive at the time of the surgery, thus making it difficult later to understand whether the observed death of the fetus was due to the surgical procedure or to a general complication during the pregnancy. Additionally, it was not always possible to definitively identify the treated fetus inside the abdomen with ultrasound before euthanasia. For this reason, the ultrasound measurements have not been included here.

Localization of the repaired defects in the whole harvested uteri demonstrate intact uterine walls in 51.5% of the uteri; 16.5% of the sacs were absorbed and 19% had ruptured (summarized in Table II and shown in Fig. 5.a). In nanofilm-repaired uteri, 59% of the fetuses were alive at the time of euthanasia. In 63% of the cases, the size of the uterus had increased in accordance with the gestational age, suggesting intrauterine development of the fetuses (Fig. 5.a). A 15% decrease in the maternal weight was measured in less than 5% of the animals. The nanofilm covered defects in normally developing fetuses appeared smooth and sealed even if the wound in the tissues did not fully close. These finding are in

accordance with recently reported observations (26) of wound healing without significant growth of new tissues at the site of the defect (Fig. 5.b). The nanofilm enabled fetal growth and survival as confirmed by hematoxylin and eosin staining of the fetal tissue in 59% of the treated fetuses (an average value of the 4 groups); these data are summarized in Table II (Fig. 6). During surgery it was possible to confirm nanofilm adhesion on the fetal membranes and sufficient sealing of a defined size rupture, summarized in Table III. These in vivo data confirm the ex vivo evaluation using human CA membranes that a successful sealing of the defect is observed when the size of the defect is equal to or smaller than 3 mm (Fig. 5.b). This conclusion does not consider the ratio between size of the defect and size of the whole amniotic sac in the rabbit, which is consistently smaller than human amniotic sacs. However, these data suggest the possible use of this polymeric film for minimally invasive procedures performed using 12G or smaller needles or catheters smaller than 9-Fr.

Analysis of the formalin-fixed tissues revealed smooth margins of the repaired defect in normally maturing fetuses (Figure 6). Initiation of fibrosis could be observed and the different fetal membrane layers, including the amniotic epithelial cell layer and syncytiotrophoblasts, could be distinguished. Limited bleeding and inflammation in the endometrium and between the decidua and the placenta was also observed. For larger and non-sealed defects, as shown in Fig. 5b, tissues were necrotic and chorion-amnion and the endometrium were severely inflamed.

Discussion

Fetal membrane repair represents a significant limiting factor in the advancement of fetal surgery, especially before the use of endoscopic procedures and minimally invasive diagnostic techniques during pregnancy become widely accepted. Maternal risks (irreversible premature rupture of the amnion and the chorion, membrane separation,

oligohydroamnios infection and premature labor) together with fetal complications (premature birth or death) are still considered high with a suboptimal risk-to-reward ratio remaining for these procedures.

Here, we aimed to test a self-adhesive, biocompatible patch for fetal membranes. Reliable animal models for ex vivo and in vivo uterine wound repair experiments are difficult to find. The encountered difficulty with exposing and handling the rabbit chorion-amnion membranes due to the extremely fragile nature of these tissues caused us to test the PLLA nanofilm directly on the uterine wall, a slight modification to the traditional protocol proposed by Deprest (15).

We have shown stable adhesion of the film to fetal membranes and confirmed in our animal model the possibility of sealing defects in the uterine wall. Application of the nanofilm patch enabled fetal development and survival with a 65.5% success rate. The defect size limit is acceptable considering the size of instrumentation currently used- an 18G needle for chorion villi sampling, 1.2-3.5 mm endoscopes or balloon trocars, or 2-3 mm instruments (29). In terms of adoption of the film clinically for surgical procedures, we conclude that the film can adhere to the chorion and seal fetal membrane defects smaller than 2 mm in diameter, corresponding to a 12G needle or an 8.4 French port.

Based on these initial studies, transition to clinical human use will require evaluation of the nanofilm's performance in a more appropriate animal model, such as sheep. Larger animal models that are more similar to humans would allow verification of direct application of the patch to the chorion-amnion membranes and to evaluate size limitations. Given the handling and adhesive properties of the film, together with its wound healing and biocompatibility, the nanofilm patch should be considered for application as a protective and sealing material for the fetal tissues during intrauterine interventions.

Among existing procedures, intrauterine surgery for myelomeningocele may benefit from adoption of this film during treatment. Myelomeningocele is one of the most common congenital anomalies of the central nervous system, with an incidence of 3.4 per 10,000 live births in the U.S (30). Evidence of the benefit for intrauterine repair of the spinal defect compared to surgical post-natal intervention has been shown (31); however, these procedures are still hindered by fetal and maternal risks (specifically PPROM (46%), membrane separation (24%), oligohydramnios (21%) and preterm labor (38%)). In order to decrease these risks, minimally invasive procedures to repair the spinal defect may prove to be helpful. One promising technique recently proposed by Jose Peiro and colleagues (32) involves single port access and a single amount of the amniotic liquid being temporarily replaced by carbon dioxide. The use of this PLLA nanofilm to close and regenerate the tissue affected by the neural defect, particularly in a partially "dried" environment would be feasible. The positioning and adhesion of the film may even be faster than the surgical sealant described by Peiro et al.

Conclusions

A biocompatible, polymeric ultrathin film has been evaluated in this study as an alternative approach for sealing fetal membranes after minimally invasive treatment of the fetus. The film showed a strong adhesion to the chorion and the amnion and ex vivo bursting tests using human membranes showed optimal sealing of up to 3 mm defects in the membranes. The PLLA films were able to seal in vivo defects in the uterine wall and perforations of the amniotic sacs in greater than 60% of the treated cases, allowing fetal growth and continuation of the gestation.

Acknowledgments

The authors would like to acknowledge the staff at Division of Surgical Research, Vanderbilt University Medical Center for their support during animal surgery, Prof. Hernan Correa and Dr. Lacey Winchester from the Department of Pathology, Microbiology, and Immunology at Vanderbilt University Medical Center for their help for histological analysis. Special thanks are due to Dr. Kellie Boyle and the Translational Pathology Shared Resource at Vanderbilt University Medical Center. The authors are also grateful to Alicia K. Crum, Registered Diagnostic Medical Sonographer from the Department of fetal medicine for her support for ultrasound, Lynne Black and Prof. Kelly Bennett from the Division of Maternal Fetal Medicine for their help for human placenta collection and IRB approval, and Emily Guess and all the nurses at Labor&Delivery at Vanderbilt University Medical Center for tissues collections.

Disclosures

All authors disclosed no financial relationships relevant to this publication. Research support for this study was provided by the Vanderbilt Initiative in Surgery and Engineering (ViSE) and Vanderbilt Institute for Clinical and Translational Research (VICTR) within the project "Self-adhesive patch for fetal and obstetrical surgery".

References

1. Mercer B.M. Preterm premature rupture of the membranes: diagnosis and management. Clin. Perinatol. 31(4):765-82, 2004.
2. Aagaard-Tillery K.M., F.S. Nuthalapaty, P.S. Ramsey, K.D. Ramin. Preterm premature rupture of membranes: perspectives surrounding controversies in management. Am. J. Perinatol. 22(6):287-97, 2005.

3. Devlieger R., L.K. Millar, G. Bryant-Greenwood, L. Lewi, J.A. Deprest. Fetal membrane healing after spontaneous and iatrogenic membrane rupture: A review of current evidence. Am. J. Obstet. Gynecol. 195: 1512–20, 2006.
4. Bilic G. et al. Injectable candidate sealants for fetal membrane repair: bonding and toxicity in vitro. Am. J. Obstet. Gynecol. 02:85.e1-9, 2001.
5. Behzad F, M.R. Dickinson, A. Charlton, J.D. Aplin. Brief communication: sliding displacement of amnion and chorion following controlled laser wounding suggests a mechanism for short-term sealing of ruptured membranes. Placenta 15:775–8, 1994.
6. Beck V et al. Preterm Prelabor Rupture of Membranes and Fetal Survival after Minimally Invasive Fetal Surgery: A Systematic Review of the Literature. Fetal Diagn. Ther. 31:1-9, 2012.
7. Okamura Y., K. Kabata, M. Kinoshita, D. Saitoh, S. Takeoka. Free-Standing Biodegradable Poly(lactic acid) Nanosheet for Sealing Operations in Surgery. Advanced Materials 21 (43): 4388–4392, 2009.
8. Fujino K., M. Kinoshita, A. Saitoh, H. Yano, K. Nishikawa, T. Fujie, K. Iwaya, M. Kakihara, S. Takeoka, D. Saitoh, Y. Tanaka. Novel technique of overlaying a poly-L-lactic acid nanosheet for adhesion prophylaxis and fixation of intraperitoneal onlay polypropylene mesh in a rabbit model. Surg. Endosc. 25 (10): 3428-3436, 2011.
9. Pensabene V., S. Taccola, L. Ricotti, G. Ciofani, A. Menciassi, F. Perut, M. Salerno, P. Dario, N. Baldini, Flexible polymeric ultrathin film for mesenchymal stem cell differentiation. Acta Biomater. 7:2883–2891, 2011.
10. Ricotti L., S. Taccola, V. Pensabene, V. Mattoli, T. Fujie, S. Takeoka, A. Menciassi, P. Dario. Adhesion and proliferation of skeletal muscle cells on single layer poly(lactic acid) ultra-thin films. Biomed Microdevices (2010) 12:809–819.

11. Taccola S., A. Desii, V. Pensabene, T. Fujie, A. Saito, S. Takeoka, P. Dario, A. Menciassi, V. Mattoli, Free-Standing Poly(L-lactic acid) Nanofilms Loaded with Superparamagnetic Nanoparticles. Langmuir 2011, 27, 5589–5595.
12. Perrini M., W. Burzle, C. Haller, N. Ochsenbein-Kolble, J. Deprest, R. Zimmermann, E. Mazza, M. Ehrbar. Contractions, a risk for premature rupture of fetal membranes: a new protocol with cyclic biaxial tension. Med. Eng. Phys. 35(6):846-51, 2013.
13. Fisk N.M., D. Ronderos-Dumit, Y. Tannirandorn, U. Nicolini, D. Talbert, C.H. Rodeck. Normal amniotic pressure throughout gestation. BJOG 99 (1): 18–22, 1992.
14. Haller C.M., W. Buerzle, C.E. Brubaker, P.B. Messersmith, E. Mazza, N. Ochsenbein-Koelble, R. Zimmermann, M. Ehrbar. Mussle-mimetic tissue adhesive for fetal membrane repair: a standardized ex vivo evaluation using elastomeric membranes. Prenat. Diagn. 31: 654-660, 2011.
15. Gratacos E., H. Yamamoto, N.A. Papadopulos, T. Andriaenssens, T. Phlips, T.E. Lerut, J.A. Deprest. The midgestational rabbit as a model for the creation of membrane defects after needle fetoscopy. Am. J. Obstet. Gynecol. 180 (5): 1263-1267, 1999.
16. Beaudoin S., P. Barbet, F. Bargy. Developmental Stages in the Rabbit Embryo: Guidelines to choose an appropriate Experimental Model. Fetal. Diagn. Ther. 18: 422-427, 2003.
17. Papadopulos N.A., P.P. Van Ballaer, J.L. Ordonez, I.J. Laermans, K. Vandenberghe, T.E. Lerut, J.A. Deprest. Fetal membrane closing techniques after hysteroamniotomy in the midgestational rabbit model. Am. J. Obstet. Gynecol. 178(5): 938-942, 1998.
18. Benirschke K., G.J. Burton, R.N. Baergen, Pathology of the Human Placenta (Anatomy and Pathology of the Placental Membranes) ed. 6, Springer-Verlag, New York, pp 249-307,2012.

19. Schmidt W. The amniotic fluid compartment: the fetal habitat. Adv. Anat. Embryol. Cell. Biol. 127:1–100, 1992.
20. Chua W.K., M.L. Oyen. Do we know the strength of the chorioamnion? A critical review and analysis. Eur. J. Obstet. Gynecol. Reprod. Biol. 1445: S128-S133, 2009.
21. Moore R.M., J.M. Mansour, R.W. Redline, B.M. Mercer, J.J. Moore. The Physiology of fetal membrane rupture: insight gained from the determination of Physical properties. Placenta 27: 1037-1051, 2006.
22. Oyen M.L., S.E. Calvin, D.V. Landers. Premature rupture of fetal membrane: is the amnion the major determinant? Am. J. Obstet. Gynecol. 195: 510-515, 2006.
23. Polishuk W.Z., S. Konhane, A. Peranio. The physical properties of fetal membranes. Obstet. Gynecol. 20:204–210, 1962.
24. Polishuk W.Z., S. Kohane, A. Hader. Fetal weight and membrane tensile strength. Am. J. Obstet. Gynecol. 20:204–250, 1964.
25. Laufer A., W.Z. Polishuk, J. Boxer, R. Ganzfried. Studies of amniotic membranes. J. Reprod. Fertil. 12:99–105, 1966.
26. MacLachlan T.B. A method for the investigation of the strength of the fetal membranes. Am. J. Obstet. Gynecol. 91:309–313, 1965.
27. Wyatt-Ashmead J., A. Ashmead, Placental membrane bursting pressures. Mod. Pathol. 17:275 (Abstract 41) 2004.
28. Gratacós E., J. Sanin-Blair, L. Lewi, N. Toran, G. Verbist, L. Cabero, J. Deprest. A histological study of fetoscopic membrane defects to document membrane healing. Placenta 27:452–456, 2006.
29. Sydorak R., A. Nijagal, C.T. Albanese. Endoscopic techniques. Yonsei Med. J. 42(6): 695-710, 2001.

30. Boulet S.L., Q. Yang, C. Mai, et al. Trends in the postfortification prevalence of spina bifida and anencephaly in the United States. Birth Defects Res. A. Clin. Mol. Teratol. 82 (7):527–532, 2008.
31. Adzick N.S., E.A. Thom, C.Y. Spong, J.W. Brock III, P.K. Burrows, M.P. Johnson, L.J. Howell, J.A. Farrell, M.E. Dabrowiak, L.N. Sutton, N. Gupta, N.B. Tulipan, M.E. D'Alton, D.L. Farmer. A Randomized Trial of Prenatal versus Postnatal Repair of Myelomeningocele. N. Engl. J. Med. 364(11):993-1004, 2011.
32. Peiro J.L., C.G. Fontecha, R. Ruano, M. Esteves, C. Fonseca, M. Marotta, S. Haeri, M.A. Belfort. Single Access Fetal Endoscopy (SAFE) for myelomeningocele in sheep model I: amniotic carbon dioxide gas approach. Surg. Endosc. 27: 3835-3840, 2013.

Tables

Table I animal numbers

Group number	Number of animals	Average number of fetuses	Number of treated fetuses
1	5 (4)	6	3*
2	5	6	22
3	5 (4)	6	25**
4	5 (4)	6	5***
5	5 (4)	6	4***
Total	25	30	59

*One first rabbit sacrificed earlier for procedure definition.

**One animal was used for testing adhesion of the film on the fetus skin after MCC defect.

***One animal from each group was euthanized earlier than planned for unexpected issues.

Table II: Fetal survival summary

Group	Live fetuses/ treated fetuses	Live fetuses/ total number of fetuses	Fetuses treated with patch
1	-	-	-
2	68% (15/22)	76% (23/30)	18
3	60% (15/25)	62.5% (15/24)	24
4	75% (3/4)	75% (18/24)	4
5	75% (3/4)	21% (5/24)	4

Table III: In Vivo Results Summary

Properties Being Considered:	Results
Adhesion to the fetal membrane	Achieved on chorion Achieved on uterus
Instantaneous sealing of the defect	Achieved on uterus
Therapeutic potential	Nanofilm can be handled and positioned with tweezers Nanofilm adhesion can be assured by spraying water with a syringe
Timing	Nanofilm adheres to the tissues in 2 minute
Maximum size of repairable defects	3 mm

Figure legends

Figure 1. Preparation of nanofilms. For ex vivo and in vivo testing: the films are obtained by spin coating fragments of silicon wafer (a). The PLLA film, together with the PVA sacrificial layer on top, can be cut with a razor to the preferred size and shape, peeled off of the wafer (b), and thus handled with tweezers (c).

Figure 2. The biaxial stretching device. The device is shown before (a) and after assembly (b). Calibration was completed using 0.5 mm thick elastomeric tape (c) bonded together to form 1.0 and 1.5 mm in thickness. Picture (d) shows the membrane during inflation inside the device. Achieved critical pressure increased significantly in relation to thickness. Tested groups are all significant between each other (unpaired t-test, $^{**}p \leq 0.01$).

Figure 3. Amnion and chorion mechanical characterization (a). Bursting measurements of intact fetal membrane (b) and of different size fetal membrane punctures (1, 2 and 3 mm) sealed with either 10x10 mm or 20x20 mm nanofilms (c) as indicated. Change error bars and explain.

Figure 4. Surgical application. The nanofilm was handled with tweezers and applied on exposed uterine wall after the surgeon punctured the fetal membranes (left). During this step, the fetal sac was kept still by the surgeon in order to prevent leakage of amniotic fluid from the wound. After removal of the PVA, the film adhered completed on the tissues and no fluid leakage was observed from the wound upon agitation of the fetal sac while placing it back inside the abdomen. The 2 mm size defect was easily visualized under the nanofilm which is completely transparent (right).

Figure 5. Efficiency of the fetal patch at healing fetal membrane defects. The whole uterus was harvested 7 days after surgery in order to measure the size of the fetal sacs and to evaluate the growth of the fetuses over the 7 days. Identification of treated fetal sacs and

counting of the live and dead fetuses was thus possible (a). Different sizes of repaired wound were easily observed in the uterine wall after the uterus was harvested. Since the nanofilm is transparent, only the defects can be visualized. For defects smaller than 3 mm, the sacs appeared completely smooth and well-sealed. For bigger wounds, sealing did not occur and the fetal skin was exposed (b).

Figure 6. Histological analysis. A sagittal section of the uterus shows a repaired defect with tissue covering the fetus (labelled with an asterisk (*)) and granulation tissues (corresponding to the two black arrowhead). An example of failure in the same procedure is visible in figure (b) where the uterine wall remained open and did not heal (white arrowheads). Fig. a 2X, Fig. b 20X.