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A collagen plug with shape memory to seal iatrogenic fetal membrane defects after fetoscopic surgery

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ABSTRACT

Iatrogenic preterm premature rupture of fetal membranes (iPPROM) after fetal surgery remains a strong trigger for premature birth. As fetal membrane defects do not heal spontaneously and amniotic fluid leakage and chorioamniotic membrane separation may occur, we developed a biocompatible, fetoscopically-applicable collagen plug with shape memory to prevent leakage. This plug expands directly upon employment and seals fetal membranes, hence preventing amniotic fluid leakage and potentially iPPROM.

Lyophilized type I collagen plugs were given shape memory and crimped to fit through a fetoscopic cannula (Ø 3 mm). Expansion of the plug was examined in phosphate buffered saline (PBS). Its sealing capacity was studied *ex vivo* using human fetal membranes, and *in situ* in a porcine bladder model.

The crimped plug with shape memory expanded and tripled in diameter within 1 min when placed into PBS, whereas a crimped plug without shape memory did not. In both human fetal membranes and porcine bladder, the plug expanded in the defect, secured itself and sealed the defect without membrane rupture.

In conclusion, collagen plugs with shape memory are promising as medical device for rapid sealing of fetoscopic defects in fetal membranes at the endoscopic entry point.

1. Introduction

Endoscopic fetal surgery using endoscopy is a rising medical technology to treat children before birth [1]. Compared to open fetal surgery, the endoscopic approach is less invasive and results in a reduction of the number and severity of maternal complications [2]. Fetoscopic surgery is already being used for treatment of severe conditions, including laser therapy of the placenta in monochorionic diamniotic twins with twin-twin transfusion syndrome (TTTS) [3] and cord coagulation in twins with twin reversed arterial perfusion sequence (TRAP) [4]. In an experimental setting, fetoscopy is also used for closure of defects in fetuses with spina bifida [5] and for fetal tracheal balloon occlusion (FETO) in moderate/severe congenital diaphragmatic hernia (CDH) patients [6–8]. Although fetoscopy has many advantages, it also introduces a high risk for iatrogenic preterm premature rupture of the membranes (iPPROM). iPPROM is a strong trigger for premature birth and puts the mother and fetus at risk for infection and sepsis [9,10]. During fetoscopy, the maternal abdominal wall, uterine wall and fetal membranes are punctured by the endoscope. Puncturing the chorion and amnion results in defects that do not heal spontaneously and may lead to leakage of amniotic fluid and chorioamniotic membrane separation, which are all adding to the risk of iPPROM. The risk for iPPROM is estimated to be as high as 30% [9,11–15]. With this considerable hazard, the benefits of the fetoscopic surgical procedure must outweigh the drawbacks and could be much more successful if iPPROM could be reduced or prevented. For this reason an urgent need exists for a technique to minimize the risk of iPPROM occurrence. A suitable medical device to close the fetoscopic entry point should be easy to use and seal the defect instantaneously upon application.

Several techniques have been clinically applied to seal the membrane

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defect after fetoscopy and prevent iPPROM and its possible risk of e.g. premature birth. A number of groups studied the effect of collagen or gelatin plugs [16–19]. For instance, Engels et al. studied the effect of a plug made out of a Lyostypt collagen sheet, rolled and placed into an 8 Fr cannula (outer diameter 2.7 mm) [16]. The plug was placed following a FETO procedure, by keeping the obturator in place and withdrawing the surrounding cannula. Compared to a control group that was left unsealed, no significant reduction of membrane rupture or delayed delivery were observed. In two retrospective studies, Papanna et al. evaluated the effect of a gelatin sponge made of a rolled sheet of Surgifoam, which was used to fill the endoscopic entry point after fetoscopic laser coagulation [17,18]. The plug was placed in a comparable way as described before and did not reduce iPPROM occurrence either. Only Chang et al. claimed a reduction in iPPROM rates following plugging defects with a gelatin sponge made of Gelfoam, but this study did not include a control group [19]. Engels et al. and Papanna et al. both hypothesized that the plug may have dissolved too rapidly [16,17]. This may partly correspond to the data of Chang et al., who were able to identify the inserted gelatin plug for two to three weeks using ultrasound and found remnants of the plug on the placenta in only one case at the time of delivery, 5 weeks post operation [19]. In these examples, the clinically applied biomaterial sheets were used off-label and rolled up to fit the fetoscope. The field may benefit from a material that is solely developed as a fetal membranes plug.

In pre-clinical studies in rabbits, *ex vivo* studies with human fetal membranes and *in vitro* studies, various techniques have been assessed for fetal membrane sealing. Besides collagen or gelatin only plugs [20,21], other materials or additional compounds were studied. Papadopoulos et al. found that the use of allogeneic amnion cells from amnion membrane biopsies on a collagen scaffold improved membrane integrity in rabbits compared to similar scaffolds without cells [22]. Also collagen plugs supplemented with platelet-rich plasma assessed for proper sealing *ex vivo* [23]. Liekens et al. showed by immunohistochemistry that enrichment of collagen plugs with platelets and allogeneic amniotic fluid cells increased cell proliferation in the center of collagen plugs sealing fetal membrane defects in rabbits, but did not assess sealing properties [24]. Engels et al. reported that a collagen plug imbued with fibrinogen and plasma reduced amniotic fluid leakage in an *ex vivo* set-up approximately 35% better than a control plug [25]. Other studies used polymer based patches like Tissuepatch *in vivo* [26], bioadhesive-coated silicone patches [27] and fetal membrane patches *ex vivo* [28]. Alternative techniques included the use of sealants and tissue adhesives, such as Duraseal in rabbits [26], fibrin glue and mussel mimetic tissue adhesive both *in vivo* and *ex vivo* [29–31]. In addition, more mechanical approaches were evaluated in *ex vivo* experiments, such as an umbrella-shaped device to cover the defect in combination with a sealant [32]. Although most studies showed promising results to seal the defect with or without additional compounds, clinical translation to assess the prevention of iPPROM after fetoscopy has not been described so far.

Previously, we communicated how shape memory can be given to hollow tubular scaffolds made of exclusively type I collagen fibrils [33–35]. In this study, we constructed a solid, non-hollow collagen plug with shape memory to expand directly upon employment in order to seal fetal membranes after a fetoscopic procedure. We studied the expansion rate and time to return to the original shape in a model without any impediments for the plug and, as a proof of concept, the ability of the plug to seal an endoscopic entry point in an *in situ* and *ex vivo* model.

2. Materials and methods

All chemicals used during the experiments were obtained from Merck, Darmstadt, Germany, unless stated otherwise.

2.1. Construction of collagen plugs

Purified type I collagen fibrils were suspended in 0.25 M acetic acid to obtain a 1.5% (w/v) collagen suspension and swollen overnight. For homogenization, the suspension was pressed through the nozzle of a 50 ml syringe with an inner diameter of 1.5 mm. The homogenized collagen suspension was casted in tubes of Ø 9.9 mm. Large air bubbles were removed using a needle and syringe. Filled tubes were frozen at −20 °C for over 4 h and lyophilized (Zirbus Sublimator 500, Bad Grund, Germany). Lyophilized plugs were crosslinked using a zero-length crosslinking method applying 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Fluka Chemie AG, Buchs, Switzerland) [36]. In short, the plugs were wetted overnight in 50 mM 2-morpholinoethane sulfonic acid (USB, Ohio, USA) containing 40% (v/v) ethanol (MES buffer, pH 5.0), followed by crosslinking for 3 h at ambient temperature in 33 mM EDC and 6 mM NHS in MES buffer, and washed with 0.1 M Na_2HPO_4, 1 M NaCl, 2 M NaCl, and demineralized water, after which plugs were frozen and lyophilized again. As a final step, plugs of approximately 6 cm were cut to a length of 4.5 cm. As controls, untreated plugs were used that were not included in the crosslinking procedure. Crosslinked and untreated plugs were radially crimped for 3 × 30 s at 552 kPa using a Model RVJ Pneumatically-Actuated Crimping Machine (Blockwise, Tempe, AZ, USA).

2.2. Evaluation of collagen plugs

2.2.1. Extent of crosslinking

The degree of crosslinking was assayed by evaluation of the loss of primary amine groups as a result of crosslinking. Amine groups were measured in collagen plugs before the crimping procedure using 2,4,6-trinitrobenzenesulfonic acid (TNBS) [37]. In short, after incubation of the samples in 4% Na_2HPO_4, they were incubated in a 0.08% (w/v) TNBS solution for 2 h at 40 °C, followed by hydrolysis with 6 M HCl for 1.5 h at 60 °C. Samples were diluted with MilliQ water and measured spectrophotometrically (Bio-Tek, Bad Friedrichshall, Germany) at 420 nm, using a glycine calibration curve. Analyses were performed in triplicate for three independent experiments.

2.2.2. Morphology and pore structure

Scanning electron microscopy (SEM) was used to analyze the morphology and structure of the collagen plugs. After lyophilization, specimens were fixed on a stub with double-sided carbon tape, sputtered with gold for 60 s (Scancoat Six Sputter Coater, Edwards, Crawley, United Kingdom) and examined with a Zeiss Sigma 300 Field Emission Scanning Electron microscope (Zeiss, Jena, Germany) at an accelerating voltage of 5 kV.

2.2.3. Expansion

The diameter of crimped plugs was recorded using a caliper after both lyophilizing steps and after crimping. For the expansion, crimped plugs were wetted in phosphate buffered saline (PBS) and the diameter of the center of the plug was measured at 0 s, 10 s, 30 s, 1 min, 5 min and 60 min after wetting. To visualize the expansion in a membrane, a cylinder (Ø 7.4 cm) was covered with a low-density polyethylene (LDPE) membrane (20 μm thick) and filled with PBS. The membrane was punctured by a fetoscopic instrument with a diameter of 9 Fr (3 mm) and an untreated and expandable collagen plug was inserted in the defect by pushing the plug through the cannula partly into the PBS and withdrawing the surrounding cannula.

In a separate experiment, plugs were exposed to fluids with different polarities to study the mechanism of shape recovery (n = 3). The following solvents were used: trichloromethane, propan-2-one, butan-1-ol, propan-1-ol, ethanol, methanol, ethane-1,2-diol and PBS. Please see Table 1 for the relative polarity of the solvents [38]. To dehydrate non-polar fluids, an excess of $Cu(II)SO_4$ crystals was added before use.

Table 1
Relative polarities of studied solvents [38].

Solvent	Trivial name	Relative polarity
Trichloromethane	Chloroform	0.259
Propan-2-one	Acetone	0.355
Butan-1-ol	Butanol	0.586
Propan-1-ol	Propanol	0.617
Ethanol		0.654
Methanol		0.762
Ethane-1,2-diol	Ethylene glycol	0.79
PBS		~1

The ability of the crosslinked plug to expand was measured after 1 h and compared to untreated controls.

In another experimental setup, a two-layer system of water with 4% (w/v) $Cu(II)SO_4$ and chloroform was used to visualize the effect of polarity on the expansion of the plug [33]. $Cu(II)SO_4$ was used to increase the contrast between both layers. The expansion of the plug was monitored until the plug was expanded over its full length.

2.3. In situ model of porcine urinary bladder

For qualitative results, a urinary bladder from a landrace pig cadaver was partly exposed and used as *in situ* high-pressure model (n = 3). The bladder was completely filled with 0.1% (w/v) 1,9-dimethyl-methylene blue in PBS and punctured by a sharp trocar used for fetoscopy with a cannula diameter of 9 Fr (3 mm), while compressing the bladder manually to increase the pressure. For this proof of concept, a 1% (w/v) collagen plug was prepared and crosslinked as described in section 2.1. The crosslinked collagen plug was inserted in the defect by pushing the plug through the cannula partly into the PBS and withdrawing the surrounding sheath. As a positive control, another defect made in the bladder using the same instrument was left open.

2.4. Ex vivo model of fresh human fetal membranes

The efficacy of the expandable collagen plug for use in fetoscopy was tested in an *ex vivo* setup with human fetal membranes. The human fetal membranes, chorion and amnion, were obtained from postpartum placentas at Radboud university medical center, without the use of any patient data. Here, two setups were used to show the efficacy in sealing the defect and preventing it from leaking. In the first setup, fetal membranes > 8 and < 24 h were obtained of which a sac was formed and filled with water (n = 1). The second setup was based on a model described by Mann et al. [28]. Here, membranes were collected after a Caesarean section and used within 4 h after birth. A piece of the fetal membranes was tightened around a plastic cylinder (Ø 21 mm) using a rubber band and without the addition of any layer to support the membranes. The cylinder was filled with 0.01% (w/v) Azure A in PBS to visualize the fluid uptake by the plug within 1 h (n = 10). For these experiments, plugs were prepared from either a 1.5% (n = 8) or 1% (w/v) (n = 2) collagen suspension and crosslinked as described in section 2.1. In both setups, a trocar used for fetoscopy with a cannula diameter of 9 Fr (3 mm) loaded with a sharp awl was used to puncture the membranes. The trocar was removed from the fetoscopic cannula and the crosslinked collagen plug was pushed through the cannula using an obturator. When the plug was partly pushed out of the cannula into the fluid, the cannula was withdrawn, while keeping the obturator and collagen plug in place.

2.5. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 25 (IBM, New York, USA). All data were presented as mean ± standard deviation (SD). For the comparison in diameters between the different conditions of the collagen plug at different time points, a Students t-test was used. In the case of more than two groups a one-way ANOVA with Bonferroni post-hoc testing was applied.

3. Results

3.1. Construction of collagen plugs

The consecutive steps of freezing, lyophilization, crosslinking, freezing, lyophilization and crimping of collagen (Fig. 1A) resulted in the formation of expandable plugs (Fig. 1B). After the second lyophilization step and before crimping, the crosslinked plugs were significantly smaller in diameter than the untreated controls, 5.8 ± 0.7 mm (n = 154) vs. 8.5 ± 0.3 mm (n = 90), respectively ($p < 0.05$). After crimping, the diameter of the crosslinked plugs was 1.8 ± 0.2 mm (n = 122), while the untreated controls had a mean diameter of 1.7 ± 0.2 mm (n = 66) ($p < 0.05$).

3.2. Evaluation of collagen plugs

3.2.1. Extent of crosslinking

The extent of crosslinking was determined using a TNBS assay. Untreated plugs showed an average of 212 ± 49 nmol of primary amine groups per mg collagen (n = 27), whereas crosslinked plugs had 123 ± 20 nmol primary amine groups per mg collagen (n = 26), corresponding to a degree of crosslinking of ~41%.

3.2.2. Morphology and pore structure

The pore structure of the 1.5% (w/v) collagen plug was examined by SEM. Overall, the pore structure of the collagen plugs showed two phenotypes (Fig. 1C1-2): a more condensed and a more open pore structure. Mostly, plugs showed the condensed pore structure for the full length or the open pore structure for the top half of the plug and the condensed pore structure for the bottom half. Collagen plugs with a more condensed pore structure contained more small and round pores with a diameter of approximately 100 μm. The collagen plugs with the open pore structure contained elongated pores with a diameter of up to approximately 400 μm in the short axis. Collagen plugs with both phenotypes were included in the following experiments. Fig. 1C3 shows the plug in its crimped state, where the compressed pores can be appreciated.

3.2.3. Expansion

After crimping, plugs were wetted in PBS and the diameter measured at several time points. Immediately upon exposure to PBS, the plug started expanding, as can be seen in Fig. 1D and Movie 1. Within 10 s, the crosslinked plug reached a diameter of 3.6 ± 1.0 mm (n = 123) and thereby exceeded the diameter of the trocar used for fetoscopy of 9 Fr (3 mm), and thus the diameter of the opening in the fetal membranes. By 1 h, the crosslinked plug had expanded from a diameter of 1.8 ± 0.2 mm (n = 122) to 6.5 ± 0.6 mm (n = 123), as compared to the diameter of the untreated plug which expanded from 1.7 ± 0.2 mm (n = 66) to 2.6 ± 0.3 mm (n = 114), still not exceeding the trocar diameter. At all time points, the mean diameter of crosslinked plugs was significantly different from untreated plugs.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bioactmat.2022.06.007

Upon placement of the crosslinked plug using the clinical instruments (Fig. 2A) in a plastic membrane, the plug started expanding and sealed the defect by forming an hourglass shape around the trocar entry point (Fig. 2B). The untreated plug did not expand enough to fix itself in the defect and was driven out by the pressure of the fluid (Fig. 2C). As a result, the defect remained open.

The different polarities of the solvents demonstrated an altered expansion of the plug within 1 h (n = 3). The crosslinked plugs did not return to their original diameter in apolar solvents, such as trichloromethane, propan-2-one and butan-1-ol (Fig. 3A). After 1 h in methanol,

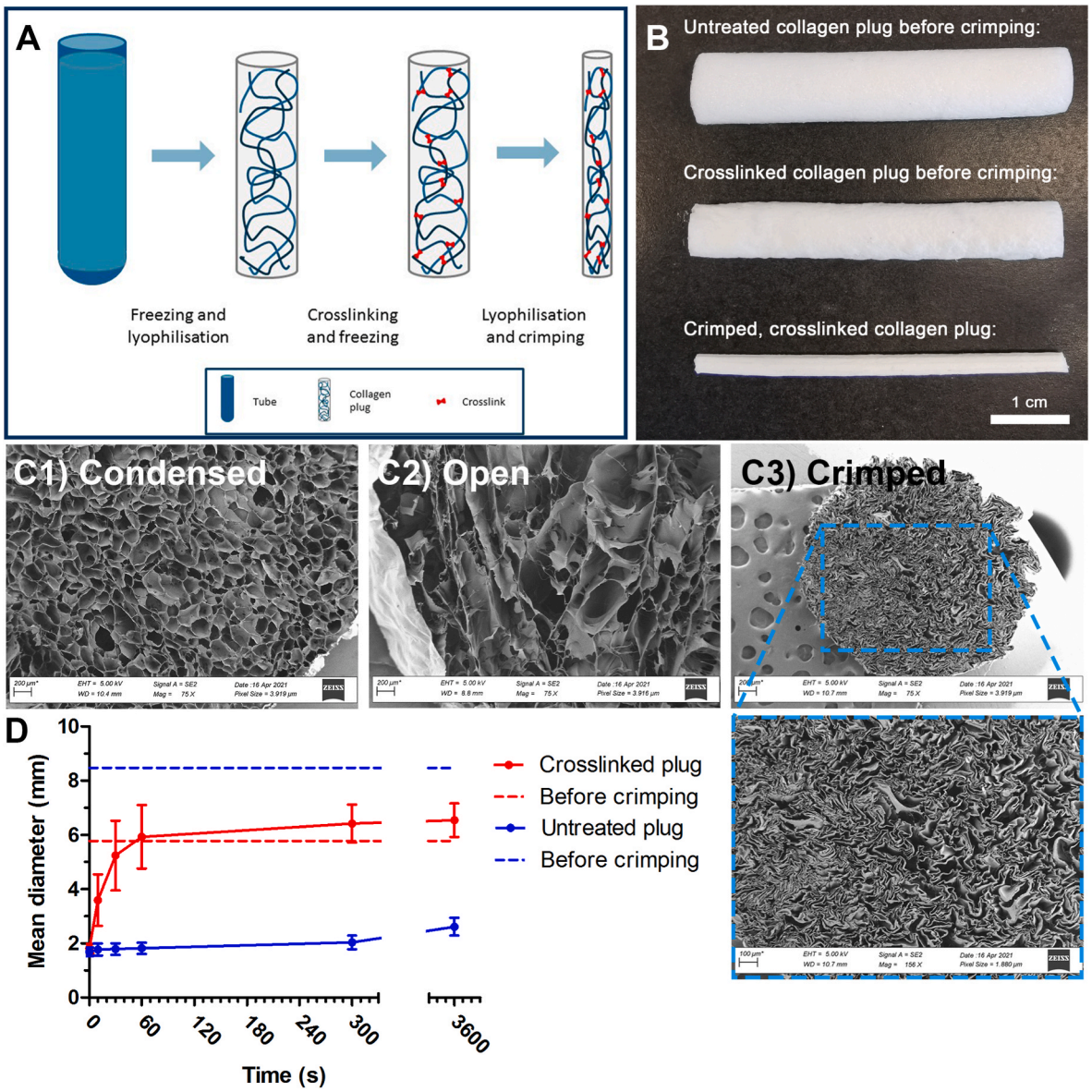


Fig. 1. Construction, morphological characteristics and expansion rate of 1.5% (w/v) collagen plugs. A) Schematic overview of the construction of shape memory plugs. B) Macroscopic view of 1.5% (w/v) collagen plugs after the first lyophilization step (= untreated plug before crimping), after the second lyophilization step (= crosslinked collagen plug before crimping) and after crimping (= crimped, crosslinked collagen plug). Bar is 1 cm. C) Scanning electron microscopical images of crosslinked collagen plugs before (C1-2) and after crimping (C3). In the plugs, pore structure varies from a more condensed pore structure (C1) to a more open pore structure (C2). Bar is 200 μm and 100 μm in the magnification. D) Mean diameter of untreated (n = 66–114) vs. crosslinked (n = 122–123) collagen plugs after swelling in PBS for various times. The crosslinked shape recovery plugs returned to more than their initial dry diameter after crosslinking, whereas the untreated plugs did not reach 3 mm. For crosslinked plugs, ANOVA results indicated that the diameter significantly increased from 0, 10, 30, 60 to 300 s ($p < 0.05$). The dashed lines indicate the mean diameter of dry plugs before crimping in both conditions. Results represent the mean diameter ± standard deviation.

ethane-1,2-diol or PBS, the plugs expanded to a diameter of about 6 mm. Untreated plugs did not reach a diameter of 3.8 mm in any of the tested solvents.

In a two-layer system of water and trichloromethane, the effect of the different polarities on the crosslinked plug were further visualized (Fig. 3B). The part of the plug that came into contact with water started to expand, whereas the rest of the plug in trichloromethane did not. After 70 min, the plug had mostly migrated into the water compartment and was fully expanded.

3.3. *In situ model of porcine urinary bladder*

The efficacy of the plug was studied using a porcine urinary bladder as a high-pressure model. After puncturing a defect in the bladder, an expandable plug could be placed into the defect (Fig. 4A). The crosslinked plug immediately started expanding, fixed itself and sealed the opening (Fig. 4A and B, black arrowhead). The plug was wetted with the stained PBS, however no substantial leakage nor rupture were observed. Without the collagen plug, the blue fluid squirted from the puncture site in this high-pressure model (Fig. 4B, white arrowhead).

3.4. *Ex vivo model of fresh human fetal membranes*

The efficacy of the crosslinked collagen plug was also shown in fresh human fetal membranes. In two different fetal membrane models, the plug started expanding when placed in the defect and no substantial leakage was observed after 1 h. In the first setup, the plug sealed the defect in the formed sac of fetal membranes (Ø~20 cm) filled with water

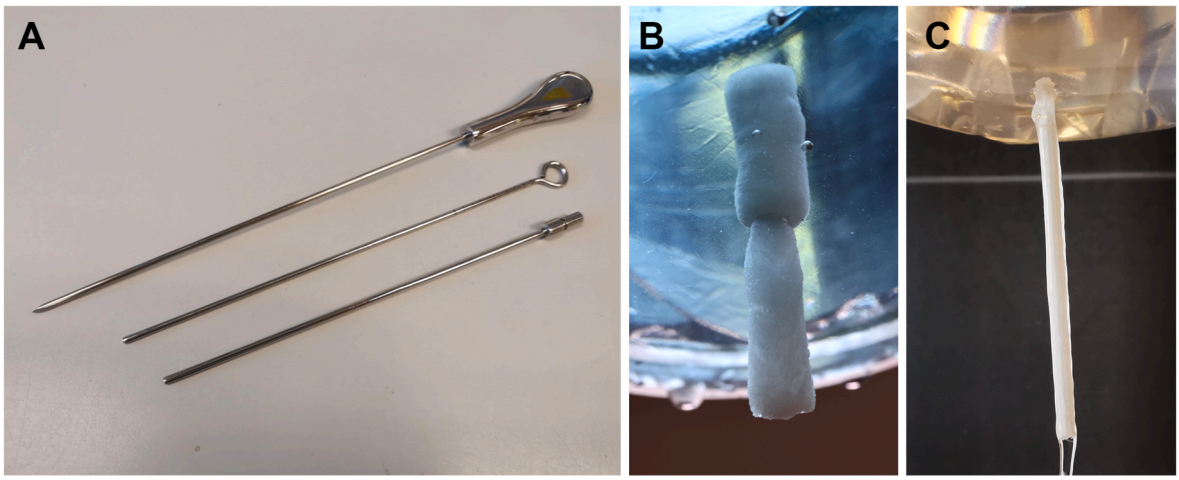
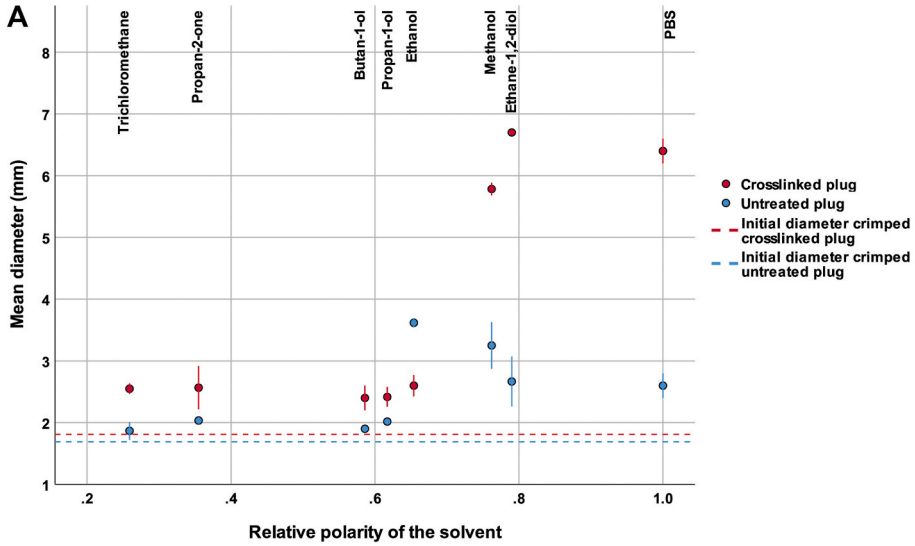


Fig. 2. A) Collagen plugs were placed using a 9 Fr fetoscopic trocar with sharp awl, obturator and metal cannula. B) Expandable collagen plug fixed in plastic membrane with PBS in upper compartment. C) Untreated collagen plug is pushed out of the plastic membrane by PBS in upper compartment.



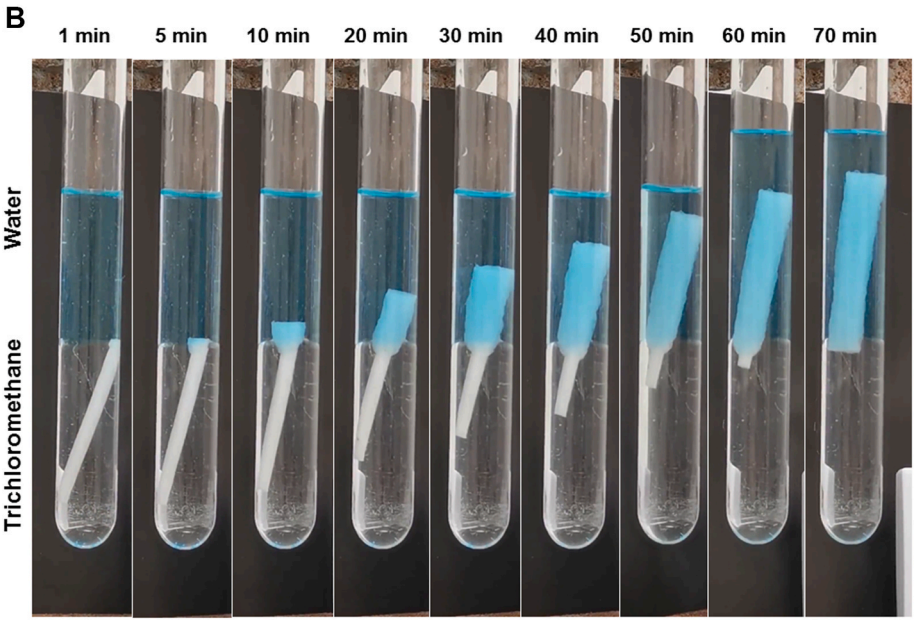


Fig. 3. Behavior of collagen plugs in solvent with varying polarities. A) Mean diameter of crosslinked and untreated plugs after 1 h in solvents with different relative polarities (n = 3). The dashed lines indicate the mean diameter of the crimped plug of untreated and crosslinked plugs. Results represent mean ± standard deviation of the diameter (in mm). B) Time-lapse of a crosslinked plug in a two-layer system of water (containing 4% $Cu(II)SO_4$) and trichloromethane to visualize the effect of solvent polarity on the plug's shape memory. The part of the plug in water started expanding while the part in the trichloromethane remained crimped. Over time, the plug migrated to the blue water compartment and fully expanded.

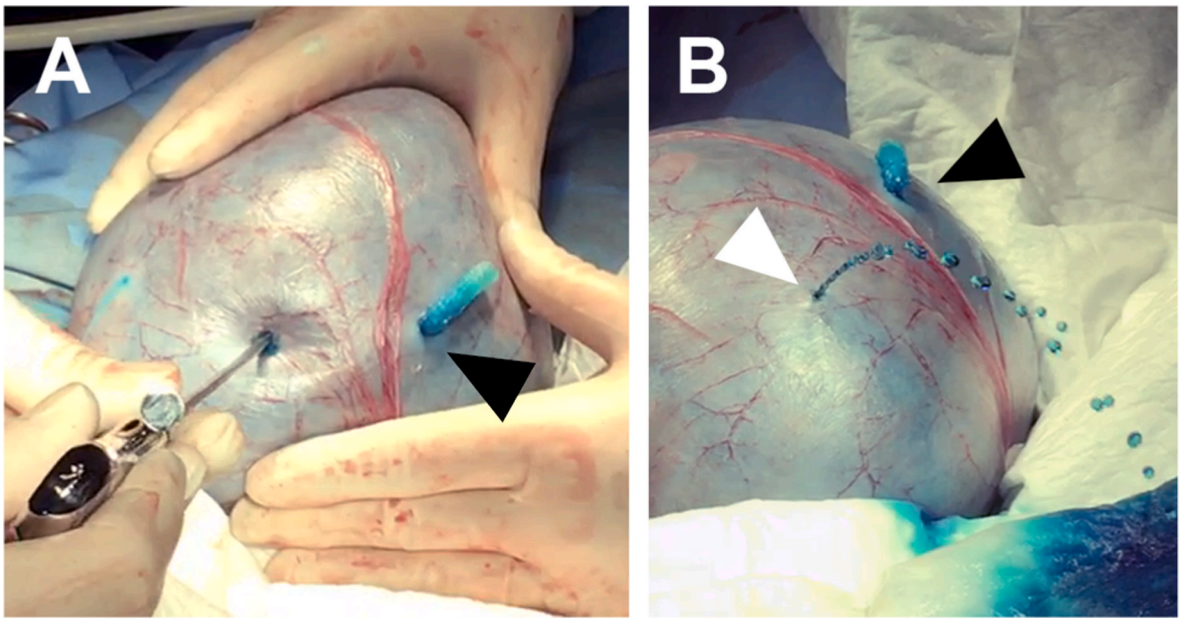


Fig. 4. Expandable plug sealing the defect in a porcine urine bladder filled with 0.1% (w/v) 1,9-dimethyl-methylene blue in PBS (A–B). Compared to the non-plugged control (B, white arrowhead), the crosslinked collagen plug (black arrowhead) successfully sealed the defect and prevented leakage.

and can be seen inside the membranes (Fig. 5A). Next to the sealing of the membranes, the second setup (Fig. 5B) visualized the little amount of dyed solution that was absorbed by the plug. The crosslinked plug expanded when it came into contact with the dyed solution. For most plugs, the plug turned only partly blue and did not leak for 1 h, indicating a sealed defect. Just three plugs stained blue over the full length of the plug, of which only two seeped a few drops. Overall, the crosslinked collagen plugs were able to close the defect in the punctured human fetal membranes *ex vivo* and prevented substantial leakage. Also no further rupture of the membranes caused by the expansion of the plug was observed in either of the two models.

4. Discussion

In this study, we investigated the potential of shape memory in a solid collagen plug to expand directly upon employment in order to seal

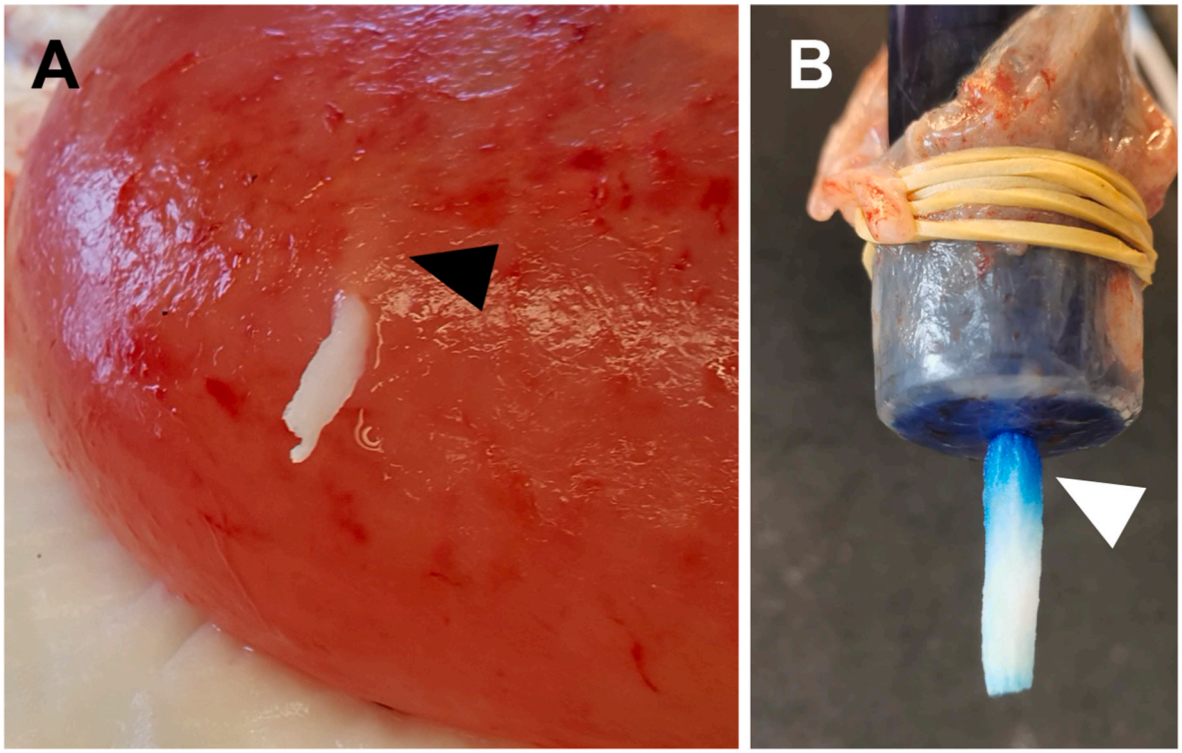


Fig. 5. Two *ex vivo* setups show the efficacy of a crosslinked collagen plug in fresh human fetal membranes. A) A plug sealed the defect and can be observed inside the fetal membranes filled with water (black arrowhead). B) The stained PBS visualizes the little amount of fluid absorbed by the collagen plug after 1 h (white arrowhead). In both setups no substantial leakage was observed.

the fetal membranes after a fetoscopic procedure. Currently, the adverse effects of fetoscopic surgery result in a hesitant approach towards surgery on the unborn child. When iPPROM can be reduced, it may open the field for a wider implementation in less severe conditions as well. For instance, fetal tracheal balloon occlusion (FETO) has recently been shown to significantly increase survival to discharge in severe cases of congenital diaphragmatic hernia (CDH) in a randomized trial [7]. However, in moderate cases of CDH, no significant benefit of FETO performed at 30–32 weeks of gestation was shown compared to the expectant care [8]. With a lower incidence of iPPROM, FETO treatment would most likely be beneficial in moderate cases as well.

For our medical device to plug the fetoscopic defect, we rely on the shape memory that can be given to a collagen plug by the consecutive steps of crosslinking, freezing, lyophilization and crimping. When a crosslinked plug comes into contact with a polar fluid, such as PBS, the plug immediately starts to rapidly expand. In its crimped state, the plug is small enough to be applied using a 9 Fr trocar used in fetoscopy, while upon employment the plug directly expands to seal the defect, exceeding the diameter of the fetoscopic entry-point within 10 s and tripling its own diameter within a minute. In the *in situ* and *ex vivo* setups the plug fixed itself in the defect in both the porcine bladder and the human fetal membranes by expanding and thereby prevented substantial fluid leakage. These experiments demonstrate the ability of the plug to seal the defect for at least 1 h, although a long-term model has to be setup. Also a supportive tissue may be included to better mimic the clinical situation with the uterine wall.

To close a defect induced by fetoscopy in the fetal membranes one needs a plug that enlarges itself after exposition to amniotic fluid. When our crimped plug with shape memory comes into contact with PBS, it fully returns to the shape in which it was crosslinked, while the untreated plugs did not show any shape recovery. The mechanism of the shape memory has been proposed before by our laboratory in both self-closing and self-expandable tubular scaffolds [33–35]. The degree of expansion of the crosslinked solid plugs in solvents with different polarities showed comparable results with the expandable tubular scaffolds [33]. During chemical crosslinking with EDC and NHS, amide bonds are formed between primary amine and carboxylic groups on the collagen fibrils. The loss of charged, hydrophilic carboxylic and amine groups result in the creation of hydrophobic areas in the collagen and thereby formation of an energetically favorable state of the plug. When the plug is crimped, it will result in the deformation of the original interfaces between hydrophobic and hydrophilic areas, and this will bring the plug in an energetically unfavorable state. Placing the plug in a polar fluid will cause a polar surrounding for the hydrophobic areas in the plug, which increases the thermodynamically unfavorable interfaces and results in a loss of entropy. An entropy-driven force will return the plug to its original expanded form. In a non-polar (hydrophobic) fluid, this will not happen and the plug will not expand.

Many attempts to close an endoscopic induced defect in fetal membranes used devices based on type I collagen. Our plug also consists of this type of material as the sole ingredient. Type I collagen has many benefits such as biocompatibility, easy availability and biodegradability [39]. Type I collagen has been shown to stimulate migration of human amnion mesenchymal cells *ex vivo* [40]. Besides, the plug's porosity allows cellular ingrowth from the surrounding tissue, which may further tighten the plug over time and stimulate regeneration of membrane tissue [41]. Highly purified collagen used for the plug will elicit minimal inflammatory response. Chemical crosslinking strengthens the plug and increases the degradation time ensuring it endures during the further duration of pregnancy [42,43], which may have been the issue in earlier studies where the collagenous biomaterial could not be retrieved [16,17]. The biodegradable expandable collagen plug can be degraded by the body, which may be important for subsequent pregnancies. Future *ex vivo* and *in vivo* experiments will evaluate the cellular ingrowth and biodegradation time of the plug.

The addition of shape memory is an interesting feature which may overcome shortcomings of earlier used collagen plugs to seal fetoscopic defects, prevent amniotic fluid leakage and stimulate regeneration of the fetal membranes. Thereby it is hypothesized that the expandable collagen plug with shape memory may prevent iPPROM without the need for additional compounds, like platelets or fibrinogen, as used in previous studies [24,25]. The shape memory of the collagen plug makes it possible to easily fit a large diameter plug in small fetoscopic surgical instruments. In fetoscopy, the crimped plug with a diameter of 1.8 mm can be inserted through the cannula and placed at the endoscopic entry point by keeping the obturator in place and withdrawing the surrounding cannula, as has been described [16,17]. When the plug comes in contact with a polar fluid, it starts expanding, seals the defect and the sheath and cannula can be removed. After expansion the plug will be tightly fixed within the defect, without the need for sutures. Next to sealing the defect, it may also keep the membranes together and thereby possibly prevent chorioamniotic membrane separation. By stimulating regeneration of the membranes, preventing leakage of amniotic fluid and keeping the membranes together, important factors may be taken away by the use of the collagen plug with shape memory and reduce the risk for iPPROM.

In theory, the expandable plugs can be used in all types of fetoscopic surgery. Here, it has to be taken into account that there are variations between the different fetoscopic procedures, like the size of the endoscopic entry point. During the fetoscopic procedure the defect may become two to three times larger in diameter than the trocar shaft itself [44]. Therefore, the plug may have to expand to a larger proportion to cover this enlarged area. In our case, the plug expanded from 1.8 mm to more than 6 mm in 60 s, so more than three times its diameter. In our experiments with a defect of 9 Fr in diameter, the force of this expansion did not lead to rupture of the membranes. The plug can be adapted to be suitable for membrane defects of various sizes by constructing collagen plugs with different initial diameters or by adjusting other variables like the percentage of collagen suspension or the procedure of crosslinking/crimping to change the expansion rate. This way, multiple types of expandable plugs can be generated to fit specific defects.

Our early results warrant further *in vivo* experimentation to study safety and efficacy. This includes further research to the quality of sealing by and potential cellular infiltration in the plugs *ex vivo* using fresh human fetal membranes, *in vivo* in preclinical models and eventually in humans.

5. Conclusion

The type I collagen plug with shape memory is a promising medical device that can be inserted through a cannula of a trocar used in fetoscopy and rapidly expands to its original diameter when in contact with aqueous solutions. These characteristics make it suitable for use in fetoscopic surgery to seal the defect in fetal membranes at the endoscopic entry point and to prevent iPPROM.

Ethics approval and consent to participate

For the *ex vivo* studies, all fetal membranes were obtained anonymously from Radboud university medical center. The membranes were waste material after vaginal or Caesarean delivery and no patient data were collected, so there was no need for approval of the patient according to Dutch law.

For the *in situ* study, the pig cadavers were remnants from another study and provided by the Radboud university medical center Animal Research Facility.

CRediT authorship contribution statement

Rob T.C. Meuwese: Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Elly M.M. Versteeg:** Methodology, Investigation. **Joris van Drongelen:**

Conceptualization, Resources. **Daniëlle de Hoog:** Investigation. **Debora Bouwhuis:** Investigation. **Frank P.H.A. Vandenbussche:** Conceptualization, Funding acquisition. **Toin H. van Kuppevelt:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Willeke F. Daamen:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition, All authors have reviewed the manuscript and approved to submit for publication.

Declaration of competing interest

WD, RM, and TvK are mentioned as inventors on related patent applications. Other authors: no conflicts of interest.

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